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Neuropeptides, Trophic Factors, and Other Substances Providing Morphofunctional and Metabolic Protection in Experimental Models of Diabetic Retinopathy

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Abstract

Vision is the most important sensory modality for many species, including humans. Damage to the retina results in vision loss or even blindness. One of the most serious complications of diabetes, a disease that has seen a worldwide increase in prevalence, is diabetic retinopathy. This condition stems from consequences of pathological metabolism and develops in 75% of patients with type 1 and 50% with type 2 diabetes. The development of novel protective drugs is essential. In this review we provide a description of the disease and conclude that type 1 diabetes and type 2 diabetes lead to the same retinopathy. We evaluate existing experimental models and recent developments in finding effective compounds against this disorder. In our opinion, the best models are the long-term streptozotocin-induced diabetes and Otsuka Long–Evans Tokushima Fatty and spontaneously diabetic Torii rats, while the most promising substances are topically administered somatostatin and pigment epithelium-derived factor analogs, antivasculogenic substances, and systemic antioxidants. Future drug development should focus on these.

ABBREVIATIONS

ACE angiotensin-converting enzyme
ACEI angiotensin-converting enzyme inhibitor
AG aminoguanidine
AGE advanced glycosylated end product
AIF apoptosis-inducing factor
ALE advanced lipoxidation end product
AQP aquaporin
BB/W Bio–Breeding/Worchester (rat)
BDNF brain-derived neurotrophic factor
BRB blood–retina barrier
BSA bovine serum albumin
CAV-1 caveolin 1
CBD cannabidiol
CGA chlorogenic acid
CNTF ciliary neurotrophic factor
CTGF connective tissue growth factor
DAG diacylglycerol
DR diabetic retinopathy
E4 exendin-4
EPO erythropoietin
ERK extracellular signal-regulated kinases
ERG electroretinogram
FDP-lysine Nε-(3-formyl-3,4-dehydropiperidino)lysine
GABA γ-aminobutyric acid
GBE *Ginkgo biloba* leaf extract
GCL ganglion cell layer
GDNF glial cell line-derived neurotrophic factor

GFAP glial fibrillary acidic protein
GK Goto–Kakizaki (rat)
GLAST glutamate aspartate transporter
GLP-1R cognate receptor
GLUT1 glucose transporter 1
Grx1 glutaredoxin 1
GS glutamine synthase
GSPE grape seed proanthocyanidin extracts
GT green tea
HIF1 α hypoxia-inducible factor-1 α
H₂S hydrogen sulfide
ICAM-1 intercellular adhesion molecule 1
IGF-1 insulin-like growth factor 1
IL-1 interleukin-1
ILM inner limiting membrane
INL inner nuclear layer
IOP intraocular pressure
IPL inner plexiform layer
JNK c-Jun N-terminal kinase
LDL low-density lipoprotein
LETO Long–Evans Tokushima Otsuka
LPA lysophosphatidic acid
MAPK mitogen-activated protein kinase
mtDNA mitochondrial DNA
NF- κ B nuclear factor κ B
NFL nerve fiber layer
NGF nerve growth factor
NMDA N-methyl-D-aspartate
nNOS neuronal nitric oxide synthase
OLETF Otsuka Long–Evans Tokushima Fatty (rat)
OLM outer limiting membrane
ONL outer nuclear layer
OP oscillatory potential
OPL outer plexiform layer
OS outer segment
PACAP pituitary adenylate cyclase-activating polypeptide
PARP poly-(ADP)-ribose polymerase
PEDF pigment epithelium-derived factor
PI3K phosphatidylinositol 3-kinase
PKC protein kinase C
PKC ζ protein kinase C ζ
PPAR peroxisome proliferator-activated receptor
PRL photoreceptor layer
RA retinoic acid
RAGE receptor of AGEs
RAS renin–angiotensin system
RGC retinal ganglion cell

ROS reactive oxygen species
RPE retinal pigment epithelium
SDT spontaneously diabetic Torii (rat)
SST somatostatin
STZ streptozotocin
TC *Tinospora cordifolia*
TNF α tumor necrosis factor- α
TH tyrosine hydroxylase
TUNEL terminal dUTP nick-end labeling
UCP-2 uncoupling protein 2
VEGF vascular endothelial growth factor
VEP visually evoked potential
VIP vasoactive intestinal peptide
ZDF Zucker diabetic fatty (rat)
ZF Zucker fatty (rat)



1. PREVALENCE AND CAUSES OF DIABETIC RETINOPATHY

1.1. Why experimental models are needed

The visual world is the most important environmental information source for many species, including humans. None of the other sensory signals reaches the brain in such variety and none is processed by as many cortical areas as the visual cues. The first steps of visual processing are performed by a thin sheath of neural tissue at the back of the eye, called retina. After phototransduction, light information is translated into neural signals and shaped by the retinal interneurons. Bipolar cells transmit the processed signal to ganglion cells that project to the brain and this is the sole source of visual signals arriving there. Therefore, any damage to the retinal tissue immediately results in vision loss and, in the worst case, causes total blindness.

Many retinodegenerative disorders such as glaucoma, ischemia, and diabetic retinopathy (DR) are thought to be consequences of pathological metabolic processes ([Osborne et al., 2004](#)). Metabolic insults vary and include exposure to extremely strong light and changes in hormone/metabolite levels or in blood/aqueous humor pressure. These processes lead to elevated extracellular glutamate levels and can provoke excitotoxic insults ([Atlasz et al., 2008](#)). The balance between the neurotoxic and neuroprotective factors is crucial in determining the survival of retinal neurons ([Hernandez and Simo, 2012](#)). DR, a common complication of diabetes, develops in 75% of patients with type 1 and 50% with type 2 diabetes, progressing to legal

blindness in about 5% (Engerman and Kern, 1995). Type 1 diabetes occurs most commonly in children and young adults and constitutes 5–10% of the diagnosed diabetics (Maahs et al., 2010). Type 2 diabetes accounts for 90–95% of diagnosed diabetes cases globally and typically develops in middle-aged adults. Both type 1 and type 2 diabetic patients may develop retinopathy; in particular, almost everyone with type 1 diabetes will develop it over a 15–20-year period and greater than 60% of type 2 diabetes patients will have retinopathy after 20 years (Hazin et al., 2011).

The aforementioned data call for increased efforts to learn more from the pathogenesis of this disease and search for possible treatments/cures. Apart from the increased diagnostic opportunities (e.g., the use of optical coherence tomography) and the carefully analyzed results of clinical trials regarding invasive and noninvasive treatment options (Gabriel, 2013; Hammes, 2013), experimental models are also necessary to utilize. However, one has to be careful when translating results obtained in commonly used animal models, because, besides similarities in the pathogenesis, profound differences can also be found (vascular symptoms and proliferativity) between animal models and human disease. Despite these differences, several animal models have been developed and used successfully in revealing basic mechanisms both in the pathogenesis of DR and in studying possible protective treatments. This is because the underlying physiological and biochemical processes in humans are identical or very similar to those of experimental animals.

The same statement applies also for the causes, as hyperglycemia is a major risk factor for the development and progression of DR. It plays an important role in the pathogenesis of diabetic complications by increasing the levels of advanced glycosylated end products (AGEs), which are late products of nonenzymatic glycation. The accumulation of AGEs appears to be a key factor in the development of DR. AGEs are thought to promote many diabetic complications, including retinopathy, nephropathy, neuropathy, and cardiovascular disease (Brownlee, 2005; Huebschmann et al., 2006).

Capillary occlusions are also characteristic features of early DR and are presumed to initiate neovascularization. Diabetic rats (2–9 months into the disease) showed capillary occlusions by leukocytes (especially monocytes), endothelial cell damage, extravascular macrophage accumulation, and tissue disintegration. In both induced diabetes and genetically diabetic mice, the development of diabetes is macrophage-dependent. The presence of numerous interstitial macrophages is characteristic to DR. Leukocytes in general and monocytes/macrophages in particular may not only be involved

in the pathogenesis of early DR but may also, at least in part, initiate the microvascular pathology observed at later stages. Occluding monocytes or granulocytes were found only in diabetic retinas. In rat models, areas of capillary loss and neovascularization were associated with the presence of monocytes or macrophages. Phagocytes are known to be necessary for the removal of products of pathological processes. Capillary disintegration is also a major factor in disease development. Accordingly, pericytes are absent from the postmortem diabetic retinas. Some blood vessels that still contained intact endothelial cells and vessels with microaneurysms tended not to contain pericytes, suggesting that their loss permits uncontrolled proliferation of endothelial cells. Pericyte dropout has been used as an index of DR (Kern and Engerman, 1994). Permeability increase occurs in many vessels simultaneously, first occurring in the larger superficial vessels and then progressing to the capillaries of the outer retina within 2 months from the onset of diabetes. The vascular permeability increase is a consequence of regulatory changes in tight junction proteins within a broad population of endothelial cells rather than the apoptosis of a small number of endothelial cells. Increased permeability causes edema in the nearby tissue. Edema is the main reason of impaired vision in nonproliferative DR. Water transport through aquaporins (AQPs) facilitates the development of ischemic edema in the retina. Experimental diabetes is associated with the altered regulation of AQPs in the pigment epithelium and the outer retinal layers. These alterations might be involved in the adaptation of retinal cells to hyperglycemic conditions and the development and/or resolution of retinal edema (Hollborn et al., 2011).

1.2. Aims

As it can clearly be seen from the aforementioned facts, there are several areas of research that have led and may further lead to breakthroughs in either the diagnosis or treatment of DR. This review focuses on the recent advances and current hopes in the area of experimental DR from the following aspects: (i) What are the primary mechanisms driving the pathogenesis? How do different neuronal cell types change during DR and what may help to possibly rescue them? (ii) Which molecular pathways lead to cell death? What is the contribution of nonneuronal cell types of the retina to these signaling pathways? (iii) Are there any differences between type 1 diabetes and type 2 diabetes in the course of pathogenesis in developing DR? Can animal models be established to study those? (iv) Which animal models are the best

for translational research? Finally, we will also give an overview of a number of endogenous compounds and list several other (synthetic or natural) substances that showed discernible protection against DR. In some cases, their mode of action will also be discussed.



2. DIABETIC RETINOPATHY: EARLY SIGNS AND LATE-DEVELOPING SYMPTOMS

As the worldwide prevalence of diabetes continues to increase, DR is a leading cause of vision loss in developed countries (Fong et al., 2004). The inability of the retina to adapt to metabolic stress leads to a glucose-mediated microvascular disease along with chronic inflammation, which finally causes neurodegeneration and dysfunction in the retina. The retina is one of the most metabolically demanding tissues in the body, and therefore, it is highly vulnerable. The interplay between the neuroretina and the vasculature is critical in developing neurological symptoms of disease. In DR, the rate of neuronal loss in the retina is slow, leading to a gradual, cumulative reduction mostly in amacrine and ganglion cells (Liu et al., 2008). Two forms of DR can be clearly distinguished: an early, nonproliferative DR, when neovascularization of the macula is not evident, and proliferative DR where the symptoms include macular neovascularization. Other vascular symptoms involve microangiopathy, formation of microaneurysms, flame hemorrhages, leukocyte adherence to the vascular wall, and formation of exudates in the extravascular space. These observations have led to the theory that DR is primarily a vascular disorder whose degrading effects are due to the consequences of vascular failure: ischemia followed by increased reactive oxygen species (ROS) production, as in the case of the retinal ischemic diseases (Fulton et al., 2009).

However, it has also been described that neuronal damage may precede any detectable microvascular change (Barber et al., 1998). The deterioration of the intrinsic time and also of oscillatory potentials (OPs) in the electroretinogram (ERG) starts early, in some cases as early as 2 days after induction of diabetes in experimental animals (Li et al., 2002). These observations led to the formulation of the “neurodegeneration-first” hypothesis (Villaroel et al., 2010). Patients suffering from DR experience gradual vision loss; after ERG deterioration, the visually evoked potentials (VEPs) also start to decrease (Wolff et al., 2010), which indicates cortical dysfunction. However, recent observations led to the conclusion that inflammation may

precede or at least runs parallel with the vascular and neural events (Joussen et al., 2004; Liu et al., 2008; Tang and Kern, 2011).

Inflammatory molecules in the retina can be produced not only by leukocytes but also by glial cells; many of them are produced by the Müller glia (Bringmann and Wiedemann, 2012). Müller cells are primarily responsible for ion and volume regulation of the retina and also control extracellular glutamate levels through their excitatory amino acid transporters. They also participate in protection against free radicals and hypoxic damage through glutathione synthesis. Usually the first sign of the Müller cell stress is upregulation of glial fibrillary acidic protein (GFAP) content, which may be accompanied by hypertrophy and proliferation under certain damaging conditions. Diabetes itself is able to upregulate GFAP in Müller cells without the presence of other symptoms of DR in humans (Villaroel et al., 2010), and this goes along well with what we see in experimental models where after 2 days of diabetes induction, GFAP upregulation is already apparent (K. Szabadfi, unpublished observation). All the aforementioned observations may lead to the formation of a fourth hypothesis of the initiation of DR, the “glial cells-first” scenario. In this case, Müller cells, by sensing the elevated glucose level, activate their volume and ion-regulating machinery and release vasoproliferative vascular endothelial growth factor (VEGF; Amandio et al., 2010; Eichler et al., 2000) and inflammatory substances (prostaglandins, tumor necrosis factor (TNF), and interleukins; Behl et al., 2008; Joussen et al., 2009; Tang and Kern, 2011). These would in turn initiate neurodegeneration, inflammation, and vascular growth. Although the exact order of events is unknown, all the aforementioned evidences suggest that low oxygen supply and high blood glucose level are important pro-DR parameters and all signaling pathways converge to activate VEGF production.

The intraocular concentration of VEGF is closely correlated with active neovascularization in diabetes. Hypoxia increases VEGF expression in retinal cells, which promotes retinal endothelial cell proliferation, suggesting that VEGF plays a major role in mediating intraocular neovascularization resulting from ischemic retinal diseases. An increased expression of VEGF in the retina is involved in generating vascular leakage and angiogenesis in DR. In addition to VEGF, pigment epithelium-derived factor (PEDF), a potent inhibitor of angiogenesis, is also involved in the pathogenesis of DR (Patel et al., 2006). The time course of the VEGF-to-PEDF ratio change is correlated with the development and progression of retinal neovascularization. The VEGF-to-PEDF ratio represents a dynamic balance

between the angiogenic stimulators and inhibitors; a disturbance in the balance plays a key role in the pathogenesis of DR (Chen et al., 2008; Gao et al., 2001, 2002). Vascular changes especially regarding permeability have been reported to occur as early as 8 days after onset of diabetes in rats (Do Carmo et al., 1998). Capillary dilation and increased blood flow are some of the earliest signs of diabetes both in rats (Cringle et al., 1993) and in humans (Grunwald et al., 1994), supporting a “capillaries first” hypothesis.

2.1. Histological alterations

The neural retina is composed of diverse neurons characterized by morphological and biochemical criteria and numerous neural networks formed through chemical and electrical synapses among the processes of these neurons. These neuronal components are arranged into layers. Retinal ganglion and displaced amacrine cells form the ganglion cell layer (GCL); bipolar, horizontal, and amacrine cells occupy the inner nuclear layer (INL), while the processes (and synapses) of the bipolar and amacrine cells and the ganglion cells contribute to assemble the inner plexiform layer (IPL). The somata of photoreceptors (rods and cones) gather in the outer nuclear layer (ONL), and their light-sensitive processes form the layer of the outer segments (PRL). The photoreceptors synapse with the horizontal and bipolar cells in the outer plexiform layer (OPL).

The limits of the retina from the intravitreal side are the inner limiting membrane (ILM), which is a close association of Müller glial cells end feet and the pigment epithelial cells on the opposite side. Müller cells are the major glial elements of the retina; their somata are positioned in the INL and extend their processes toward the photoreceptors and retinal ganglion cells (RGCs).

The retinal pigment epithelium (RPE) is composed of a single layer of hexagonal cells that are densely packed with pigment granules. The RPE shields the retina from excess incoming light. It supplies omega-3 fatty acids and glucose, the former for building photoreceptive membranes and the latter for energy. It also serves as a layer limiting transport, thus maintaining and protecting the retinal environment.

The diabetic damage inflicted on all retinal (neuronal and nonneuronal) cells by high glucose initiates a number of metabolic processes that altogether closely approximate the molecular basis for the loss of vision associated with this disease. Here, we give a point-to-point overview about the retinal compartments that are altered by (experimental) diabetic retinal degeneration.

2.1.1 RGCs and the optic nerve

RGCs are the output elements of the retina. Their death immediately and directly leads to loss of visual information. They have high sensitivity to cellular damage and neurotoxicity; thus, RGCs are prone to degeneration in diabetic retinas. All studied rat strains have shown RGC loss or damage in diabetes starting from 4 weeks after the onset of diabetes up until 12 months (Kern and Barber, 2008). Reduced number of RGCs was demonstrated in the retina of 15-week diabetic rats (Hammes et al., 1995b) and also in short-term (4 weeks) diabetes (Zeng et al., 2000).

In ocular conditions, RGC loss is often associated with elevated intraocular pressure (IOP). The levels of IOP in 6- and 12-week diabetic mice (C57BL/6) were significantly higher than their controls. In parallel with these phenomena, the number of RGCs was significantly decreased after only 6 weeks of diabetes. During a 12-week examination period, IOP remained constantly high. However, it has to be noted that elevated IOP-induced cell loss is not specific to induced diabetes, but rather is an accompanied symptom of several ocular diseases (Yang et al., 2012).

In diabetic retina, structural alterations in RGCs can be easily recognized. Their features include swellings on axons, often associated with constriction close to the cell body. There is structural remodeling of dendrites, including an increase in the total length, density, and number of branches. These changes surprisingly were found limited to the large ON-RGCs in short-term diabetes and did not occur in any class of OFF-RGCs (Kern and Barber, 2008). Similar changes in RGC morphology have been observed in human retinas (Meyer-Rusenberg et al., 2007). This changes in a subset of RGCs could alter the functional output of the certain subtypes of RGCs (Kern and Barber, 2008), leading to changes in VEPs (see later text). While neurons in the INL were resistant to stable and long-lasting moderate hyperglycemia during a 2-months' period, the GCL underwent both reactive and destructive changes. Chromatolytic changes to the RGCs were in the foreground, and there was a somewhat less marked increase in the number of pyknomorphic RGCs (Logvinov et al., 2010). Several molecular and cellular mechanisms may be involved in RGC loss, which will be discussed in the later text.

The number or density of axons in the rat optic nerve was found reduced in induced diabetes in some, but not in all, rodent studies (Kamijo et al., 1993). The cross-sectional area of the optic nerves was not changed at 8 or 12 weeks of diabetes, but the percentage of area occupied by glial cells increased and the axonal component decreased significantly. These

alterations showed highly significant correlations with the associated prolongation of the latencies of the VEPs, suggesting that axoglial disjunction and axonal atrophy are major determinants for impaired optic nerve function (Kamijo et al., 1993).

2.1.2 Amacrine, horizontal, and bipolar cells

These classes of cells represent the interneurons of the retina. Bipolar cells transmit the signals from the photoreceptors to the RGCs, while horizontal and amacrine cells are the constituents of the lateral signaling pathways in the OPL and IPL, respectively. Despite the fact that bipolar cells are thought to dominantly contribute to the b wave of the ERG, which is a major functional marker of retinal health, there are currently no data available elucidating the bipolar cell reaction to diabetic conditions. The same also applies to horizontal cells.

The most prominent change in the ERG seen in the early stages of diabetes involves the loss of OPs that are thought to derive from dopaminergic amacrine cells (Shirao and Kawasaki, 1998). Therefore, the alterations in the dopaminergic system seem to be among the first significant events in the development of DR. Seki et al. (2004) named several possible mechanisms underlying the degeneration of dopaminergic amacrine cells in diabetic animals: (i) severe insulin deprivation, (ii) hyperglycemia, and (iii) dysfunction of Müller glial cells. The retina of the insulin-deficient *Ins2^{Akita}* mice contained 16% less dopaminergic amacrine cells than nondiabetic age-matched control littermates (Gastinger et al., 2006). An extensive loss of cells, dendritic varicosities, and tyrosine hydroxylase (TH) content was seen in acute (3 weeks) diabetes in rats (Szabadfi et al., 2012; see also Fig. 1.1).

The total number of cholinergic amacrine cells in the diabetic mice was 20% less than that in nondiabetic animals, but they were lost in a random pattern. Diabetes leads to a greater reduction of cholinergic amacrine cell density in the peripheral retina than in the central regions (Gastinger et al., 2006). In the rat retina, most of the neurons containing neuronal nitric oxide synthase (nNOS) appear to be amacrine cells, residing in the INL or displaced to the GCL and closely related to vessels. The number of these nNOS-containing cells was found to decrease by 32% as early as 1 week after the streptozotocin (STZ)-induced diabetes and remained reduced for up to 8 months (Goto et al., 2005). These results suggest that some amacrine cell types may play pivotal roles in maintaining retinal integrity and if these cells are damaged, retinal circuitry suffers nonreparable consequences. Also, these cells may not be the most numerous—dopaminergic and nitrergic cells both

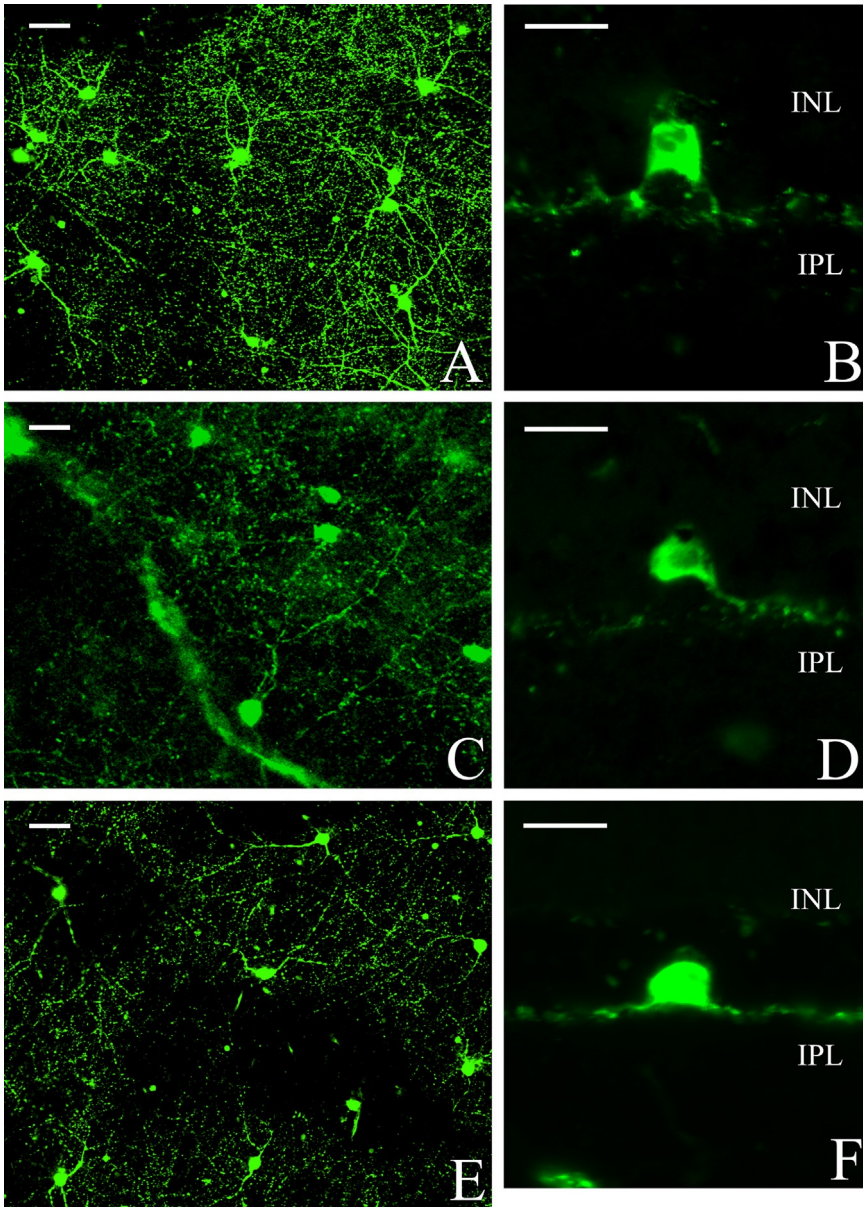


Figure 1.1 Tyrosine hydroxylase (TH) immunoreactivity in healthy and diabetic rat retinas. The dopaminergic amacrine cells are situated in the inner retinal layers, at the border of INL and IPL of the retina as were shown in the control vertical retinal section (B). Representative fields of whole-mount retinal preparations of control (A), 3-week diabetic (C), and insulin-treated chronic diabetic animals (4 months of diabetes; E) and retinal cross sections from the same three groups (B, D, F). TH immunoreactivity was in higher density in the control retinas (A, B) compared with the two diabetic groups (C, D and E, F, respectively), where the arborization of the TH-positive cells was also reduced. Scale bars are 200 μm in pictures A, C, and E and 20 μm in pictures B, D, and F. Abbreviations: INL, inner nuclear layer; IPL, inner plexiform layer.

represent less than 1% of amacrine cells but may confer widespread regulatory functions.

2.1.3 Photoreceptors

Various aspects of visual information are transmitted in parallel from photoreceptors to the output neurons of the retina, which code for many different signals, for example, brightness, darkness, contrast, color, and motion. Photoreceptors are one of the most metabolically active cell types of the retina (Wassle, 2004). There are several factors such as metabolic changes associated with diabetes that may lead to outer retinal dysfunction. Aizu and coworkers (2002) provided the first evidence showing changes of photoreceptor layer (PRL) during DR; the shortening of the photoreceptor outer segment (OSs) and the increase in the number of fragmented cone photoreceptors and a disrupted photoreceptor–RPE cell complex were shown by electron microscopy in rats with very early stage of diabetes (1 month after the onset of diabetes). Since RPE cells produce a variety of trophic factors and have an important role in renewing the photoreceptor OSs, their damage will also influence the phototransduction process. A 2-month diabetic period induced destructive changes in the neurosensory cells. Fragmentation and vacuolar degeneration of the OSs are characteristic, as well as nuclear pyknosis, edema of the perikaryon, and enlargement of the scleral processes of Müller cells, which actively phagocytose degraded OSs. Quantitative analysis demonstrated a threefold increase in the proportion of photoreceptors with pyknotic nuclei (Logvinov et al., 2010).

As a consequence of the aforementioned structural damage after diabetes-induced dysfunction, the retinal phototransduction is apparently hindered even in early-stage diabetes, including deterioration of the flash response and light adaptation (Kim et al., 2005).

2.1.4 Synaptic layers

The signal transmission of various classes of retinal neurons is the physiological bases for visual processing and requires a range of synapses with different kinetics, such as electrical synapses, conventional chemical synapses, and ribbon synapses. Diabetes impacts a broad population of retinal synapses, which may explain significant thinning of the IPL, after a longer duration of diabetes (Barber et al., 1998, 2005). Ribbon synapses in both plexiform layers are especially strongly affected (Fig. 1.2). The thickness of the IPL in the 6-month diabetic rats was markedly reduced (Aizu et al., 2002). There was a 22% decrease in the thickness of the IPL in rats after 7.5 months of

STZ-induced diabetes, suggesting a cumulative loss of neural dendrites and synapses in the inner retina (Barber et al., 1998).

After 1 month of STZ-induced diabetes, synaptophysin content was found reduced in the whole retina and also in isolated retinal synaptosomes. The size and density of synaptophysin-immunoreactive puncta were reduced in the plexiform layers. The loss of synaptic proteins in retinal synaptosomes was accompanied by decreased mRNA content after 1 month of diabetes; therefore, diabetes may increase local degradation rate of presynaptic proteins at retinal synapses and also that of their mRNA (VanGuilder et al., 2008). Indeed, synaptophysin mRNA translation continues at a higher rate after the induction of diabetes as a compensatory mechanism of the reduction of the mature protein. Nevertheless, there is also an increase in its degradation during an early stage of its maturation, possibly during its posttranslational processing (D'Cruz et al., 2012). These findings suggest multiple potential regulatory mechanisms for synapse disintegration.

2.1.5 Müller glial and retinal pigment epithelial cells

Although often considered as nonprincipal components, these cell types are integral constituents of the retinal metabolism.

2.1.5.1 Müller glial cells

Müller glial cell is the principal glia of the retina, which expresses a diversity of ion channels and transporters, releases a variety of cytokines and survival factors, and has receptors for numerous neurotransmitters and growth factors. Müller cells play an active, dynamic role in the retina. They are radially oriented and span the depth of the retina from the vitreal border to the interphotoreceptor matrix of the subretinal space. Their processes are in close apposition to neuronal cell bodies, neurites, and synapses, and blood vessels and their processes make contact with most neural cells (Sarthy and Harris, 2001). They also form end feet on both large vessels and capillaries in the inner and outer retinal vessel beds. Müller glial cells are vital for maintaining normal neuronal and vascular function in the retina. Their mechanical role is stabilizing the retinal architecture, and they are involved in regulating retinal glucose metabolism, modulating the ionic and molecular composition of the retinal microenvironment, altering blood flow to match the local metabolic needs, and contributing to the maintenance of the inner blood–retina barrier (BRB; Bringmann et al., 2006).

In normal conditions, the end feet of the glia express GFAP. Increased GFAP expression has been widely used as a cellular marker for several retinal

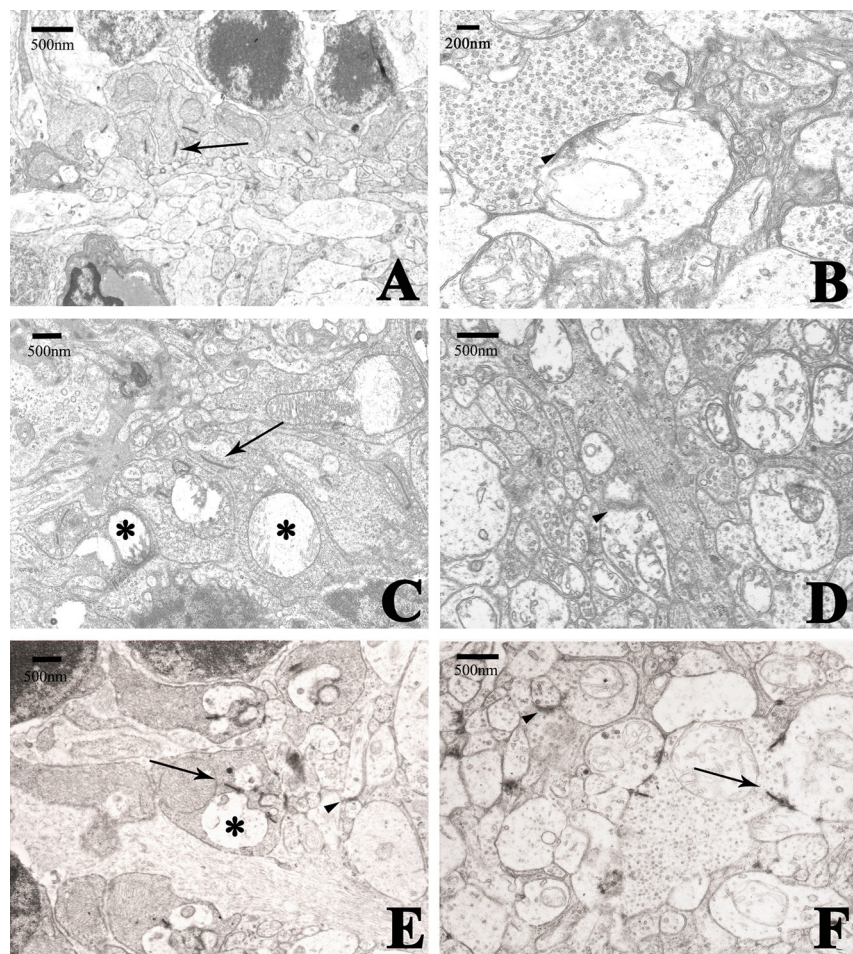


Figure 1.2 Ultrastructure of the outer and inner plexiform layers (OPL and IPL) of control, early diabetic (3 weeks) and chronic diabetic (4 months) rat retinas. Although ribbons of the photoreceptors are still present in the OPL of both diabetic retinas (arrows: C, E), they are less densely packed than those of the controls (A). Some degenerative profiles appear (asterisks: C, E). The processes are often devoid of synaptic vesicles. In the IPL, the ribbon and conventional synapses (arrowheads) are healthy in the control retinas (B) if compared with the same structures of the diabetic retinas (D, F). The shape of the ribbons was distorted in diabetic animals; they did not have sharp contours as found in control retinas. Almost no synapses were present in the IPL compared with the control (B); some unusual ribbons and degenerating synaptic profiles were seen in the IPL (arrowheads: D, F). Scale bars: indicated in the images.

pathologies. It has been suggested that elevated expression of GFAP in the cell body and processes is an aspecific metabolic stress signal (Lundkvist et al., 2004). Müller glial cells regulate the extracellular environment of neurons in the retina by clearing glutamate from synaptic clefts and by providing glutamine to neurons for the resynthesis of glutamate to be used in neurotransmission. This ability of glia in diabetic rat retina is reduced to only 65%, which results in an overall increase of total extracellular glutamate levels that is toxic to retinal neurons. Müller glial cells in diabetes showed reduced glutamate aspartate transporter (GLAST) function (Li and Puro, 2002) and impaired glutamine synthesis. The adverse effects of diabetes on the function of Müller cells in transporting glutamate by GLAST or in metabolizing glutamate by glutamine synthase (GS) have been widely studied (Kowluru et al., 2001; Li and Puro, 2002; Lieth et al., 2000). Although alterations in GLAST activity during diabetes remain controversial, impairment of GS activity is convincingly evident (Mysona et al., 2009; Ward et al., 2005; Zeng et al., 2009). A positive-feedback loop involving oxidative stress and dysfunction of GLAST may be important in the progression of DR (Puro, 2002). These dysfunctions also participate in creating elevated glutamate levels in the microenvironment of diabetic retinas, which might induce excitotoxicity in amacrine cells and RGCs (Lieth et al., 1998).

Glial cells not only send signals to other components of the retina but also change their own makeup. Changes in glia are accompanied by several dysfunctional responses, including altered potassium and glutamate regulation (Li and Puro, 2002; Lieth et al., 1998) and γ -aminobutyric acid (GABA) accumulation (Ishikawa et al., 1996). One of the early histological alterations in the retina of the diabetic rats was the increase in GFAP expression of the Müller cell processes as early as few days after the onset of diabetes, which is a nonspecific response to pathophysiological conditions (Fig. 1.3). Müller glia is adversely affected early in the course of diabetes, the reactive “phenotype” characterized by hyperplasia and upregulation of GFAP (Lieth et al., 1998). Apart from the aspecific glial reactions, hyperglycemia leads to overexpression of several biologically active factors, like proinflammatory cytokines (Gerhardinger et al., 2005) and insulin-like growth factor 1 (IGF-1; Inokuchi et al., 2001).

Besides excitotoxicity-induced changes, products of carbohydrate metabolism may also cause alterations in Müller glia functions. During early experimental diabetes, there is a selective accumulation of the acrolein-derived advanced lipoxidation end products (ALEs), one of which is N ϵ -(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine), originally restricted

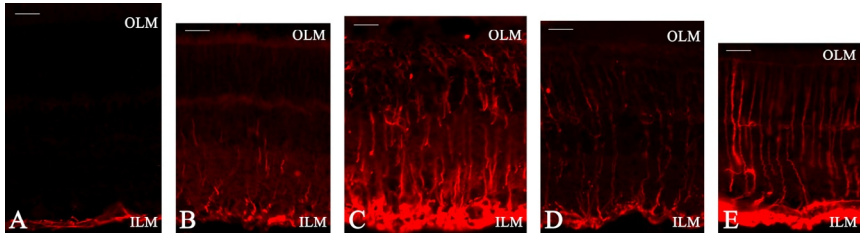


Figure 1.3 Immunofluorescent labeling of glial fibrillary acidic protein (GFAP) in the rat retinas. In normal conditions, only the end feet of the Müller glia (ILM) express GFAP (A). Damaging conditions such as diabetes are able to initiate the upregulation of GFAP in the whole extent of the cells. In STZ-induced hyperglycemia, GFAP upregulation is readily observed (B), as well as after 3 weeks of diabetes (C). Upregulation may be present even if insulin treatment is applied in chronic diabetes (4 months; E). Reduced GFAP expression is detected in PACAP-treated 3-week acute diabetic retinas (D). Scale bar: 20 μ m. Abbreviations: OLM, outer limiting membrane; ILM, inner limiting membrane.

to the end feet at the ILM, but as the disease progresses, this adduct appears in the radial fibers. This agent is pathogenic for the Müller glia itself; changes in polyamine catabolism could underlie the diabetes-induced accumulation of FDP-lysine. The observed accumulation of these adducts could potentially contribute to Müller cell dysfunction and death during long-term diabetes (Yong et al., 2010). Despite all these unfavorable processes, Müller cells are not among the retinal cell populations undergoing apoptosis early in diabetes (Ali et al., 2008).

During diabetes, hyperglycemia and oxidative stress upregulate VEGF that induces retinal neovascularization, vascular leakage, and macular edema. VEGF may also be expressed in, to a lesser extent, endothelial cells, astrocytes, RPE, and RGCs. However, Müller cells are the major source of VEGF in DR (Arjamaa and Nikinmaa, 2006; Kim et al., 2009b). VEGF plays a leading role in inducing retinal inflammation and vascular leakage that occurs as a consequence of diabetes. The loss of Müller cell-derived VEGF significantly inhibits diabetes-induced vascular leakage and attenuates capillary acellularity. It is possible that VEGF derived from other endothelial and retinal cells contributes to diabetes-induced retinal inflammation and vascular leakage in the presence of Müller cell-derived VEGF, which together may serve as a key to the “pathological threshold” (Wang et al., 2010a). While the endothelial cell-derived VEGF plays a role in the pathogenesis of DR (Huang and Sheibani, 2008), the RPE-derived VEGF rather contributes to the RPE–choriocapillaris interaction and regulates outer

BRB (Hartnett et al., 2003), which may influence the osmotic balance and the nutrient supply of the outer retina.

Diabetes also alters osmotic swelling characteristics of glial cells. Glial cells in diabetic retinas are more sensitive to osmotic stress. The functional K^+ channels in retinal glial cells undergo alterations in the course of experimental diabetic conditions, and these are associated with the changed swelling characteristics of retinal glial cells, which contribute to the development of edema. Retinal edema also develops under ischemic–hypoxic and/or inflammatory conditions. Osmotic glial cell swelling has been linked to the decrease of the main K^+ conductance of the cells and to endogenous formation of arachidonic acid in response to osmotic stress (Pannicke et al., 2006), which may in turn regulate the inflammatory responses. This aspect is critical to retinal integrity, since inflammatory cells to the retina mostly arrive through the ILM, which is formed by the end feet of Müller cells. Indeed, Müller cells form two discreet barriers at two strategic locations. Apart from the aforementioned ILM, the second barrier is formed at the top of the ONL, which is a discontinuous, lace-like structure surrounding the photoreceptor cell bodies. It can be disrupted during diseases such as DR (Omri et al., 2010). The apical processes of Müller cells are attached to each other and to inner segments of the photoreceptor cells that collectively form the outer limiting membrane (OLM). The junction proteins are located in the OLM. Adherens junctions, occludins, and desmosomes have been identified there. OLM could serve as a gate for macromolecule transport into the retina and act as a semipermeable barrier. During DR, the Müller cells are not only swollen, but they lose their occluding power at the OLM level, which leads to cyst formation (Omri et al., 2010).

Since astrocytes are also present in the nerve fiber layer (NFL) and around the capillaries, here, early changes in astrocytes are coincident with inner retinal hypoxia and RGC functional deficits. Therefore, astrocytes may contribute to changes in retinal vasculature and inner retinal dysfunction in diabetes (Ly et al., 2011).

2.1.5.2 Pigment epithelial cells

The inner BRB is formed by tight junctions between vascular endothelial cells, whereas the outer retinal barrier is specific to the eye and consists of the tight junction present in a monolayer epithelium, the RPE cells. The apical side of RPE faces the photoreceptor OSs of the neuroretina, and

the basolateral side lies on Bruch's membrane, which separates the RPE from the fenestrated endothelium of the choriocapillaris (Simo et al., 2010). The RPE is a selective exchange platform between the systemic circulation and the retina, and the subretinal space between RPE and the neuroretina is considered as an immune-privileged site (Xu et al., 2009). Glucose entry into the retina occurs at two major anatomical interfaces: the retinal capillaries and the RPE. These cells form part of the BRB. Glucose entry into the retina through the BRB is mediated by a saturable, facilitated transport process involving glucose transporter 1 (GLUT1), a member of a family of sodium-independent glucose transporter proteins (Kumagai, 1999). It has already been demonstrated that GLUT1 has asymmetrical distribution between the luminal and abluminal membranes (with a 4-to-1 ratio) of the inner BRB in normal rats (Fernandes et al., 2003). Glucose transport in the retinal endothelial cells is mediated by a number of different factors, including hypoxia, hyperglycemia, and cytokines, through a variety of different signaling pathways (DeBosch et al., 2002). Several factors associated with the development and progression of DR, such as hypoxia, VEGF, and IGF-1, have been demonstrated to increase GLUT1 expression and/or glucose transport (DeBosch et al., 2002; Sone et al., 1997).

High blood glucose results in high glucose levels in the retina by transporting more glucose with the GLUT1 between the blood and retina. Changes in retinal endothelial cell GLUT1 expression and glucose transport may have a major impact in providing substrate to the various pathogenic processes. It has also been thought to underlie the development of DR and be the ultimate cause of it (Kumagai, 1999). To protect the intracellular environment from excessive glucose flux and/or diabetes, most tissues downregulate glucose transport in response to elevated extracellular glucose concentration. In STZ-induced diabetic rats, Badr et al. (2000) reported a 50% decrease in total retinal GLUT1 and retinal microvascular GLUT1 after 8 weeks of diabetes. Persistently high glucose levels may induce the proliferation of RPE cells. RPE cells also secrete VEGF, which is believed to play an important role in the neovascularization of the retina. RPE showed deepened hollows in the basal infoldings 1 month after the onset of diabetes. After 6 months, the RPE contained large concavities, which were created by depressions of basal infoldings. Both the microvilli and the basal infoldings were degenerated, which suggests the destruction of the transport pathway between the choroid and the outer retina suggesting a possible breakdown of the BRB (Aizu et al., 2002; Fig. 1.4).

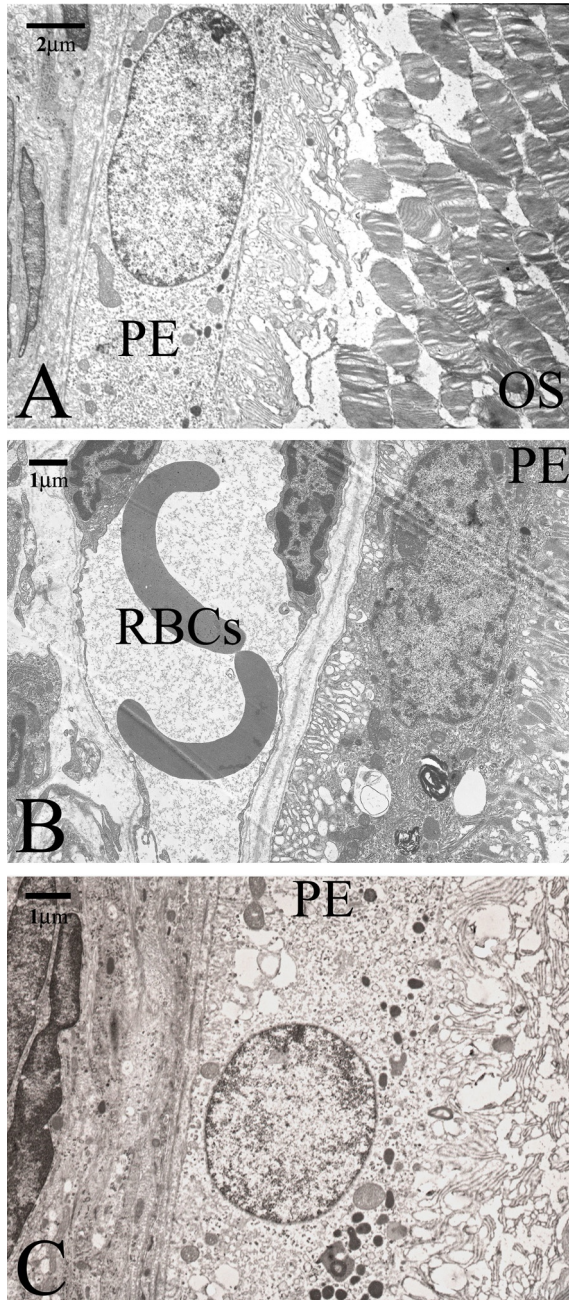


Figure 1.4 Electron micrographs of pigment epithelial cells (RPE) and photoreceptor outer segments (OS) in control, early diabetic (3 weeks) and chronic diabetic (4 months)

2.1.6 Cells migrating to the retina in response to diabetes

DR is a chronic inflammatory disorder; low-grade inflammation has been observed in the retinas of both diabetic animals and human patients (Kern, 2007; Krady et al., 2005; Zeng et al., 2008).

Microglial cells, the main resident sentinel immune cells in the healthy retina, are located in the inner retina, around blood vessels. These cells become activated and migrate in the subretinal space in several retinopathies, including DR (Zeng et al., 2000, 2008). The activation of microglia induced by hyperglycemia has been associated with the early development of DR and occurs as early as electroretinographic modifications (Gaucher et al., 2007; Kern, 2007). Cytokines, among other cells released by activated microglia, were shown to contribute to neuronal cell death (Krady et al., 2005). Activated displaced microglial cells have a pathogenic role in the time course of DR. Indeed, activated microglia produce cytotoxic substances, such as $\text{TNF}\alpha$, ROS, proteases, and excitatory amino acids, which may induce neuronal degeneration. *In vivo*, the time course of diabetes in noninsulin-dependent Goto–Kakizaki (GK) rats is modified by microglia/macrophage trafficking, leading to subretinal accumulation and potential toxicity mediated by these cells. “Pores” in RPE cells are formed at the early stages of DR, when tight junctions between RPE cells are still intact and serve as a migratory pathway for inflammatory cells (microglia/macrophages). This “transcellular” route is also used by leukocytes or lymphocytes for migration through the endothelial cell bodies. The transcellular pathway is established in RPE cells in the following steps: (i) the identification of “tunnel structures” crossing the whole thickness of the cell with preserved tight junctions, (ii) the recruitment of intercellular adhesion molecule 1 (ICAM-1) and caveolin 1 (CAV-1) around the pores and getting protein kinase $\text{C}\zeta$ (PKC ζ) involved in the pore formation, and (iii) the identification of ionized calcium-binding adaptor molecule 1-positive cells (resident microglial cells) crossing the RPE through the pore. The presence of microglia/macrophages in the retina of diabetic GK rat could be related to the expression of ICAM-1, which is

rat retinas. In normal retinas, intact structures of the RPE and the OSs were seen (A). Early diabetic retinas contained abnormal blood vessels with red blood cells, nearby the RPE. The somata of RPE were pycnotic; the membranes between the RPE and choroid are swollen (B). RPE had intact appearance in chronic diabetic retinas, and it seemed like the control retinas; however, their microvilli were missing and several degenerative structures were seen at the junction of the RPE and OS (C). Scale bars: indicated in the images. Abbreviations: OS, outer segment; RPE, pigment epithelium; RBCs, red blood cells.

essential for inflammatory cell migration. At the early stages of diabetes (5 months of hyperglycemia), the RPE transcellular pathway is functional. Later in the course of diabetes (12 months), ICAM-1 and CAV-1 protein expressions in RPE cells decrease and the accumulation of subretinal microglia/macrophages is observed, together with significant decrease in the number of RPE pores. Morphological abnormalities are observed in the outer retina, with RPE vacuolization and loss of junction integrity as well as disorganization of photoreceptor OSs. A migration of cells from the retina toward the choroid could occur through RPE; PKC ζ is a new actor participating in this process (Omri et al., 2011). Via the inhibition of PKC ζ signaling pathway, macrophage survival and deactivation are promoted possibly by changing the intraocular immune environment (Mantovani et al., 2004). PKC ζ is induced by TNF α , suggesting its possible implication in diabetes-induced BRB breakdown (Aveleira et al., 2010).

Leukocyte-mediated retinal cell apoptosis is among the earliest pathological manifestations of DR and results in the formation of acellular-occluded capillaries, microaneurysms, and vascular basement membrane thickening (Engerman and Kern, 1995). The role of VEGF in the development of these diabetic complications in the eye is well established. In human DR, all types of macrophages could be detected regardless of clinical history and duration of the disease. In the eye of a 47-year-old man with simple DR, numerous spherical cells with pseudopodal protrusions were observed throughout the retina. They were identified as macrophages in light, scanning electron and transmission electron microscopic studies. The macrophages were particularly dense and were arranged in a circinate pattern corresponding to circinate lesions within the retina. The macrophages also densely populated retinal areas with hard exudates. In areas adjacent to the macrophages, the inner retinal surface lost its smooth and continuous structure. The matrix in the ILM disappeared in such areas so that a coarse network of filaments lay bare on the retinal surface (Kishi et al., 1982). In the sclera of alloxan-induced diabetic mice, the number of mast cells was significantly increased. No increase in the mast cell number could be observed in the sclera of the obese hyperglycemic mice. The lack of insulin may be of importance for the accumulation of mast cells in the sclera of mice with hyperglycemia (Jansson and Naeser, 1987).

2.2. Molecular alterations

As we have shown in the preceding text, DR is a multifactorial progressive disease where its pathogenesis is extremely complex involving many

different cells, molecules, and factors. Diabetes-induced metabolic changes in the retina induce a range of molecules and pathways involved early in the pathophysiology of DR.

2.2.1 Apoptotic markers

Many kinds of retinal cells undergo accelerated apoptosis before other histopathologic changes are detectable in diabetes. One of the most perplexing biochemical changes in DR is the persistent, slow apoptosis of vascular and neural cells, which has been observed in retinal tissues from humans with diabetes and diabetic animal models. The relationship between apoptoses of vascular and neural cells is unclear, with the possibility that loss of these different classes of the cells occurs over different time frames and by unrelated mechanisms (Barber et al., 2011). Significant neural apoptosis is an earlier event than vascular apoptosis, and the rate of neural apoptosis remains constant throughout the duration of diabetes. Since neurons are unable to proliferate, apoptosis of these cells will result in cumulative loss leading to chronic retinal degeneration (Barber et al., 2011).

In rats, a 10-fold increase in the frequency of the apoptosis was observed compared with control animals after only 1 month of STZ-induced diabetes, continuing with the same frequency for at least 12 months. The vast majority of apoptotic cells appeared to be RGCs (Barber et al., 1998; Lorenzi and Gerhardinger, 2001). Apoptotic events can be revealed by *in situ* DNA terminal dUTP nick-end labeling (TUNEL) or measuring active caspase 3. After only 2 weeks, STZ-induced diabetic rat retinas, besides 2.5-fold more TUNEL-positive nuclei, had the highest density of caspase 3-immunopositive cells compared to the control (Gastinger et al., 2006). Labeling could be found in photoreceptors and dopaminergic amacrine cells as well. Both methods showed significantly more apoptotic cells in the retinal layer in 6-week diabetic mouse retinas, and in addition, electron microscopic analysis revealed cells in the GCL that had the classical morphological characteristics of apoptosis (i.e., disintegrating nuclei) (Martin et al., 2004).

Several studies demonstrated that oxidative stress induced by hyperglycemia is closely linked to apoptosis in a variety of retinal cell types (Abu El-Asrar et al., 2007). This may partly be linked with the changes of capillary structure and distribution. Engerman and Kern (1995) had found a small but significant increase in the number of TUNEL-positive apoptotic cells in the capillaries of postmortem human diabetic retinas (who had diabetes for an average of 10 years) compared with normal retinas. A similarly modest increase in vascular cell death was found in rats after 31 weeks of STZ-induced diabetes. The increase in vascular cell apoptosis suggests a potential

mechanism for the appearance of acellular capillaries given that the endothelial cell bodies and nuclei were thought to disappear while leaving their intact basement membranes behind (Mizutani et al., 1996). Vascular cell dropout can be a response to reduced metabolic demands from the surrounding neurosensory retina, as in photoreceptor degeneration; hence, it is conceivable that vascular apoptosis represents a final response to localized cell death in the surrounding neural tissue (Barber et al., 2011).

2.2.2 Glutamate excitotoxicity-induced apoptosis in diabetic retina

Diabetes may impair glutamate uptake and metabolism resulting in accumulation of extracellular glutamate, leading to excitotoxicity in which excess glutamate stimulation causes an uncontrolled intracellular calcium response in postsynaptic neurons (Laabich and Cooper, 2000). This is primarily true for RGCs, which are known to possess N-methyl-D-aspartate (NMDA) receptors (Thoreson and Witkovsky, 1999). The elevated aspartate immunoreactivity of Müller cells in diabetic rats suggests slower clearance of glutamate, whereas excised Müller cells from STZ-treated rats after 1 month of diabetes demonstrated reduced glutamate transporter activity (Li and Puro, 2002; Puro, 2002; Ward et al., 2005). Diabetes may also alter the regulation of glutamate receptor expression. The combined changes in the glutamate signaling system may ultimately increase apoptosis despite compensatory mechanisms designed to protect neurons, and the resultant imbalance in neurotransmission could also lead to dysfunction in visual signal processing in the retina. Thus, glutamate excitotoxicity suggests a mechanism for not only the increases in cell death but also the altered ERG response and the loss of visual function in diabetes (Barber et al., 2011).

2.2.3 Pathways

Hyperglycemia has been considered the key initiator of retinal damage associated with DR by activation or dysregulation of several (glycolytic, protein kinase C (PKC), polyol, poly-(ADP)-ribose polymerase (PARP), and hexosamine) pathways. All these converge to increase the production of ROS, which induces apoptosis and inflammatory responses and promotes angiogenesis (Ola et al., 2012). It also induces the diacylglycerol (DAG) pathway *de novo*, initiating its synthesis through actions of phospholipase C. The elevated level of DAG has been linked to vascular dysfunctions and pathogenesis of DR (Geraldes and King, 2010). The activity of PKCs is greatly enhanced by DAG, which has been implicated in several cellular

and structural abnormalities occurring in DR (Curtis and Scholfield, 2004; Das Evcimen and King, 2007).

The overexpression of the pro nerve growth factor (NGF) mimics diabetes action resulting in retinal neurodegeneration *in vivo* and *in vitro*. RGCs are more sensitive to death signals in response to proNGF. The direct apoptotic effect of proNGF on RGCs delineates the apoptotic role of RhoA activation in retinal neurodegeneration. The significant activation of RhoA/p38 mitogen-activated protein kinase (MAPK) pathway causes neuronal death with increasing phosphorylation of c-Jun N-terminal kinase (JNK) and p38 MAPK in response to overexpression of proNGF in the diabetic rat retina (Al-Gayyar et al., 2013).

The polyol pathway of glucose metabolism becomes active when intracellular glucose levels are elevated. The polyol pathway is the only mechanism of glucose toxicity currently proved to be responsible for the spectrum of neural, glial, and vascular abnormalities detectable during the development of DR (Dagher et al., 2004). Aldose reductase is the key and rate-limiting enzyme in polyol pathway, and galactose and glucose are substrates to this enzyme. Under diabetic conditions, the increased level of glucose activates this pathway, which causes reduced NADPH level in the cytoplasm. The resulted decrease in glutathione and increased oxidative stress are the major factors of the retinal damage (Barba et al., 2010). The polyol pathway is therefore a rational candidate mechanism for the ganglion cell apoptosis and Müller glial cell activation that occur early in both human and rat diabetes (Lorenzi and Gerhardinger, 2001). Further, the polyol pathway initiates and multiplies several mechanisms of cellular damage by activation of aldose reductase and other pathogenetic factors such as advanced glycosylated end product (AGE), activation of oxidative–nitrosative stress, and PKC pathway that may lead to initiation of inflammation and growth factor imbalances (Obrosova and Kador, 2011). PARP activation in diabetic retinas causes DNA breaks, thus exacerbating oxidative and nitrosative stress (Drel et al., 2009). The progression of axonal atrophy and axoglial disjunction in the optic nerve in 12-month diabetic Bio-Breeding/Worcester (BB/W) rats is a polyol pathway-related mechanism activated by hyperglycemia and galactosemia. Hexosamine has been shown to impair insulin signaling in retina (Nakamura et al., 2001), therefore being also considered as a potential pathway implicated in DR (Ola et al., 2012).

The high glucose and the diabetic state stimulate different pathways to produce excess levels of ROS. It is still unclear whether oxidative stress has a primary role in the pathogenesis of diabetic complication occurring

at an early stage in diabetes or whether it is a consequence of the tissue damage (Fernandes et al., 2011; Izuta et al., 2010). Mitochondria play a key role in the control of apoptotic cell death. Bad is a proapoptotic member of Bcl-2 family that promotes apoptosis by binding to and inhibiting functions of antiapoptotic proteins Bcl-2 and Bcl-xL. Early during the apoptotic process, mitochondria can release several apoptogenic proteins, such as cytochrome *c* and apoptosis-inducing factor (AIF), into the cytosol (van Gurp et al., 2003). Increased ratio of Bax to Bcl-2 occurs early in the DR and plays a role in the apoptosis of retinal cells (Gao et al., 2009b). Retinal mitochondria become leaky during diabetes (8 months). When capillary cell apoptosis is observed, cytochrome *c* starts to accumulate in the cytosol and Bax is secreted into the mitochondria. The inhibition of superoxides in turn decreases the glucose-induced release of cytochrome *c* and Bax and suppresses apoptosis in both endothelial cells and pericytes (Kowluru and Abbas, 2003).

Mitochondria are also the major endogenous source of superoxides and hydroxyl radicals. Reactive oxidant intermediates can trigger mitochondria to release cytochrome *c*, resulting in activation of caspase 3 (Sandbach et al., 2001). Overproduction of superoxides by mitochondria is considered a causal link between elevated glucose and the major biochemical pathways postulated to be involved in the development of diabetic complications (Brownlee, 2001). According to a recent report, mitochondrial structure, mitochondrial function, and mitochondrial DNA (mtDNA) are damaged in the diabetic retina and its vasculature, and the mtDNA repair machinery is also compromised. Diabetes also facilitates epigenetic modifications, which contribute to the mitochondria damage. Diabetes-induced abnormalities in mitochondria continue even when the hyperglycemic insult is terminated and are implicated in the metabolic memory phenomenon associated with the continued progression of DR (Kowluru, 2013).

An increase in peroxynitrite as indicated by tyrosine nitration correlates with accelerated retinal endothelial cell death, breakdown of the BRB, and accelerated neuronal cell death in experimental models of diabetes, inflammation, and neurotoxicity (Ali et al., 2008; Du et al., 2002; El-Remessy et al., 2003a,b, 2005). Peroxynitrite plays a key role in mediating different aspects of DR. In response to hyperglycemia-induced oxidative stress, both microglial and macroglial cells are activated, and the function of macroglia in transporting glutamate by glutamate transporters and in metabolizing glutamate by GS may be impaired (Li and Puro, 2002; Lieth et al., 1998, 2000). This may lead to glutamate accumulation, such as that reported in the vitreous humor of diabetic patients (Ambati et al., 1997) and in the retina of

diabetic animals (Kowluru et al., 2001; Lieth et al., 1998). GS is susceptible to tyrosine nitration, which subsequently can impair the enzyme activity (Gorg et al., 2005, 2007). This vicious cycle of glial dysfunction will result in cell death and the injury of adjacent retinal neurons.

2.2.4 Metabolic end products

Glycation is an important event that occurs in physiological and pathophysiological circumstances. Acute hyperglycemia and chronic hyperglycemia are known to enhance early, intermediate, and advanced glycation. The deposition of hyperglycemia-induced AGEs in retinal blood vessels plays an important role in the onset and development of DR *in vivo*. AGEs are late products of nonenzymatic glycation. The pathogenic significance of ALEs in DR is less well known (Shanmugam et al., 2008; Stitt et al., 2004) than those of AGEs (Denis et al., 2002). AGEs/ALEs can form on the amino groups of proteins, lipids, and DNA through a number of complex pathways including nonenzymatic glycation by glucose and reaction with metabolic intermediates and reactive dicarbonyl intermediates. AGEs/ALEs are known to accumulate in the diabetic retina where they are thought to influence retinal vascular cell function (Stitt et al., 2004). A possible pathway of oxidative stress in the development of DR that may contribute to retinal cell dysfunction and degenerative changes is the formation of ALEs. ALEs may induce a variety of cytopathologic effects, including cross-linking of cell surface proteins, inactivation of enzymes, and stimulation of proinflammatory and proapoptotic signaling pathways.

During the pathological action, AGEs promote the upregulation of SLIT-ROBO signaling, which acts as a cue in neuronal guidance during development and plays a role in vasculogenesis and angiogenesis throughout life. Hereby, it may actively participate in the progression of DR (Zhou et al., 2011). The SLIT-ROBO signaling cascade includes Slit family of secreted proteins (Slit1, 2, and 3) and their corresponding receptors (Robo1, 2, 3, and 4). Robo1 and 4 are involved in retinal vasculogenesis and angiogenesis (Huang et al., 2009a,b, 2010) and are expressed in the choroid and the retinal endothelial and RPE cells. RPE cells autosecrete Slit2, which then binds the single-pass transmembrane receptor, Robo1, under conditions of DR. AGEs promote the upregulation in Robo1 and Slit2 expressions, which may be involved in the pathological action of AGE. N-Slit2 (the recombinant protein) results in increased proliferation, attachment, and migration of RPE cells, which increases VEGF mRNA expression and VEGF secretion (Zhou et al., 2011).

AGEs initiate the onset of DR through both receptor- and nonreceptor-mediated pathways. AGE cross-linking causes proteins that are normally flexible to become rigid, resulting in their dysfunction. The binding of AGEs with receptors in macrophages and endothelial cells initiates a release of a large number of cytokines, inducing endothelial cell proliferation and damaging retinal blood vessels (Qazi et al., 2009). Glucose irreversibly modifies long-lived macromolecules by forming AGEs during periods of normal glucose homeostasis, which may explain the phenomenon of hyperglycemic memory. AGEs cause qualitative and quantitative changes in extracellular matrix components such as collagen and laminin and can affect cell adhesion and growth and matrix accumulation, thus altering cell function. AGEs can attack the nucleus directly and block DNA synthesis, thus interfering with the proliferation of pericytes. AGEs disturb microvascular homeostasis through interaction with the receptor of AGEs (RAGE). This AGE–RAGE axis plays a central role in the inflammation, neurodegeneration, and microvascular dysfunction in DR (Zong et al., 2011).

Increased AGEs formation and accumulation have been found in retinal blood vessels and vitreous of diabetic patients and animals, which correlate with a degree of DR (Goh and Cooper, 2008). Retinal pericytes accumulate AGEs during diabetes, which are implicated in endothelial cell injury and BRB dysfunction (Stitt et al., 2000). AGEs increase VEGF, monocyte chemoattractant protein 1, and ICAM-1 expressions in microvascular endothelial cells through increased intracellular ROS generation causing apoptosis of pericytes and other retinal cells (Ibrahim et al., 2011; Yamagishi et al., 2008a,b). Luo et al. (2012) found no significant differences in the amount of AGEs between 3- and 6-month STZ-induced diabetic rats, suggesting that AGE deposition in the retinal blood vessels occurs prior to obvious morphological changes in DR; thus, the damage to blood vessels by AGEs is a chronic process. The degree of AGE deposition was negatively correlated with pericyte number, but positively correlated with endothelial cell number, suggesting that AGEs damage pericytes in a cumulative manner. Without pericyte support, retinal capillaries could expand to form microaneurysms and acellular capillaries, which are closely linked with nonperfusion areas and could potentially lead to the onset and development of proliferative DR (Luo et al., 2012).

2.3. Electrophysiological changes

Tissue injuries and neurochemical changes of the retina are reflected in electrophysiological changes at all levels of visual information processing. Two

such electrophysiological recordings are practiced routinely: ERG that reflects retinal functions and VEP that shows cortical processes.

The ERG recordings had long been believed to be useless for the detection of early DR, because the classical ERG components at that time, such as the a and b waves, had been proven to be normal at early stages of DR. Only after introducing better recording techniques (Galloway et al., 1971) did it become possible to detect fine differences between signals derived from healthy and diseased tissues. The a wave arises principally from photoreceptors, whereas the b wave originates mainly from ON bipolar cells. OPs represent the contribution of certain amacrine cells. Abnormal ERG in diabetic rats suggests the dysfunction of the retinal neuronal processing and reflects deterioration of function (Li et al., 2002). ERG deficit was found as early as 2 weeks after induction of diabetes indicating that the functional integrity of the diabetic retina had been compromised (Li et al., 2002).

After the first clinically validated change of the OPs, they were widely accepted as one of the earliest manifestations of DR (Galloway et al., 1971). The OP generator is presumed to lie in the intraretinal feedback neuronal circuitry, which consist of the interneurons. The disturbance of dopamine metabolism in the retina may play a role in the OP abnormality in diabetes. Morphological changes of the neural cells are seen 10 weeks after the induction of diabetes in mice and rats. This suggested that the alterations of the OPs probably represent metabolic disturbances affecting inner retinal function at the early stages of DR: the depletion of dopamine and abnormal dopamine metabolism (Gibson, 1988). In STZ-induced diabetic rats, the OPs have significantly prolonged implicit times and reduced amplitudes as early as 2 weeks following the induction of hyperglycemia (Hancock and Kraft, 2004). These abnormalities are restored by the administration of insulin, suggesting that the abnormal OPs seen in early DR represent only metabolic and/or functional changes of the neurons. During 6 weeks of diabetes, the peak latency of the OPs was prolonged (Shirao and Kawasaki, 1998).

The amplitudes of a and b waves and OP1, OP2 and OP3 of ERG were reduced in rats 1 month after the onset of diabetes. The peak latencies of a and b waves were not delayed, but the OP2 and OP3 were (Aizu et al., 2002). The RGC-dominated positive scotopic threshold response was reduced following a single episode of acute IOP elevation in STZ-induced diabetic rats, but not in control rats after 11 weeks of diabetes. These data indicate that hyperglycemia renders the inner retina more susceptible (Kohzaki et al., 2012). The peak latency of the OPs is one of the most sensitive markers for the early phase of DR, while in later phases, the intensity

response curves of the ERG b wave of diabetic rats during the 25 weeks' diabetic period were shifted to higher intensities. This, along with significant reductions in the maximal amplitude of the b wave, means reduced sensitivity. The maximal amplitude of the b wave was more reduced than the corresponding parameter of the a wave. These observations suggest that the photoreceptors are relatively resistant to the pathological processes in early diabetes (Li et al., 2002; Samuels et al., 2012).

The loss of amacrine cells may play a role in the reduction of the OPs in animals and humans with diabetes (Dong et al., 2004). The source of abnormal or missing OPs may be from the altered synaptic activity between amacrine and bipolar cells or RGCs. These deficits could be explained by the degeneration in synaptic neurotransmission and/or combined loss of amacrine neurons and RGCs (Kern and Barber, 2008). The function of RGCs is compromised when there is a loss of dopaminergic or cholinergic signaling in the retina (Amthor et al., 2002). Loss of dopaminergic and cholinergic neurons may cause changes to visual processing that play a role in the vision loss associated with diabetes (Gastinger et al., 2006). The significantly prolonged peak latency of the OPs in the ERG and the increased VEGF level are correlated with the increased serum AGE concentration (Segawa et al., 1998).

In diabetic patients, a functional loss of the inner retina has been detected by ERG and VEP recordings before vasculopathy (Moreo et al., 1995). Spontaneously, diabetic BB/W rats develop central sensory neuropathy, characterized functionally by modification of VEP and structurally by dystrophy in the RGCs and optic nerve fibers without visible vascular changes. The amplitude alterations of ERG and VEP begin 1 week after STZ injection in rats and progress week by week reaching a stable reduction by the end of the first month of diabetes. The b wave and VEP latency changes, however, were significant only after 1 month of diabetes (Biro et al., 1998). Similar VEP latency changes appeared to be parallel with RGC dystrophy and optic nerve axonopathy in 6-month diabetic BB/W rats (Kamijo et al., 1993).



3. EXPERIMENTAL MODELS OF DIABETIC RETINOPATHY

This field became a major focus area in the recent years. Journals and academic departments devoted to target this area of research exclusively. Experimental models of any kind of human disorder undergo vigorous evaluation from time to time. DR is no exception from the rule. In this section,

besides reviewing scientific achievements in the field, this aspect is put to the forefront. To understand the pathophysiological mechanisms of diseases and to test the pharmacological effects of new drugs, animal models for the diseases have been developed and widely utilized. These efforts called to life a new scientific discipline, translational medicine.

3.1. Type 1 diabetes

Due to the early discovery of easy inducibility of this disorder (Szkudelski, 2001), an enormous body of data has accumulated over the years. Therefore, most of the solid knowledge on diabetes has been gained through this approach.

3.1.1 Chemical induction

Alloxan and STZ are widely used as diabetogenic agents in experimental animal models that include rodents, dogs, and primates (Szkudelski, 2001). This approach replicates some of the early symptoms of DR and has the advantage that the onset of diabetes can be defined as the time of injection of the toxin. However, toxin-induced diabetes in mice has been less successful because of strain-dependent resistance to STZ (Rossini et al., 1977).

3.1.1.1 Alloxan

Alloxan is synthesized by uric acid oxidation and exerts its diabetogenic action by intravenous, intraperitoneal, or subcutaneous administration. Alloxan evokes insulin release for a short duration inducing complete suppression of the islet response to glucose. The mechanism of alloxan action is through the formation of ROS, and in the pancreas, it is preceded by rapid uptake by the β cells (Weaver et al., 1978). Alloxan elevates cytosolic free Ca^{2+} concentrations in pancreatic β cells (Park et al., 1995) and also targets DNA; its fragmentation takes place in β cells exposed to alloxan (Sakurai and Ogiso, 1995).

3.1.1.2 Streptozotocin

STZ is synthesized by *Streptomyces achromogenes* and is used to induce insulin-dependent diabetes mellitus (Szkudelski, 2001). STZ-induced diabetic rats are useful models of human type 1 (insulin-dependent) diabetes mellitus with hyperglycemia. STZ action in β cells is accompanied by characteristic alterations in blood insulin and glucose concentrations. This is reflected in the abnormalities in β cell function. STZ is taken up by pancreatic β cells via the glucose transporter GLUT2. It impairs glucose oxidation and decreases

insulin biosynthesis and secretion. At first, it abolishes the β cell response to glucose. Intracellular action results in changes in DNA in pancreatic β cells comprising its fragmentation and alkylation (Elsner et al., 2000). Some ancillary factors are involved in the process of the STZ effects: nitric oxide, ROS, and superoxide anions. Inhibition of the Krebs cycle and limited mitochondrial ATP production activate poly-ADP ribosylation (Sandler and Swenne, 1983) and are also characteristic of this condition.

Although STZ-injected rats provide an intensively studied model of diabetes, this is not identical to the clinical situation. One reason for this is that uncontrolled hyperglycemia is not typical in patients. Also, of course, rats may respond differentially to hyperglycemia and hypoinsulinemia than humans. Additionally, a chemically induced loss of β cell function is very rare in humans (Puro, 2002). Still, the most widely accepted animal model for the evaluation of retinal complication of diabetes is the STZ-induced diabetic rats. It has been shown that in STZ-treated rats, significantly more cells undergo apoptosis, resulting in cell loss in the retinas of diabetic rats and not in control animals (Asnaghi et al., 2003; Barber et al., 1998). However, some typical clinical symptoms (neovascularization and macular edema) could not be detected in rats.

During the preclinical phase of diabetes drug research, it is indispensable to perform experiments on a reliable animal model to mimic diabetes-related complications. Recently, several reviews have been published summarizing the existing methods for the investigation of diabetic eye disorders in rodents (Islam, 2013; Jo et al., 2013; Kong et al., 2013; Robinson et al., 2012). Nowadays, a number of diabetes models are well known and widely used, but only a few of them offer a thorough examination of the diabetic manifestations in their complexity. Such diabetic state should be extended for a time interval, long enough to allow the formation of accurately detectable functional and morphological diabetes-related complications in various severities. However, the main limitation of the currently available diabetes models is that the experimental subjects cannot tolerate the severe general conditions in the absence of any antidiabetic therapy (Fox et al., 1999). Therefore, it is essential to apply an experimental design enabling tolerable general states of the animals. Maintenance of a suboptimal glycemic control mimics the diabetic conditions and permits the development of chronic complications. Our group has recently developed a novel chronic, insulin-controlled method for the investigation of diabetic ocular changes. Diabetes was induced by single-dose STZ injection and maintained for 12 or 16 weeks in male Sprague–Dawley rats. Diabetic rats were divided into four

groups according to the different doses of insulin replacement with subcutaneously applied implants. In groups where blood glucose levels were between 5–10 mmol/l and around 20 mmol/l, resulting in mild and medium severe forms of diabetes, we found only minimal functional and histopathologic changes in the eye. When the blood glucose concentration was over 30 mmol/l, severe pathophysiological signs developed. Marked corneal neovascularization, retinal degeneration, and cataract formation were observed. This chronic, insulin-controlled animal model is available for the preclinical studies of promising drug candidates against diabetic complications (Hajna et al., 2014).

3.1.2 Genetic defects

Using the advantages of the widespread controlled breeding of laboratory animals (primarily rats and mice), a number of inbred strains were selected with metabolic deficiencies, several of those bearing the hallmarks of diabetes.

3.1.2.1 BB/W rats

The BB/W Wistar rat develops autoimmune diabetes similar to type 1 human diabetes mellitus. The syndrome is characterized by sudden onset of hyperglycemia, ketonemia, and pancreatic β cell destruction; therefore, BB/W rat strain is a model of type 1 diabetes. In these animals, diabetes develops spontaneously. It is an excellent laboratory model of type 1 juvenile-onset diabetes mellitus from both a metabolic point of view and an immunological point of view. The diabetic syndrome of these rats spans a spectrum of increasing severity from insulinitis without glucoregulatory changes to insulin-dependent diabetes with massive β cell destruction. Obesity is absent, both sexes are affected, and peak incidence of diabetes occurs around the age of sexual maturation (80–100 days). Sublines with expected zero or high diabetes incidence (40–100% overt type 1 diabetes) have been developed. However, these animals have never been examined extensively for DR syndromes. It remains to be shown that these animals develop DR at all (Yale and Marliss, 1984).

3.2. Type 2 diabetes

Type 2 diabetes is a complex, heterogeneous, polygenic disease characterized mainly by insulin resistance and pancreatic β cell dysfunction. Appropriate experimental models are essential tools for understanding the molecular basis, pathogenesis of the vascular and neural lesions, actions of therapeutic agents, and genetic or environmental influences that increase

the risks of type 2 diabetes. In contrast to type 1 diabetes, where rats are used most often, there are several mouse models of type 2 diabetes too.

3.2.1 *OLETF rats*

A spontaneously diabetic rat with polyuria, polydipsia, and mild obesity was discovered in 1984 in an outbred colony of Long–Evans rats. A strain of rats developed from this rat by selective breeding has since been maintained at the Tokushima Research Institute and named Otsuka Long–Evans Tokushima Fatty (OLETF) strain. They became a widely used model of noninsulin-dependent diabetes. The characteristic features of OLETF rats are late onset of hyperglycemia (after 18 weeks of age), a chronic course of disease, mild obesity, inheritance by males, hyperplastic foci of pancreatic islets, and renal complication (nodular lesions). Histologically, the changes in pancreatic islets can be classified into three stages: (1) an early stage (6–20 weeks of age) of cellular infiltration and degeneration, (2) a hyperplastic stage (20–40 weeks of age), and (3) a final stage (at >40 weeks of age). These clinical and pathological features of disease in OLETF rats resemble those of human noninsulin-dependent diabetes mellitus. Architectural defects of the islet capillaries can cause impaired β cell function in aged OLETF rats (Mizuno et al., 1999).

OLETF rats exhibited a significantly reduced total retina thickness, especially that of the NFL at 28, 36, and 40 weeks of age. Histological examination revealed increased apoptosis (active caspase 3-positive and TUNEL-positive cells in NFL) and a decrease in the number of RGCs (Yang et al., 2013a). GFAP immunofluorescence staining was upregulated in vertical sections and showed a more ramified pattern in whole-mount retinas. VEGF expression extended into the OPL (Jung et al., 2013). The INL decreased from 3–4 rows to 2 rows, whereas the photoreceptor cell nuclei decreased from 8 rows to 3–6 rows. These results suggest that retinal neurodegeneration occurs in type 2 diabetic OLETF rats. RPE decreased in height, and basal infoldings were poorly developed. Retinal capillary basement membranes were significantly thicker in the OLETF rats than in the Long–Evans Tokushima Otsuka (LETO) rats, and endothelial cell damage was observed (Lu et al., 2003). In the 64-week-old OLETF rats without treatment, corrosion cast revealed diabetic retinal and choroidal vascular changes: tortuosity of the vessels, variations in caliber, narrowing of arteries, arterio-arterial anastomoses and hairpin loop formation in precapillary arterioles, sparse collecting venules in the choroid, and marked capillary changes such as caliber irregularity, narrowing, tortuosity, loop formation and

decreased capillaries, outpouching, and microaneurysms (Bhutto et al., 2002). However, they are not the most suitable animal model for the study of angiopathic DR because retinal capillaries of OLETF rats were found to remain morphologically normal and pericyte ghosts were barely detectable. There was no difference in the number of acellular capillaries in the retinas between OLETF and control LETO rats. Formation of acellular capillaries and pericyte ghosts, the characteristic morphological changes in early DR, is not accelerated in OLETF rats (Matsuura et al., 2005).

3.2.2 Zucker diabetic fatty rats

The Zucker diabetic fatty (ZDF) rat is most widely used for studying type 2 diabetes associated with obesity. The initial characterization of a novel type 2 diabetes model derived from Zucker fatty (ZF) rats. Further inbreeding resulted in new phenotypes.

Zucker fatty diabetes mellitus rats develop diabetes as early as 10 weeks of age, which reaches 100% incidence at around 20 weeks of age. This rat strain possesses high reproductive efficiency and therefore should serve as a useful model of young- to middle-aged adult-onset type 2 diabetes in the studies of the pathophysiology, therapeutic interventions, and complications of the disease (Yokoi et al., 2013).

Retinal VEGF mRNA and protein expression increased in ZDF rats after 2 months of disease, but not in ZF rats. Some oxidative stress and inflammatory markers (TNF α , IL-6, ICAM-1, and IL-1 β) were upregulated in the retina of ZDF rats after 4 months of the disease. In contrast, activation of nuclear factor κ B (NF- κ B) in the retina was observed in ZF and ZDF rats (Mima et al., 2012). ZDF rats had thicker basement membranes and more cells per unit capillary length (Yang et al., 2000). Diabetes increased anticellular activated caspase 3- and TUNEL-positive microvascular cell numbers and acellular capillary formation were reduced in ZDF rats (Behl et al., 2008). Acute hyperglycemia did not have an effect on control rats, while chronic hyperglycemia in ZDF rats was associated with enhanced scotopic ERG amplitudes, which were up to 20% higher than those of age-matched controls (8–22 weeks of age), and a reduction in a wave amplitudes and maximum slopes of about 30%. The electrophysiological differences between untreated ZDF rats and controls preceded an activation of Müller cells in the ZDF rats (upregulation of GFAP), which was attenuated by insulin treatment. There were otherwise no signs of cell death or morphological alterations in ZDF groups (Johnson et al., 2013). These data show that under chronic hyperglycemia, the ZDF rat retina became abnormally sensitive to

variations in substrate supply. In diabetes, a similar inability to cope with intensive glucose lowering could render the retina susceptible to damage.

3.2.3 Spontaneously diabetic Torii rats

A new spontaneously diabetic strain of the Sprague–Dawley rat was established in 1997 and named the spontaneously diabetic Torii (SDT) rats. The SDT rat strain should be a useful model to understand the pathology of the diabetic disease. An SDT rat spontaneously develops hyperglycemia resulting from a defect of insulin secretion due to tissue damage and dysfunction of the pancreatic islets. Glucose tolerance in SDT rats was impaired in correspondence with the decreased insulin secretion. Diabetes in SDT rats directly results from absolute decrease of insulin secreted from pancreatic β cell. The body mass of the SDT rats decreased after the onset of hyperglycemia, but the SDT rats did not show obesity throughout their lives. The SDT rats exhibited reduced pancreatic insulin content at 20 weeks and a decreased mass of β cell at 10 weeks. The β cells of the SDT rats are in the sense of “overworking” to fulfill the insulin demand of the peripheral tissues with a limited number of β cells and increased body mass before the onset of diabetes. Males spontaneously develop hyperglycemia predominantly due to an insulin secretory defect resulting from pathological damage to the pancreatic islets (Masuyama et al., 2004). Glucose tolerance in SDT rats was impaired in correspondence with the decreased insulin secretion. The age of onset of diabetes in SDT rats depends on the magnitude of glucose intolerance observed prior to the onset of diabetes (Masuyama et al., 2003). Inflammation could be observed in the SDT rats, such as lymphocyte infiltration consistently observed in autoimmune diabetes (Komeda et al., 1998; Like et al., 1982). Male SDT rats spontaneously develop hyperglycemia without obesity after 20 weeks of age with an incident rate reaching 100% at 40 weeks of age. At 38 weeks, almost all the β cells disappear from the pancreatic islets of SDT rats that show overt hyperglycemia and marked hypoinsulinemia accompanied by decreased body weight and body mass index (Masuyama et al., 2004). SDT rats develop severe diabetic ocular complications such as cataract and proliferative retinopathy (by 40 weeks of age) and tractional retinal detachment with fibrous proliferation (by 70 weeks of age) and massive hemorrhaging in the anterior chamber (by 77 weeks of age), which resemble human diabetic ocular complications (Shinohara et al., 2000). The SDT rat is a useful animal model to elucidate the underlying mechanism of nonobese type 2 diabetes mellitus where the pancreatic islet tissue is thought to play the main role in the development of diabetes.

The SDT rat is considered to be a potentially useful model for studies of DR encountered in humans (Shinohara et al., 2000). This notion is supported by the fact that large retinal folds mimicking diabetic tractional retinal detachment were observed in SDT rat retinas like retinal hemorrhages, a neovascular fibrous membrane around the iris, a massive anterior chamber hemorrhage, an area of nonperfusion and/or extensive hyperfluorescence, acellular capillaries and pericyte loss, and retinal changes (Takehashi et al., 2006). Apoptotic cells in the GCL and the INL were numerous in 40-week-old SDT rat retinas; GFAP immunoreactivity spanned the whole retina in SDT rats. The perivascular AQPs shifted from AQP4 to AQP1 in 40-week-old SDT rats that exhibited marked hyperglycemia. Thus, the development of diabetes increases neuroretinal apoptosis and coincides with an altered expression pattern of GFAP and water-selective channels AQP1 and AQP4 in SDT rats (Fukuda et al., 2010). In 44-week-old animals, a and b waves and the OPs were significantly reduced with prolonged implicit times in the SDT rats compared with Sprague–Dawley rats (Okuno et al., 2008). The depressed ERG may reflect vascular and neuronal damage throughout the retina as are seen in the advanced stages of human DR. Further, in SDT rat, hyperglycemia-induced abnormal retinal vascular bed was formed and the optic disc protruded (Sasase et al., 2009). Thus, the SDT rat can be used to study the physiology of DR.

3.2.4 Goto-Kakizaki rats

The GK rat is a spontaneous model of noninsulin-dependent diabetes mellitus without obesity, which was developed by repeated selective breeding of normal Wistar rats using glucose intolerance as a selection index (Goto and Kakizaki, 1981). Glucose intolerance appears after 2 weeks of age (Goto and Kakizaki, 1981) and a significant hyperglycemia is found as early as 4 weeks of age, and the animals show hyperinsulinemia and decreased pancreatic insulin stores. Development of type 2 diabetes mellitus in the GK model results from the complex interaction of multiple events such as (i) the presence of several susceptibility loci containing genes responsible for some diabetic traits, (ii) gestational metabolic impairment inducing an epigenetic programming of the offspring pancreas and the major insulin target tissues, and (iii) environmentally induced loss of β cell differentiation due to chronic exposure to hyperglycemia/hyperlipidemia, inflammation, and oxidative stress (Portha et al., 2012). The adult GK body weight is 10–30% lower than that of age- and sex-matched control animals. Signs of early neuropathy (2 months) have been reported in GK adult rats, while

nephropathy and retinopathy develop late (12 months; [Portha, 2005](#)). In the NFL, RPE, and choroid, strong VEGF immunoreactivity was noted only in the GK rat. Increased VEGF production in certain ocular tissue, similar to that in humans, is observed quite early, at least before the appearance of observable retinal changes in the diabetic GK rat ([Sone et al., 1997](#)).

GK rats do not exhibit cataracts for a long time because of their moderate diabetic state. However, they provide a useful model for the investigation of the retinal microcirculatory changes of diabetes mellitus over an extended time, since they show decreased retinal blood flow with reduced oxygen consumption without serious retinopathy; at the same time, decreased retinal metabolism, increased blood viscosity, and increased vascular resistance to flow can be detected in them ([Miyamoto et al., 1996](#)).

In the GK rats, immunoreactivity to L-glutamate and GABA was observed in the Müller and photoreceptor cells in addition to the immunoreactivity in normal rats. These immunoreactivity patterns in the GK rat retina might be induced by ischemia associated with diabetes mellitus ([Takeo-Goto et al., 2002](#)). Morphological changes could be demonstrated in retinal vessel preparations of GK rats; the endothelial/pericyte ratio was found to be higher in GK rats aged 8 months and after 24–30 months compared with their matched controls. Furthermore, in 24- to 30-month-old GK rats, the endothelial/pericyte ratio was higher than in 8-month-old GK rats ([Agardh et al., 1997](#)). *In vivo* studies indicated that in GK rats, the BRB permeability was increased; *ex vivo* studies showed that in retinas from GK rats, NOS activity was also higher; there is an increased production of NO, which may contribute to the BRB breakdown ([Carmo et al., 2000](#)).

The amplitudes of ERG a and b waves and also the OPs of the GK rats were reduced between 4 and 48 weeks of age. The a wave latencies in GK rats were significantly prolonged, but not the implicit times of OPs. At 14 days of age, the a wave amplitudes were significantly smaller in GK rats than in Wistar rats. Functional abnormalities of photoreceptors might be induced by inheritable degeneration at an early age in the GK rat. Although hyperglycemia would cause retinal hypoxia, it would not be severe enough to disturb the generation of OPs ([Matsubara et al., 2006](#)). Taken together, the GK rat appears to be a suitable model for experimental studies of chronic complications of diabetes, including DR. These results also suggest that the GK rat can be used as a model of initial-phase or latent-phase DR.

3.2.5 *ob/ob* mice

ob/ob mice are hyperphagic, obese, hyperinsulinemic, and hyperglycemic, so they are used as a model for diabetes and obesity. *ob/ob* mice are

indistinguishable from their lean littermates at birth, but later, they become heavier and develop hyperinsulinemia. These differences are much more pronounced after weaning, and overt hyperglycemia is observed during the fourth week. The blood glucose rises to reach the peak after 3–5 months when the mice also have very high food intake and a rapid growth. After that, blood glucose values decrease and eventually become nearly normal at old age. The animals remain insulin-resistant, but impaired glucose tolerance and glycosuria after a glucose load are observed mostly in the postweaning period of rapid growth, and this usually becomes normalized when the mice get older (Lindstrom, 2007). In ob/ob mice, insulin receptor, insulin receptor substrate 1, and insulin receptor substrate 2 proteins and phosphorylation were maintained or increased, while protein levels and phosphorylation of pyruvate dehydrogenase kinase and Akt were decreased in the retina. Interestingly, phosphorylations of p38 MAPK and extracellular signal-regulated kinase 1 (ERK1) were responsive to insulin in the retina. At the same time, hypoxia-inducible factor (HIF)-1 α and VEGF were increased and endothelial NOS was decreased there (Kondo and Kahn, 2004).

3.2.6 db/db mice

The db/db mice are perfect animal models of type 2 diabetes. The phenotypes of severe obesity, hyperphagia, polydipsia, and polyuria are due to a spontaneous mutation of leptin receptor. The db/db mice are differentiated by the spontaneous mutations in different sites of leptin receptors. The db/db mice have abnormal phenotypes in their metabolic, reproductive, and immune systems (Wua et al., 2013). The genetically diabetic db/db mouse is a model of type 2 diabetes, where nephropathy and neuropathy, but not retinopathy, were observed in early examinations. Midena and coworkers (1989) showed a marked increase in the ratio of endothelial cells to intramural pericytes in diabetic mice compared with controls. This increase resulted from a selective and highly significant loss of pericytes in db/db mice. Some strand-like and relatively acellular capillaries were also observed. Basement membrane thickening and an accumulation of basement membrane material in the capillaries of the OPL of retinas from diabetic db/db mice were observed. When the thickness of the whole retina, in particular the INL and ONL, and the integrity of the RPE and the RGC numbers were examined, all were found decreased in db/db mice (Tang et al., 2011). At the same time, expression levels of Nox4 and VEGF were significantly increased in their retinas (Li et al., 2010).

In 15-month-old db/db mice, signs of DR, including BRB breakdown, loss of pericytes, neuroretinal apoptosis, glial reactivation, and proliferation

of blood vessels, were evident. These changes in the diabetic retina were associated with increased expression of aldose reductase (Cheung et al., 2005). The db/db mice at the age of 19 weeks exhibited significantly increased retinal vascular leakage and decreased tight junction protein level in the retina. Moreover, the expression of proinflammatory factors, for example, ICAM-1 and TNF α , was drastically upregulated in diabetic retina (Li et al., 2009).

The altered visual functions of RGCs were characterized by the reduced receptive field center size, elevated luminance response, and attenuated contrast gain in 12- and 20-week db/db mice, respectively. These altered visual functions could, at least partly, be due to oxidative stress (Xiao et al., 2012a). Therefore, the db/db mouse may represent an adequate model for studies on the pathogenesis of DR.

3.2.7 *Ins2^{Akita}* mice

An autosomal-dominant mutation that produces juvenile-onset hyperglycemia and insulinopenia in the absence of obesity was discovered in C57BL/6 mice. The *Ins2^{Akita}* mutation disrupts normal insulin processing and causes a failure in secretion of mature insulins, which results in the early development of hyperglycemia (Mathews et al., 2002). The *Ins2^{Akita}* mutation results in a single amino acid substitution in the insulin 2 gene and replaces a cysteine with tyrosine at the seventh amino acid of the A chain of the insulin 2 gene product, blocking the formation of an essential disulfide bond between the A and B chains of the mature protein, that causes misfolding of the insulin protein. The mutation arose and is maintained on the C57BL/6J background. Male mice heterozygous for this mutation have progressive loss of β cell function and decreased density with significant hyperglycemia as early as 4 weeks of age (Barber et al., 2005).

As we have mentioned earlier, dopaminergic and cholinergic amacrine cells are lost during the early stages of retinal neuropathy in diabetes. Loss of these neurons may play a critical role in the development of visual deficits (Gastinger et al., 2006). Within the first 3 months of diabetes, RGCs are lost from the peripheral retina of *Ins2^{Akita}* mice, and the dendrites of surviving large ON-type cells undergo morphological changes. These abnormalities may explain some of the early anomalies in visual function induced by diabetes (Gastinger et al., 2008). *Ins2^{Akita}* diabetic models are characterized by upregulation of α -, β -, and γ -crystallins in the retina. Despite being over-expressed, the molecular properties of α -crystallins are disrupted by diabetes and contribute to the loss of neuroprotective function. Identification and

prevention of these alterations could lead to the emergence of new therapies for DR (Losiewicz and Fort, 2011). Although both choroidal and retinal blood flow and vision were altered after prolonged diabetes in the $\text{Ins2}^{\text{Akita}}$ mouse, choroidal blood flow was reduced early, suggesting that ocular blood flow deficit could be an early pathological change in DR (Muir et al., 2012).

Diabetes increases retinal vascular permeability in mice. The number of leukocytes adherent to the vascular wall was significantly elevated in the $\text{Ins2}^{\text{Akita}}$ mouse, confirming that the vascular inflammatory component of DR is present in this model. Astrocytes in some regions of the $\text{Ins2}^{\text{Akita}}$ mouse retinas contained reduced GFAP immunoreactivity with shorter processes, suggesting atrophy or loss of contact with blood vessels, but increased GFAP expression was not observed. Regions of the $\text{Ins2}^{\text{Akita}}$ mouse diabetic retinas contained microglia with swollen and contracted processes, suggesting a reactive state. Thinning of the inner layers of the retina suggests that chronic degeneration occurs after 22 weeks of hyperglycemia. The significantly reduced thickness of the peripheral INL suggests loss of horizontal, bipolar, and amacrine cell bodies. The thickness of the IPL was significantly reduced in all regions measured, indicating atrophy of the processes between neurons in all parts of the retina. There was no significant loss of the ONL and the OSs of the photoreceptors, suggesting that the degeneration predominantly occurs in the inner retina. Increased apoptosis was found in $\text{Ins2}^{\text{Akita}}$ mice after 4 weeks of hyperglycemia (Barber et al., 2005). No retinal thinning or disruption of retinal architecture was observed by optical coherence tomography or resin histology up to 6 months of age. In addition, no vascular changes were detected by fluorescein angiography or by scanning laser ophthalmoscopy. With the exception of microglial activation, reduced GFAP expression in astrocytes, and an increase in GFAP expression by Müller cells, no other changes were observed in the $\text{Ins2}^{\text{Akita}}$ mouse retina. These observations indicate that the classical clinical correlates of human DR are absent in $\text{Ins2}^{\text{Akita}}$ mice up to 6 months of age suggesting either that the histopathologic processes underlying the development of DR in this model require longer than 5 months of hyperglycemia to result in disruption of retinal architecture or that advanced DR is not a feature of the $\text{Ins2}^{\text{Akita}}$ diabetic mouse (McLenachan et al., 2013).

As can be seen from the aforementioned facts, the $\text{Ins2}^{\text{Akita}}$ mouse has several important advantages over other animal models. First, the autosomal-dominant mutation provides the opportunity to study heterozygotic animals. Second, the mice are fertile and breed well. Third, they have stable diabetes with insulin deficiency and can be maintained in a noncatabolic state without

exogenous insulin. Fourth, the mechanism of diabetes onset does not involve systemic immunologic alterations, and it is therefore possible to evaluate the metabolic impact on the retina (Barber et al., 2005). Therefore, the use of the Ins2^{Akita} mouse is an excellent choice to explore the molecular mechanisms involved in the initiation and progression of DR in older ages.

3.3. *Ex vivo* and *in vitro* models

There is increasing pressure from the society to find viable alternatives for animal research. Along with this requirement, scientists tried to set up experimental models for DR, which did not involve long-term keeping of sickened animals. In the succeeding text, we collected the results obtained in these models and evaluate how well they fare compared with *in vivo* models. Also, we try to pinpoint those aspects of DR, which can be studied using these approaches.

3.3.1 *Retinal ganglion cell lines*

The retinal ganglion cell line RGC-5 is a transformed line obtained from postnatal Sprague–Dawley rats. For studies of the effect of high glucose levels, cells were grown in a medium supplemented with 11 mM glucose (control) plus 34 mM mannitol or 28 mM glucose (medium glucose) plus 17 mM mannitol, 45 mM glucose (high glucose), and 10 μ M succinate. Mannitol was added to control for osmolar effects (Hu et al., 2013; Ola et al., 2002). After incubation with succinate and various concentrations of glucose, the expression of VEGF in RGC-5 cells was elevated. The phosphorylation levels of ERK1/2, p38 MAPK, and JNK in RGC-5 cells after exposure to high glucose were increased (Hu et al., 2013). The type 1 sigma receptor (sigmaR1) has numerous pharmacological and physiological functions. This sigmaR1 is expressed under hyperglycemic conditions both *in vitro* and *in vivo* (Ola et al., 2002), and its expression has been demonstrated recently in RGCs, which undergo apoptosis early in DR via NMDA receptor-mediated overstimulation. It can be concluded that this model is suitable to study the molecular events of neuronal apoptosis in DR.

3.3.2 *Müller glial cell lines*

Isolated rat retinal Müller cells (rMC-1) (from 5-day-old Wistar rats) were cultured, and passaged cells were seeded for 2 weeks in a culture to mimic diabetic conditions. In other experiments, rMC-1s were plated on tissue culture plastic. When the cells reached 70% confluence, they were treated with different concentrations of glucose (normal group, 5 mM glucose, and

high-glucose group, 25 mM glucose) in test medium for an indicated time (Du et al., 2003; Zhang et al., 2011). The cells in the glucose-treated group showed elevated VEGF immunoreactivity and significantly more VEGF mRNA than the control cells. The expression of VEGF in Müller cells increased after a short 3-day exposure to high glucose in culture (Ke et al., 2012). Hyperglycemia significantly increased the amount of GAPDH protein in the nucleus above normal within the first 48 h and induced apoptosis. The nuclear translocation of GAPDH is closely associated with the induction of apoptosis (Kusner et al., 2004). Glutaredoxin (Grx1) plays a key role in such regulation because it is a specific and efficient catalyst of deglutathionylation, and an increased level of Grx1 could be observed in retina of diabetic rats and in rMC-1 cultured in high glucose. Diabetes or incubation in elevated glucose concentration significantly increased superoxide production, which contributes to impaired viability and increased cell death under those circumstances (Du et al., 2003). The upregulation of Grx1 was concomitant with NF- κ B activation and induction of ICAM-1, which play a central role in diabetic complications *in vivo* and also in these cell cultures (Shelton et al., 2009).

It is also possible to prepare freshly isolated human retinal Müller cell culture. The eyes were enucleated, packed on wet ice, and received in the laboratory within 12 h after death. Cells were plated on tissue culture plastic in the medium and allowed to attach and grow at 37 °C in a humidified chamber. Müller cells were isolated after the second passage of cells; cultures were 95% pure for Müller cells as assessed by immunohistochemistry using antibodies to GS, GFAP, and vimentin. Cells were switched to normal (7.8 mM) or high glucose (25 mM glucose). Cells were grown for up to 5 days (Kusner et al., 2004). These cell cultures are suitable to study Müller cell reactions to hyperglycemia and diabetes but obviously not fit for studying retinal changes as a whole, not to speak about the functional integrity of the retinal tissue.

3.3.3 Retinal pigment epithelial cell lines

Human retinal pigment epithelial (ARPE-19) cells were cultured at 37 °C, after the cells were grown to 90% confluence; they were cultured for another 48 h in the presence of D-glucose at a concentration of 5, 7.5, 12.5, or 17.5 mM, which refer to as very low glucose, low glucose, medium glucose, or high-glucose culture medium, respectively. ARPE-19 cells were cultured for 72 h (Xie et al., 2012; Yokouchi et al., 2013). Other cultures were exposed to D-glucose at a final concentration of 25 and 100 mM

(corresponding to 2 h after meal plasma glucose level of diabetic patients and glucose level in uncontrolled diabetic patients, respectively) and compared with cultures exposed to 5.5 mM D-glucose as control (corresponding to fasting plasma glucose level of diabetes-free people). After exposure for at least 3 weeks, the monolayer cultures were used for further analysis (Chen et al., 2012b). A significant cellular damage was seen in ARPE-19 cells after a 48 h treatment with high glucose, accompanied by a decrease in SOD activity and glutathione concentration; high glucose also caused ARPE-19 cell apoptosis and activation of p38 MAPK and ERK (Xie et al., 2012). ANGPTL4, the major angiogenic factor released by ARPE-19, was induced by high glucose in RPE cells (Yokouchi et al., 2013). Fifty-six proteins showed significant changes in expression in cultured ARPE-19 cells. Significant changes in thiol reactivity were seen in response to high glucose concentration. Some of the identified proteins have been validated with clinical samples and provide potential targets for the prognosis and diagnosis of DR (Chen et al., 2012b). Thus, this methodology may provide molecular markers for improving diagnostic possibilities in DR.

3.3.4 Retinal endothelial cell lines

Bovine primary retinal endothelial cells were incubated in 5 or 25 mM glucose (Du et al., 2003). For high-glucose treatment, endothelial cells were cultured for 10 days in media supplemented with additional glucose to a final concentration of 25 mM. Control cells were simultaneously treated with either 25 mM L-glucose or 5 mM D-glucose. Primary retinal endothelial cells were used between passages 10 and 13 (Aranda et al., 2012). In another study, a transformed endothelial cell line was isolated from the BRB of transgenic mice. The cells were cultured in 5 or 25 mM glucose (Shelton et al., 2009). Rhesus retinal vascular endothelial cell line RF/6A is also thought to be a useful cell line for modeling DR (Hu et al., 2013). High-glucose treatment of the aforementioned retinal explants mimicked the diabetic phenotype. Similarly, primary retinal endothelial cells, which were subjected to high-glucose treatment, organized into tubes that were resistant to lysophosphatidic acid (LPA). Hyperglycemia caused LPA resistance within retinal endothelial cells by elevating ROS, which stimulated the ERK pathway, which antagonized LPA-mediated signaling events that were required for regression. This ROS/ERK pathway mechanism appeared to be the same route by which diabetes-induced LPA resistance is conferred to retinal neovessels. It is concluded that diabetes/hyperglycemia reprograms signaling pathways in retinal endothelial cells to induce a state of LPA resistance

(Aranda et al., 2012). This and similar molecular pathways can be successfully studied to understand endothelial cell reactions in DR.

3.3.5 Retinal explants

Retinal explants were obtained by gently peeling the choroid away from the RPE, leaving the RPE attached to the neurosensory retina. Control retinas were cut into 1 mm² pieces and placed in a collagen sandwich. Vessel formation was observed after 2–3 weeks. In some experiments to model diabetes, the medium was altered to achieve a final concentration of 25 mM D-glucose or L-glucose during the 2–3-week period of tube formation (Aranda et al., 2012; Lecleire-Collet et al., 2005). For other studies, rats were killed by CO₂ inhalation after 3 weeks of diabetes. Retinas were isolated 1.5 mm from the optic nerve head, excised into 36 small pieces, and placed in a liquid collagen solution. The retinal explants were cultured to examine the effect of diabetes on the number of regenerating neurites. The number of neurites per retinal explant was counted under a phase-contrast microscope on day 6 at which time point, their number was greatly increased (Oshitari and Roy, 2005).

In an 8-week-old female, C57BL/6 mouse diabetes was induced by STZ. Retinas were dissected from the eyeballs of both 10-week-old diabetic and control mice. Retinal pieces located at a distance of 1.0–1.5 mm from the optic disc were excised and cut into 16 pieces (approximately 500 μm²) with a sharp razor blade. These retinal pieces were embedded in collagen gel and were cultured in a medium that contained 7 mM of glucose; for high-glucose medium, 50 mM of glucose was added (the final glucose concentration was 57 mM). The number of outgrowing neurites from the explants was counted under a phase-contrast light microscope after 3, 6, and 10 days in culture. TUNEL- and cleaved caspase 3-positive cells increased significantly in retinal explants incubated in glycated bovine serum albumin (BSA) (by 2.2- and 2.5-fold, respectively). The GCL was the most sensitive retinal layer to the glycated BSA. Neuronal damage was confirmed by the increased GFAP labeling in Müller glial cells from retinal explants (Lecleire-Collet et al., 2005). The number of regenerating neurites in the retinal explants of diabetic rats was significantly reduced (Oshitari and Roy, 2005). Treatment of retinal explants with high glucose concentration (57 mM) significantly diminished the number of regenerating neurites in the control mice, but not in the diabetic mice. These results suggest that retina in diabetic mice has impaired capability of neurite

regeneration in a normal glucose environment but is adaptable to a high-glucose environment *in vitro*.

3.3.6 Choroidal explants

Choroidal explants can be prepared after removing the cornea, lens, corpus vitreum, and retina from the inside of the globe. The posterior segment containing the sclera and the choroid is sectioned into either quadrants or thirds. After breaking any adhesions between the choroid and the sclera, the isolated segment of choroid was then sectioned into 1–2 mm² explants and placed in the collagen gel (Lameynardie et al., 2005). Angiogenesis was triggered by the injury of the dissection procedure and did not require stimulation by exogenous growth factors. Angiogenesis is enhanced in diabetic rats when compared with Wistar rats, in the presence of fetal bovine serum. Thereafter, explants prepared as described in the preceding text were cultured with different glucose concentration (Lameynardie et al., 2005). The assay model of choroidal angiogenesis was thus established by determining the number and length of microvessels in cultured choroidal explants. The STZ-induced diabetic states of Wistar and GK rats enhanced hyperglycemia-induced choroidal angiogenesis. This assay model is useful for determining angiogenic activity of growth factors and effective drugs in diabetic choroidopathy and retinopathy.



4. EXPERIMENTAL APPROACHES TO THE TREATMENT OF DIABETIC RETINOPATHY

Several experimental therapies have been found to inhibit the development of DR in animal models. These included treatments with endogenous protective compounds, natural substances, and fully synthetic drugs (Table 1.1). If one tries to understand this enormous body of data, a more systematic approach is needed. Recently, Lu et al. (2013) had shown that suppression of GLUT1 is a new strategy to prevent diabetic complications. If one considers just the protection of RGCs, a plethora of approaches can be collected from the literature. For example, anti-inflammatory drugs such as nonsteroidal cyclooxygenase inhibitor nepafenac (Kern et al., 2007; Krady et al., 2005; Vincent and Mohr, 2007); several salicylates (Zhang et al., 2007); the glutamate NMDA receptor antagonist, memantine (Kusari et al., 2007); cannabidiol (CBD) (El-Remessy et al., 2006); NGF (Hammes et al., 1995b); IGF-1 (Seigel et al., 2006); aldose reductase inhibitors (Asnaghi et al., 2003); erythropoietin (EPO) (Zhang et al., 2008); and

Table 1.1 Effects of different compounds in experimental diabetic retinopathy.

Effects on		Compound/drug	Type 1 diabetes	Type 2 diabetes
Vascular	VEGF ↓	Insulin	Wang et al. (2007)	
		Telmisartan/valsartan	Nagai et al. (2007)	
		Candesartan		Fukumoto et al. (2008) Sugiyama et al. (2007)
		Perindopril	Zheng et al. (2009)	
		Prorenin receptor blocker	Satofuka et al. (2009)	
		Angiostatin	Sima et al. (2006)	
		Bevacizumab	Ma et al. (2010)	
		EPO	Mitsubishi et al. (2013)	
		Melatonin		Salido et al. (2013)
		PEDF	Boehm et al. (2003) Liu et al. (2004) Yu et al. (2010)	
		Purearin	Chen et al. (2012a)	
		Aminoguanidine	Luo et al. (2012)	
		Baicalein	Yang et al. (2009)	

Continued

Table 1.1 Effects of different compounds in experimental diabetic retinopathy.—cont'd

Effects on	Compound/drug	Type 1 diabetes	Type 2 diabetes
	Eriodictyol	Bucolo et al. (2012)	
	Hesperidin	Shi et al. (2012)	
	GT	Kumar et al. (2012)	
	CGA	Shin et al. (2013)	
	Arctiin	Lu et al. (2012)	
	TC	Agrawal et al. (2012)	
	Oat diet	Al-Malki (2013)	
	GBE	Bucolo et al. (2013)	
	Zeaxanthin	Kowluru et al. (2008)	
	Cilostazol		Jung et al. (2013)
	Resveratrol	Kim et al. (2012)	
	Astragalin	Ke et al. (2012)	
	Fenugreek	Gupta et al. (2014)	
	Memantine	Kusari et al. (2007)	
	Vitamin D	Ren et al. (2012)	Ren et al. (2012)
	H ₂ S	Si et al. (2013)	

ICAM-1 ↓	Telmisartan/valsartan	Nagai et al. (2007)
	Prorenin receptor blocker	Satofuka et al. (2009)
	PEDF	Yamagishi et al. (2006b) Yu et al. (2010)
	Eriodictyol	Bucolo et al. (2012)
	Hesperidin	Shi et al. (2012)
	Zeaxanthin	Kowluru et al. (2008)
	Fasudil	Arita et al. (2009)
	Photobiomodulation	Tang et al. (2013)
Occludin ↑	PACAP	Scuderi et al. (2013)
	VIP	Scuderi et al. (2013)
	PEDF	Yu et al. (2010)
	GT	Silva et al. (2013)
	CGA	Shin et al. (2013)
	Sitagliptin	Gonçalves et al. (2012)

Continued

Table 1.1 Effects of different compounds in experimental diabetic retinopathy.—cont'd

Effects on	Compound/drug	Type 1 diabetes	Type 2 diabetes
Vascular permeability ↓	Angiotensin(1–7)	Verma et al. (2012)	
	Candesartan	Phipps et al. (2009)	
	Angiostatin	Sima et al. (2006)	
	PEDF	Boehm et al. (2003) Inagaki et al. (2003) Liu et al. (2004) Yamagishi et al. (2006a)	
	H(2) saline	Xiao et al. (2012b)	
Neovascularization ↓ Neoangiogenesis ↓	SST and SST analogs	Casini et al. (2005) Davis et al. (2001) Grant and Caballero (2002)	
	Angiostatin	Sima et al. (2004)	
	Decursin	Ahn et al. (1996) Yang et al. (2013b)	
	EPO	Watanabe (2007)	
	PEDF	Boehm et al. (2003)	
	Hesperidin	Shi et al. (2012)	
	TC	Agrawal et al. (2012) Rathi et al. (2002)	
	Vitamin D	Aksoy et al. (2000) Albert et al. (2007) Kaur et al. (2011)	Aksoy et al. (2000) Albert et al. (2007) Kaur et al. (2011)

BRB breakdown ↓ BRB dysfunction ↓	PACAP	Scuderi et al. (2013)
	VIP	Scuderi et al. (2013)
	Candesartan	Phipps et al. (2009)
	EPO	Villa et al. (2003) Zhang et al. (2008)
	PEDF	Liu et al. (2004)
	Eriodictyol	Bucolo et al. (2012)
	Hesperidin	Shi et al. (2012)
	CGA	Shin et al. (2013)
	Cannabidiol	El-Remessy et al. (2006)
	RA	Miyajima et al. (2005) Nishikiori et al. (2007a)
	Sitagliptin	Gonçalves et al. (2012)
	Memantine	Kusari et al. (2007)
	H ₂ S	Si et al. (2013)
	H(2) saline	Feng et al. (2013)
Diabetes-related (retinal) edema ↓	EPO	Brines et al. (2008) McVicar et al. (2011)
	Arctiin	Lu et al. (2012)

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Table 1.1 Effects of different compounds in experimental diabetic retinopathy.—cont'd

Effects on	Compound/drug	Type 1 diabetes	Type 2 diabetes
Vascular degenerative pathology ↓	Perindopril	Zheng et al. (2009)	
	Captopril	Zhang et al. (2007)	
	Bevacizumab	Ma et al. (2010)	
	EPO	Brines and Cerami (2008) Brines et al. (2008) McVicar et al. (2011) Wang et al. (2011) Watanabe (2007) Zhang et al. (2008)	
	NGF	Colafrancesco et al. (2011) Hammes et al. (1995b)	
	Aminoguanidine	Gardiner et al. (2003) Luo et al. (2012)	
	Baicalein	Yang et al. (2009)	
	TFF	Li et al. (2013b)	
	GT	Kumar et al. (2012)	
	CGA	Shin et al. (2013)	
	TC	Agrawal et al. (2012) Rathi et al. (2002)	
	Oat diet	Al-Malki (2013)	
	Cilostazol		Jung et al. (2013)

	Resveratrol	Kim et al. (2012)
	Fenugreek	Gupta et al. (2014)
	Carnosine	Pfister et al. (2011)
	Nepafenac	Kern et al. (2007)
	Fasudil	Arita et al. (2009)
Cells and tissues Neuroprotection	Insulin	Reiter et al. (2006)
	IGF analogs	Kummer et al. (2003)
	Rosiglitazone	Li et al. (2011)
	SST	Casini et al. (2005) Hernandez et al. (2013)
	PACAP	Szabadfi et al. (2012, 2014)
	EPO	Brines and Cerami (2008) Hernandez and Simo (2012) Wang et al. (2011) Zhang et al. (2008)
	rhEPO	Zhu et al. (2008)
	Melatonin	Kanter et al. (2006) Sudnikovich et al. (2007)
	E4	Zhang et al. (2011)

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Table 1.1 Effects of different compounds in experimental diabetic retinopathy.—cont'd

Effects on	Compound/drug	Type 1 diabetes	Type 2 diabetes
	NGF	Colafrancesco et al. (2011) Hammes et al. (1995b)	
	AG	Gardiner et al. (2003)	
	Baicalein	Yang et al. (2009)	
	TFF	Li et al. (2013b)	
	4-Methyl-2,6-diisobornylphenol	Logvinov et al. (2010)	
	GSPE	Li et al. (2008b)	
	Wolfberry		Tang et al. (2011)
	CBD	El-Remessy et al. (2006)	
	RA	Nishikiori et al. (2008)	
	H ₂ S	Si et al. (2013)	
Histological alterations ↓	Rosiglitazone	Li et al. (2011)	
	PACAP	Szabadfi et al. (2012)	
	Perindopril	Tikellis et al. (2004)	
	AG	Kern and Engerman (2001)	
	Hesperidin	Shi et al. (2012)	
	TFF	Li et al. (2013b)	
	GSPE	Li et al. (2008b)	
	Wolfberry		Tang et al. (2011)

Glial activation ↓ GFAP ↓	Zeaxanthin	Kowluru et al. (2008)
	CBD	El-Remessy et al. (2010)
	Nepafenac	Kern et al. (2007)
	H(2) saline	Feng et al. (2013)
	SST	Hernandez et al. (2013)
	PACAP	Szabadfi et al. (2012)
	EPO	Brines et al. (2008) McVicar et al. (2011)
	rhEPO	Zhu et al. (2008)
	Melatonin	Salido et al. (2013)
	E4	Zhang et al. (2011)
	PEDF	Li et al. (2002)
	Phlorizin	Zhang et al. (2013)
	GT	Silva et al. (2013)
	Curcumin	Gupta et al. (2011) Zuo et al. (2013)
	Cilostazol	Jung et al. (2013)
	CBD	El-Remessy et al. (2010)

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Table 1.1 Effects of different compounds in experimental diabetic retinopathy.—cont'd

Effects on	Compound/drug	Type 1 diabetes	Type 2 diabetes
Müller cell dysfunction ↓	Baicalein	Yang et al. (2009)	
Microglial and astroglial activation ↓	Baicalein	Yang et al. (2009)	
	<i>N</i> -acetylcysteine (NAC)	Tsai et al. (2009)	
Dopaminergic amacrine cell number ↑	PACAP	Szabadfi et al. (2012)	
	BDNF	Seki et al. (2004)	
Ganglion cell number ↑	Rosiglitazone	Li et al. (2011)	
	PACAP	Szabadfi et al. (2014)	
	EPO	Zhang et al. (2008)	
	Exenatide	Fu et al. (2012) Hao et al. (2012)	
	BDNF	Seki et al. (2004)	
	NGF	Colafrancesco et al. (2011)	
	4-Methyl-2,6-diisobornylphenol	Logvinov et al. (2010)	
	Astaxanthin		Dong et al. (2013)
	CBD	El-Remessy et al. (2006)	

	SNJ-1945 (calpain inhibitor)	Shanab et al. (2012)
	Memantine	Kusari et al. (2007)
	Nepafenac	Kern et al. (2007) Kradly et al. (2005)
	Photobiomodulation	Tang et al. (2013)
Photoreceptor cells number ↑	PACAP	Szabadfi et al. (2012)
	4-Methyl-2,6-diisobornylphenol	Logvinov et al. (2010)
Loss of pericytes ↓	EPO	Garcia-Ramirez et al. (2011) Wang et al. (2010b)
	AG	Luo et al. (2012)
	Resveratrol	Kim et al. (2012)
	Nepafenac	Kern et al. (2007)
	N-acetylcysteine	Tsai et al. (2009)
Endothelial cell proliferation ↓	PACAP	Castorina et al. (2010)
	Bevacizumab	Ma et al. (2010)
	Aminoguanidine	Frank et al. (1997) Luo et al. (2012)

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Table 1.1 Effects of different compounds in experimental diabetic retinopathy.—cont'd

Effects on		Compound/drug	Type 1 diabetes	Type 2 diabetes
Protect endothelial cells		PEDF	Inagaki et al. (2003) Yamagishi et al. (2006a)	
		Curcumin	Gupta et al. (2011)	
		Nepafenac	Kern et al. (2007)	
		<i>N</i> -acetylcysteine	Tsai et al. (2009)	
		Fasudil	Arita et al. (2009)	
Protect RPE cells		PACAP	Szabadfi et al. (2014)	
		EPO	Garcia-Ramirez et al. (2011)	
		Wolfberry		Tang et al. (2011)
TUNEL-positive cells ↓		IGF-1	Barber et al. (1998, 2001)	
		SST and SST analogs	Casini et al. (2005)	
		PACAP	Szabadfi et al. (2014)	
		Cilostazol		Jung et al. (2013)
Retinal parenchyma thickening ↓		H(2) saline	Xiao et al. (2012b)	
Intracellular alterations	PI3K/Akt	Insulin/IGF-1	Barber et al. (2001) Reiter et al. (2003, 2006) Wu et al. (2004b)	
			PACAP	Szabadfi et al. (2014)
			EPO	Garcia-Ramirez et al. (2011) Wang et al. (2010b)

Bad ↓/Bax ↓	IGF-1	Barber et al. (2011) Galvan et al. (2003) Seigel et al. (2006)
	Exenatide	Fu et al. (2012) Hao et al. (2012)
	TFF	Li et al. (2013b)
Caspase 3 ↓	IGF-1	Barber et al. (2001)
	Rosiglitazone	Li et al. (2011)
	PACAP	Szabadfi et al. (2014)
	Melatonin	Li et al. (2013a)
	Exenatide	Fu et al. (2012) Hao et al. (2012)
	Nepafenac	Kern et al. (2007)
	H(2) saline	Xiao et al. (2012b)
Bcl 2 ↑	PACAP	Giunta et al. (2012) Szabadfi et al. (2014)
	Exenatide	Fu et al. (2012) Hao et al. (2012)
	TFF	Li et al. (2013b)
MAPK	PACAP	Szabadfi et al. (2014)
Misbalance between proapoptotic and survival signaling	SST	Hernandez et al. (2013)

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Table 1.1 Effects of different compounds in experimental diabetic retinopathy.—cont'd

Effects on	Compound/drug	Type 1 diabetes	Type 2 diabetes
Apoptotic process ↓	IGF-1	Barber et al. (2001) Kummer et al. (2003) Galvan et al. (2003)	
	SST	Hernandez et al. (2013)	
	Rosiglitazone	Li et al. (2011)	
	PACAP	Szabadfi et al. (2014)	
	Candesartan	Gao et al. (2009a)	
	EPO	Brines and Cerami (2005) Zhang et al. (2008)	
	Melatonin	Kanter et al. (2006) Li et al. (2013a) Sudnikovich et al. (2007)	
	E4	Zhang et al. (2009b, 2011)	
	NGF	Colafrancesco et al. (2011) Hammes et al. (1995b)	
	Phlorizin		Zhang et al. (2013)
	TFF	Li et al. (2013b)	
	Wolfberry		Tang et al. (2011)
	RA	Nishikiori et al. (2008)	
	Sitagliptin		Gonçalves et al. (2012)
	H(2) saline	Xiao et al. (2012b)	

	Cytokine level ↑	Tinospora cordifolia	Agrawal et al. (2012)	
	Cell survival pathways ↑	PACAP	Szabadfi et al. (2014)	
		Melatonin	Li et al. (2013a)	
		Wolfberry		Tang et al. (2011)
Oxidative stress	Superoxide and other radicals ↓	EPO	Garcia-Ramirez et al. (2011) Wang et al. (2010a,b)	
		Zeaxanthin	Kowluru et al. (2008)	
		Lutein	Sasaki et al. (2010)	
		Astaxanthin		Dong et al. (2013)
		Nepafenac	Kern et al. (2007)	
		Photobiomodulation	Tang et al. (2013)	
	Effect on SOD and CAT enzymatic activity ↑	Melatonin	Li et al. (2013a)	Salido et al. (2013)
		Hesperidin	Shi et al. (2012)	
		GT	Kumar et al. (2012)	
		Astaxanthin		Dong et al. (2013)
		Photobiomodulation	Tang et al. (2013)	

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Table 1.1 Effects of different compounds in experimental diabetic retinopathy.—cont'd

Effects on	Compound/drug	Type 1 diabetes	Type 2 diabetes
Antioxidant mechanisms ↑ Oxidative stress ↓	Angiotensin(1–7)	Verma et al. (2012)	
	Candesartan	Gao et al. (2009a)	
	Melatonin	Li et al. (2013a)	
	PEDF	Yoshida et al. (2009)	
	Hesperidin	Shi et al. (2012)	
	GT	Kumar et al. (2012) Silva et al. (2013)	
	TC	Agrawal et al. (2012) Rathi et al. (2002)	
	Curcumin	Gupta et al. (2011) Zuo et al. (2013)	
	GBE	Bucolo et al. (2013)	
	Zeaxanthin	Kowluru et al. (2008)	
	Astaxanthin		Dong et al. (2013)
	CBD	El-Remessy et al. (2010)	
	Ginsenosides	Sen et al. (2013)	Sen et al. (2013)
	Fenugreek	Gupta et al. (2014)	
	H ₂ S	Si et al. (2013)	
	H(2) saline	Feng et al. (2013)	
	N-acetylcysteine	Tsai et al. (2009)	

	ROS ↓	Perindopril	Zheng et al. (2009)
		GT	Silva et al. (2013)
		Oat diet	Al-Malki (2013)
		Wolfberry	Tang et al. (2011)
		H ₂ S	Si et al. (2013)
	Glutathione peroxidase ↑ Balance pro- and antioxidative factors	EPO	Garcia-Ramirez et al. (2011) Wang et al. (2010b)
		Valsartan	Fukumoto et al. (2008)
		Perindopril	Zheng et al. (2009)
	NADPH oxidase ↓	PEDF	Inagaki et al. (2003) Yamagishi et al. (2006a) Yoshida et al. (2009)
		Melatonin	Li et al. (2013a)
		Exenatide	Fu et al. (2012) Hao et al. (2012)
Glutamate/ GLAST	Glutamate accumulation ↓, GLAST downregulation	SST	Hernandez et al. (2013)
		E4	Zhang et al. (2009b, 2011)

Continued

Table 1.1 Effects of different compounds in experimental diabetic retinopathy.—cont'd

Effects on		Compound/drug	Type 1 diabetes	Type 2 diabetes
AGE/RAGE	AGE ↓	Candesartan		Sugiyama et al. (2007)
		PEDF	Yoshida et al. (2009)	
		Aminoguanidine	Hammes et al. (1995a) Kern et al. (2000) Kern and Engerman (2001) Luo et al. (2012)	
		Phlorizin		Zhang et al. (2013)
		Hesperidin	Shi et al. (2012)	
		Oat diet	Al-Malki (2013)	
		GSPE	Li et al. (2008b)	
	RAGE ↓	Purearin, pyridoxamine	Chen et al. (2012a) Luo et al. (2012)	
Retinal lipid peroxidation	↓	Melatonin		Salido et al. (2013)
		Eriodictyol	Bucolo et al. (2012)	
		Astaxanthin		Dong et al. (2013)
		Ginsenosides	Kim and Park (2003)	

Other factors	BDNF ↑ CNTF ↑	EPO	Hu et al. (2011)
	BDNF ↑	H ₂ S	Si et al. (2013)
		H(2) saline	Feng et al. (2013)
	Synaptophysin ↑	Telmisartan/valsartan	Kurihara et al. (2008)
		H ₂ S	Si et al. (2013)
		H(2) saline	Feng et al. (2013)
Inflammation	Inflammatory process ↓	Angiotensin(1–7)	Verma et al. (2012)
		Prorenin receptor blocker	Satofuka et al. (2009)
		EPO	Villa et al. (2003) Zhang et al. (2008)
		PEDF	Shen et al. (2011)
		Baicalein	Yang et al. (2009)
		Hesperidin	Shi et al. (2012)
		GT	Kumar et al. (2012)
		TC	Agrawal et al. (2012) Rathi et al. (2002)
		Curcumin	Gupta et al. (2011) Zuo et al. (2013)

Continued

Table 1.1 Effects of different compounds in experimental diabetic retinopathy.—cont'd

Effects on	Compound/drug	Type 1 diabetes	Type 2 diabetes
	GBE	Bucolo et al. (2013)	
	CBD	El-Remessy et al. (2006)	
	Fenugreek	Gupta et al. (2014)	
	Sitagliptin		Gonçalves et al. (2012)
	H ₂ S	Si et al. (2013)	
	N-acetylcysteine	Tsai et al. (2009)	
	Valsartan/telmisartan	Nagai et al. (2007)	
Leukostasis ↓ Leukocyte adhesion ↓	Captopril	Zhang et al. (2007)	
	Prorenin receptor blocker	Satofuka et al. (2009)	
	PEDF	Yamagishi et al. (2006b)	
	Fasudil	Arita et al. (2009)	
	Photobiomodulation	Tang et al. (2013)	
	Melatonin		Salido et al. (2013)
TNFα ↓ IL-1β ↓	Eriodictyol	Bucolo et al. (2012)	
	Hesperidin	Shi et al. (2012)	
	GT	Kumar et al. (2012)	
	TC	Agrawal et al. (2012)	
	Oat diet	Al-Malki (2013)	
	Fenugreek	Gupta et al. (2014)	

Functional	ERG abnormalities ↓	SST	Hernandez et al. (2013)
		Telmisartan/valsartan	Kurihara et al. (2008) Phipps et al. (2007)
		Photobiomodulation	Tang et al. (2013)
	Amplitudes of a wave ↑	Melatonin	Salido et al. (2013)
		PEDF	Li et al. (2002)
		Memantine	Kusari et al. (2007)
	Amplitudes of b wave ↑	rhEPO	Zhu et al. (2008)
		Melatonin	Salido et al. (2013)
		E4	Zhang et al. (2011)
		PEDF	Li et al. (2002)
		Memantine	Kusari et al. (2007)
		H(2) saline	Feng et al. (2013)
		Photobiomodulation	Tang et al. (2013)

Continued

Table 1.1 Effects of different compounds in experimental diabetic retinopathy.—cont'd

Effects on	Compound/drug	Type 1 diabetes	Type 2 diabetes
Amplitudes of OPs ↑	rhEPO	Zhu et al. (2008)	
	Melatonin		Salido et al. (2013)
	E4	Zhang et al. (2011)	
	Nepafenac	Kern et al. (2007)	
	H(2) saline	Feng et al. (2013)	

Abbreviations: AG, aminoguanidine; AGE, advanced glycosylated end product; BDNF, brain-derived neurotrophic factor; CBD, cannabidiol; CGA, chlorogenic acid; CNTF, ciliary neurotrophic factor; E4, exendin-4; EPO, erythropoietin; GBE, *Ginkgo biloba* leaf extract; GFAP, glial fibrillary acidic protein; GSPE, grape seed proanthocyanidin extracts; GT, green tea extract; ICAM-1, intercellular adhesion molecule 1; IGF-1, insulin-like growth factor 1; IL-1, interleukin-1; NGF, nerve growth factor; PACAP, pituitary adenylate cyclase-activating polypeptide; PEDF, pigment epithelium-derived factor; PI3K, phosphatidylinositol 3-kinase; RA, retinoic acid; RAGE, receptor of AGEs; ROS, reactive oxygen species; RPE, retinal pigment epithelium; SST, somatostatin; TC, *Tinospora cordifolia*; TNF α , tumor necrosis factor α ; TUNEL, terminal dUTP nick-end labeling; VEGF, vascular endothelial growth factor; VEP, visually evoked potential; VIP, vasoactive intestinal peptide.

peroxynitrite decomposition catalyst (El-Remessy et al., 2005) all have been found to inhibit RGC degeneration in diabetes. Aminoguanidine and aldose reductase inhibitors have been found to inhibit atrophy of optic nerve axons in diabetes (Inoue et al., 1998). Both the clinical trials and the laboratory investigations agree that besides the effectivity of the drug, the manner of administration may be the clue to treat the retinal complications, since many of the drugs do not cross the BRB, but intraocular injection may be effective. In the next paragraphs, we will therefore review both effectivity and administration of the different substances.

4.1. Insulin and IGFs

The retina is an insulin-sensitive tissue, especially regarding the anabolic effects of insulin (Reiter et al., 2003). All types of retinal cells express insulin receptors, with particularly high expression on Müller cells end feet and neuronal dendrites (Gosbell et al., 2002). However, insulin production within the retina has not been described to date. Exogenous insulin stimulation of whole retina activates the insulin receptor substrate/Akt branch of the insulin receptor signaling networks (Diaz et al., 2000; Reiter et al., 2003). Insulin provides trophic support for retinal neurons via phosphatidylinositol 3-kinase (PI3K)/Akt and P70S6 kinase pathways (Barber et al., 2001; Wu et al., 2004b), and this trophic function of insulin on retinal neurons is impaired by exposure to elevated glucose and glucosamine (Nakamura et al., 2001). Constitutive insulin receptor/Akt/p70S6K prosurvival signaling in retina is impaired by diabetes and may contribute to neuronal degeneration in DR. Vascular and neuronal survival could be compromised by reducing insulin receptor activity directly on these cells and/or indirectly by altering glial or microglial cell function, on which they depend. Systemic insulin therapy from the onset of diabetes prevents loss of retinal insulin receptor kinase activity, and importantly, for therapeutic implications, intraocular insulin injection restored that activity. Retinal insulin receptor signaling pathway provides neurotrophic support therefore DR may be considered as a neurotrophin-deficient and/or neurotrophin-resistant state (Reiter et al., 2006). Retinal apoptosis is increased 10-fold in rat retinas after at least 1 month of diabetes. Insulin implants significantly reduced the number of apoptotic cells (Barber et al., 1998). Indeed, insulin can rescue retinal neuronal cells from apoptosis through a PI3K/Akt-mediated mechanism, and systematically administered insulin activates the retinal insulin receptor, PI3K, and Akt in normal rats (Barber et al., 2001; Reiter et al., 2003). Many

growth factors, including insulin, activate p70S6K in a PI3K-dependent manner resulting in retinal neuronal cell survival. This pathway works *in vivo* via the PI3K/Akt/mTOR signal transduction (Wu et al., 2004b).

Several authors argue that the best therapy for DR is the strict control of blood glucose, because insulin can decrease VEGF expression in the retina and protect retinal vessels from impairment in early STZ-induced diabetic rats (Wang et al., 2007); a similar conclusion has been reached recently regarding human DR (Hammes, 2013).

IGFs are neurotrophic factors that have been implicated in the pathogenesis of diabetic neurological disorders. The signaling pathway involves the type 1 IGF receptor, which binds both IGF-1 and IGF-2. Early systemic treatment with IGF or its structural analogs may prevent predegenerative changes that lead to the death of retinal cells in diabetes. IGF can prevent cell death in the nervous system in the context of diabetes despite the fact that IGF-1 treatment had no effect on severity of hyperglycemia or reduced body weight in diabetic rats (Seigel et al., 2006). IGF-1 mRNA content is reduced in the eye in the early stages of clinical and experimental diabetes (Gerhardinger et al., 2001), and IGF-1 levels are elevated in the vitreous due to variable disruption of the BRB in chronic disease. IGF replacement therapy can prevent or reverse diabetic neurological complications, such as early treatment with IGF-1 analogs could counteract certain proapoptotic abnormalities that precede retinal cell degeneration (Kummer et al., 2003). Further, systemic IGF-1 administration also prevents both the increase in p-Akt and TUNEL staining in retinal cells in diabetic rats. IGF-1 or IGF analog treatment in diabetic rats may rescue retinal cells from death by the inhibition of caspase 3 activation through the PI3K/Akt pathway (Barber et al., 2001). This treatment simultaneously reduced the elevation of Bad immunoreactivity in diabetes as well (Seigel et al., 2006). IGF-1 binding to its receptor blocks apoptosis by inducing the phosphorylation and inhibition of proapoptotic proteins such as Bad in all significantly affected retinal layers (INL, ONL, and GCL; Barber et al., 2001; Galvan et al., 2003).

Drugs acting along the insulin/IGF pathway may have similar mode of action. Rosiglitazone (with the trademark name Avandia) is an antidiabetic drug in the thiazolidinedione class of drugs. It works as an insulin sensitizer, by binding to peroxisome proliferator-activated receptors (PPARs) in fat cells and making the cells more responsive to insulin. After treatment with rosiglitazone in experimental STZ-induced diabetes, the thickness of the retina and the number of cells in the GCL were significantly greater. It also attenuated the diabetes-induced apoptosis of retinal neurons and

mitochondrial metamorphosis in RGCs. Consistent with these effects, decreased cleaved caspase 3 and p-STAT3 levels and increased SOCS3 expression were observed (Li et al., 2011). Consequently, rosiglitazone might be used to prevent retinal neuronal damage in diabetes.

4.2. Neuropeptides

Apart from their well-known neuromodulatory actions in the retina, recent investigations revealed their role in retinal development (Bagnoli et al., 2003), and we have just started to appreciate their possible role in aging (Reglodi et al., 2012). When examining their role in DR, some substances of extraretinal origin must also be taken into account.

4.2.1 Somatostatin

Somatostatin (SST) is a neuropeptide widely distributed in the central and peripheral nervous system, where it plays a variety of biological roles (Blake et al., 2004; Olias et al., 2004). Two forms of SST have been identified — SST-14 and SST-28 — the former is the one preferentially expressed in the mammalian retinal amacrine cells. It interacts with five main membrane receptors (sst1–sst5) that are coupled to different transduction pathways (Olias et al., 2004). Mostly, sst2 and sst4 receptors can be found in the retina. Expression of sst2 is the most abundant; it can be detected in virtually all layers (Casini et al., 2005; Thermos, 2003). sst1 and sst5 receptors are also found (amacrine cells and RGCs). However, sst3 receptors are not expressed in the mammalian retina (Ke and Zhong, 2007; Thermos, 2003).

Potential therapeutic use of SST is based on its ability to inhibit growth hormone secretion, which was implicated in the pathogenesis of DR (Kirkegaard et al., 1990). In some points of view, the neuronal death in diabetic retinas is caused by increased extracellular glutamate level. In the retina, SST inhibits glutamate accumulation and GLAST downregulation induced by diabetes (Hernandez et al., 2013). Indeed, potential neuroprotective roles of SST or its analogs may be mediated by inhibition of glutamate release through activation of K^+ channels. Further, SST has a dual action in DR, playing against both neoangiogenesis and excitotoxic neuronal death (Casini et al., 2005). SST is downregulated in the diabetic eye; its concentration is low in the vitreous body. SST-containing eye-drop treatment prevented ERG abnormalities, glial activation, apoptosis, and misbalance between proapoptotic and survival signaling in STZ-induced diabetic rats. Topical administration of SST or its analogs has a potent effect in preventing retinal neurodegeneration induced by diabetes (Hernandez et al., 2013).

SST or SST analogs may counteract retinal damages in DR through a protective paracrine effect directly on retinal cells, which are known to express sst receptors. Several lines of clinical and experimental evidence suggest that SST analogs may be efficacious in inhibiting neovascularization associated with proliferative DR (Davis et al., 2001; Grant and Caballero, 2002).

Octreotide, a peptide analog of SST, can counteract vascular endothelial dysfunction. This drug proved to be effective in delaying or to some extent reverting symptoms of DR (Grant et al., 2000). Acting on sst2 receptors, it may possibly control inflammatory processes (Pinter et al., 2006), endothelial symptoms (Cervia et al., 2012), and also to some extent neural symptoms (Cervia et al., 2008; Vasilaki and Thermos, 2009; Vasilaki et al., 2002). Additional peptide and nonpeptide analogs should be involved in future experiments.

One of those may be cortistatin, which is produced mostly by a non-neural cell of the retina, the RPE. Its role may lie in the activation of glial cells under stress conditions (Carrasco et al., 2008). Its receptors are identical with those activated by SST. The influence of cortistatin on neural information processing is unknown at present. However, it is presumed that cortistatin is a part of a signaling system residing in the RPE of the retina, which shows some parallel properties with the hypothalamo–pituitary–adrenal axis (Zmijewski et al., 2007).

4.2.2 Pituitary adenylate cyclase-activating polypeptide

Pituitary adenylate cyclase-activating polypeptide (PACAP), a member of the vasoactive intestinal peptide (VIP)/secretin/glucagon peptide superfamily, is a neuropeptide with highly potent neuroprotective and general cytoprotective effects. PACAP and its receptors (PAC1, VPAC1, and VPAC2) occur in the retina (Atlasz et al., 2010), specifically in all retinal layers except the photoreceptors (Seki et al., 2000).

PACAP treatment has been proven to be protective in several retinal pathologies (Nakamachi et al., 2012). It attenuates retinal damage in excitotoxic, ischemic, traumatic, and UV light-induced retinal degeneration (Atlasz et al., 2010; Varga et al., 2011). Several lines of evidence suggest that PACAP might have therapeutic potential in the treatment of diabetes (Yamamoto et al., 2003) because of its effects on insulin secretion protection during β cell damage induced by various insults. Systemic PACAP treatment decreases the STZ-induced nephropathy (Li et al., 2008a) and experimental neuropathy (Dickinson et al., 1999), and PACAP inhibited the

hyperglycemia-induced microvascular endothelial cell growth *in vitro* (Castorina et al., 2010).

This peptide effectively reduced the diabetes-induced symptoms by upregulating its own receptor, PAC1-R, and protecting several cell types (including the dopaminergic amacrine cells and RGCs; Szabadfi et al., 2012). An evidence was provided that RGCs, photoreceptors, and bipolar, horizontal, Müller glial, and amacrine cells underwent apoptosis and that PACAP treatment could attenuate this degeneration. Among amacrine cells, dopaminergic cells are certainly affected. PACAP has an ameliorating effect on dopaminergic cell degeneration. It has recently been suggested in an avian model of retinal development that PACAP may support the appearance of cells with newly acquired dopaminergic phenotype (Fleming et al., 2013). Although this possibility cannot be discounted in adult rat retinas with DR, it is less likely than protection mediated by PACAP in the case of the original dopaminergic cells.

Ultrastructural analysis revealed that retinal ribbon synapses and other synaptic profiles suffered alterations in diabetes and contained nontypical elements: empty profiles and blood vessels extending into the retina were also revealed. In PACAP-treated retinas, there were still some degenerative profiles in the OPL, but more ribbon synapses were found in the IPL. Noncharacteristic elements such as immune cells and blood vessels were present in both synaptic layers, similar to diabetic retinas (K. Szabadfi, unpublished results).

Breakdown of outer BRB is one of the major complications of DR, which is associated with the reduction of claudin-1 and ZO-1 expressions. This process was reversed by PACAP and VIP. Both peptides counteract hyperglycemia-induced damage in ARPE-19 cells, suggesting that they might be relevant to the maintenance of outer BRB function (Scuderi et al., 2013).

PACAP exerts its protective effects by increasing antiapoptotic factors and decreasing proapoptotic factors in different retinal and other injuries (Martin et al., 2004; Racz et al., 2006; Szabo et al., 2012; Vaudry et al., 2009). PACAP-induced pathways attenuate apoptosis in DR. Four pathways influenced by PACAP (MAPKs, PI3K/Akt, PKC, and inhibiting ER stress) converge to minimize apoptotic damage of retinal neurons in PACAP-treated diabetic retinas. PACAP (i) downregulated the precursor and active forms of caspase 8, caspase 3, and caspase 12 in diabetic retinas. PACAP treatment (ii) suppressed the expression and the phosphorylation of p38 MAPK and activated ERK1/2 in diabetes after 3 weeks of diabetes suggesting that the activity of MAPK pathways may account for at least part

of the protection. It also potentiates (iii) PKC and Bcl-2 in early DR possibly accounting for some of the observed protection. PACAP reduces apoptosis via (iv) elevated level of p-Akt protein and its downstream target GSK3 β phosphorylation. Akt signaling seems to play an important role in the neuroprotective effects of PACAP in DR (Szabadfi et al., 2014) and also other different retinal and other injuries (Lazarovici et al., 2012; Li et al., 2005; Racz et al., 2006; Szabo et al., 2012). Most of the cytoprotective effects of PACAP are mediated through activation of PAC1-R, which can induce a signaling cascade to stimulate protective factors and block caspase activation (Seaborn et al., 2011). PAC1-R-containing cells were not TUNEL-positive in any of the diabetic retinas, suggesting that PAC1-R-containing cells are more resistant (Szabadfi et al., 2014) than those without. In addition, mRNA and protein levels for PAC1-R are higher in diabetic retinas after PACAP treatment (Szabadfi et al., 2012). Tsutsumi et al. (2002) described that activation of VPAC1-R has been implicated in elevating glucose output, whereas activation of VPAC2-R may be involved in insulin secretion. PACAP exerts an inhibitory activity on hyperglycemia-induced endothelial cell proliferation, thus suggesting that the effect might be mediated by PAC1 and VPAC2 receptors (Castorina et al., 2010). We have also found unusual cells, like pericytes, granulocytes, and macrophages, in PACAP-treated diabetic retina (Szabadfi et al., 2012). According to our preliminary data, this can be correlated with the changing mRNA and protein levels of VPAC1-R and VPAC2-R, through which receptors of PACAP and VIP may have an action in inflammation. Thus, all three PACAP receptors may have positive contribution to fighting diabetes and its consequences (Giunta et al., 2012; Szabadfi et al., 2012, 2014).

The potency of PACAP in diabetes and related conditions in addition to its retinoprotective actions suggests that PACAP might have a therapeutic potential in the treatment of DR.

4.3. Therapies with vascular targets

Despite evidence that hyperactivity of the vasodeleterious axis (ACE/angiotensin II (Ang II)/angiotensin II type 1 receptor (AT1R)) of the renin-angiotensin system (RAS) is associated with the pathogenesis of DR, use of the inhibitors of this axis has had limited success in the control of this pathophysiology. Inhibition of the RAS has been shown to provide beneficial effects against DR, and AT1R activation may be a clue to retinal vascular dysfunction.

4.3.1 Angiotensin

DR is associated with impaired balance of RAS. Increased expression of ACE2/angiotensin(1–7) overcomes this imbalance and confers protection against DR. Intraocular administration of ACE2/angiotensin(1–7) by a viral vector resulted in significant reduction in diabetes-induced retinal vascular leakage, acellular capillaries, infiltrating inflammatory cells, and oxidative damage in both diabetic mice and rats. Thus, strategies enhancing the protective ACE2/angiotensin(1–7) axis of RAS in the eye could serve as a novel therapeutic target for DR (Verma et al., 2012).

4.3.2 Angiotensin receptor blockers

Valsartan or telmisartan is an AT1R blocker. AT1R blockade significantly reversed the diabetes-induced electroretinographic changes and reduction of synaptic protein but not mRNA levels in the diabetic mouse and rat retina. Synaptophysin degradation and neuronal dysfunction ran parallel after AT1R activation in the diabetic retina, suggesting the possibility of the AT1R blockade as a novel neuroprotective treatment for DR (Kurihara et al., 2008; Phipps et al., 2007). Retinal leukocytes were significantly suppressed by AT1R blockade by valsartan. Administration of valsartan inhibited diabetes-induced retinal expression of ICAM-1 and VEGF (Nagai et al., 2007). Valsartan also decreased AQP1 expression in STZ-induced diabetic rats (Qin et al., 2012). Gao et al. (2009a) described a comprehensive proteomic analysis in diabetic mice in which the AT1R antagonist candesartan ameliorated the diabetes-induced changes of metabolism, oxidative phosphorylation, and apoptotic pathway-associated proteins. Further, candesartan treatment decreased diabetes-stimulated retinal vascular permeability suggesting that activation of AT1R contributes to BRB dysfunction (Phipps et al., 2009) and at the same time significantly reduced the levels of VEGF and NADPH oxidase subunits in type 2 DR (Fukumoto et al., 2008). Candesartan also inhibited the development of DR by reducing the accumulation of pentosidine (an AGE) and expression of VEGF (Sugiyama et al., 2007).

4.3.3 Angiotensin-converting enzyme inhibitors

Perindopril is an angiotensin-converting enzyme (ACE) inhibitor (ACEI). It exerts a protective effect in DR by decreasing VEGF-to-PEDF ratio (downregulating VEGF and upregulating PEDF). The lowering of VEGF-to-PEDF ratio is significantly correlated with the relief of the vascular damage and was a result of reduced mitochondrial ROS production by

increasing uncoupling protein 2 (UCP-2). ACEI can attenuate oxidative stress through both the NADPH oxidase pathway and the UCP-2/mitochondrial pathway. The upregulation of UCP-2 expression is mediated by PPAR γ (Zheng et al., 2009). Connective tissue growth factor (CTGF) has been postulated to have prosclerotic and angiogenic properties. DR is associated with a greater than twofold increase in CTGF mRNA levels and immunoreactivity, which was attenuated by perindopril treatment. The protective effects of ACEIs on retinal pathology may be mediated via effects on retinal CTGF expression (Tikellis et al., 2004).

The ACE inhibitor captopril completely inhibited the diabetes-induced retinal capillary degeneration. Captopril inhibited hyperglycemia-induced leukostasis in the retinal vasculature (Zhang et al., 2007).

4.3.4 (Pro)Renin receptor blockers

The receptor-associated prorenin system was implied with diabetes-induced retinal inflammation. The administration of a prorenin receptor blocker inhibited the diabetes-induced retinal expression of VEGF and ICAM-1 in rats. Retinal adherent leukocytes were significantly suppressed with a (pro)renin receptor blocker. A significant contribution of the receptor-associated prorenin system to the pathogenesis of diabetes-induced retinal inflammation suggests the possibility of the prorenin receptor as a novel molecular target for the treatment of DR (Satofuka et al., 2009).

4.3.5 Antiangiogenic treatments

One of the major problems in DR is neovascularization. Any treatment preventing the formation of new vessels could prove to be antiangiogenic. The major factor driving the vascularization and neovascularization is VEGF. Reducing VEGF levels is therefore a major target in preventing DR. Anti-VEGF therapies in the treatment of proliferative DR and the use of intravitreal anti-VEGF therapy and anti-VEGF traps in clinical practice are now encouraged as adjuncts to corrective surgery (Hayden et al., 2011). It is safe and efficacious for macular condition in humans, although vitreal administration may be associated with an increased risk of systemic thromboembolism (Zhang et al., 2009a).

4.3.5.1 Angiostatin

Angiostatin is a naturally occurring protein found in several animal species, including humans. It is an endogenous angiogenesis inhibitor, which blocks the growth of new blood vessels, effecting vascular leakage in any tissue

(Sima et al., 2004). Diabetic rats showed significant increases in vascular permeability in the retina and iris. Angiostatin could reverse this effect. Angiostatin downregulates VEGF expression and thus blocks the major cause of vascular leakage in the diabetic retina (Sima et al., 2006). Angiostatin may have a therapeutic potential in the treatment of diabetic macular edema and other diseases with vascular leakage.

4.3.5.2 Decursin

Decursin is a compound isolated from the root of *Angelica gigas Nakai* that can be a novel compound for the antiangiogenesis approach of DR (Ahn et al., 1996). Targeting VEGFR-2 with decursin inhibits second messenger signaling in retinal cells (Kim et al., 2009a). The antiangiogenic effects of decursin have been largely attributed to reduced VEGFR-2 expression in endothelial cells. Even high doses of decursin did not induce apoptosis suggesting that it may be a safe therapeutic agent (Yang et al., 2013b).

4.3.5.3 Anti-VEGF antibodies

Bevacizumab is a humanized anti-VEGF antibody. The efficacy of intravitreal injection of bevacizumab was evaluated as a preventive intervention of vascular endothelial cell proliferation in the rat retina after 2 months of diabetes. A single intraocular injection of bevacizumab may be beneficial as a therapy for preventing retinal vascular endothelial cell growth (Ma et al., 2010). Ranibizumab is a recombinant humanized monoclonal anti-VEGF antibody fragment developed for intravitreal use. It has the ability to bind to all biologically active isoforms of VEGF. These recent developments led to their widespread use in clinical trials and even in clinical practice (Gabriel, 2013; Hammes, 2013).

4.4. Hormones

There are a few compounds that have been tried in DR that had not been localized to neurons in any parts of the nervous system to date and therefore by no means can be considered neuropeptides or neurohormones. Delivered via circulation to the target organs, by classical terminology, they can be considered hormones.

4.4.1 Erythropoietin

EPO is a glycoprotein hormone and a neurotrophic and endothelial survival factor that has both neuroprotective and vascular protective functions (Ghezzi and Brines, 2004; Zhong et al., 2007). EPO is upregulated in the

eye in DR. It is considered to act in a dual way as a neuroprotective factor by inducing angiogenesis (Marti et al., 2000) and has been found in both RPE and neuroretina in diabetic eyes. Its overexpression is unrelated to mRNA expression of HIFs (Garcia-Ramirez et al., 2008; Hernandez et al., 2006). It is possible that higher concentrations of EPO can be neuroprotective under certain circumstances (Hernandez and Simo, 2012). In early diabetic retinas, upregulation of EPO-R mRNA and protein and increased expression of EPO-R in neurons in different layers were seen. In the experimental STZ-induced diabetic rat, intravitreal injection of EPO caused a dose-dependent inhibition of the breakdown of the BRB after a characteristic cytokine response (Zhang et al., 2008). The protective effects of EPO against the breakdown of BRB are related to the known anti-inflammatory effect of EPO (Villa et al., 2003). Inhibition of EPO synthesis or action reduces retinal neovascularization in proliferative DR and inhibits endothelial cell proliferation *in vitro* (Watanabe, 2007). EPO can prevent or delay neuronal apoptosis. Apoptotic neurons in the ONL were essentially undetectable in EPO-treated diabetic eyes up to 4 weeks. At 6 weeks, some cell death was detectable suggesting a therapeutic window for EPO in DR treatment. Treatment with EPO at the dose inducing erythropoiesis is beneficial not only for retinal vessels but also for retinal neuron survival (Zhang et al., 2008). Apoptosis in EPO-R-expressing cells could be aborted by rapid EPO binding (Brines and Cerami, 2005). Intravitreal injection of EPO resulted in downregulation of EPO-R, VEGF, and VEGF receptor at 4 weeks of DR (Mitsuhashi et al., 2013).

Exogenous EPO administration by intravitreal or intraperitoneal injection in early diabetes may prevent structural vascular and neuronal damage in STZ-induced diabetic rats (Wang et al., 2011; Zhang et al., 2008). Intravitreal EPO administration is able to upregulate brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) expressions (Hu et al., 2011). The EPO molecule has been successfully altered to selectively eliminate erythropoietic and prothrombotic potencies, while preserving tissue-protective activities (Brines and Cerami, 2008). Administration of the EPO helix-B domain inhibits diabetes-related edema and significantly protects against neuroglial and vascular degenerative pathology (Brines et al., 2008; McVicar et al., 2011). Administration of suberythropoietic amounts of EPO delta peptide reduces oxidative stress in target tissues and prevents pericyte loss in the diabetic retina (Wang et al., 2010b). It ameliorates prosurvival signals involving Akt and reduces the loss of pericytes in the diabetic retina. EPO can reduce oxidative stress via different

mechanisms, acting as a direct and indirect antioxidant by reducing superoxide and other radicals; inducing glutathione peroxidase, which is down-regulated in the diabetic retina, thus improving the balance between pro- and antioxidative factors; and stimulating p-Akt predominantly in cells of the INL in the diabetic retina. EPO treatment reduces pericyte dropout via reduction of angiotensin-2 upregulation. It also protects RPE cells against the increase of permeability induced by diabetic conditions, and this effect is mainly mediated by the downstream signaling of Janus kinase 2 and PI3K/Akt pathways (Garcia-Ramírez et al., 2011).

Recombinant human erythropoietin (rhEPO) has roles in preventing and reversing dysfunction of retinal neurons and glial cells in early STZ-induced diabetic rats. The amplitudes of b wave and OPs showed no decrease in diabetic rats with rhEPO injection. The administration of rhEPO may be useful in the neural treatment of DR at early stage (Zhu et al., 2008).

4.4.2 Melatonin

Indoleamine hormone melatonin, secreted by pinealocytes (Stehle et al., 2011), is a powerful free radical scavenger and lipophilic antioxidant agent (Galano et al., 2011; Reiter et al., 2002). Apart from its main biological function (regulating circadian cycle — e.g., Lanfumey et al., 2013), melatonin shows activity against oxidative stress and inflammatory and apoptotic processes in diabetic complications (Chang et al., 2008; Klepac et al., 2006; Zwirski-Korczala et al., 2005). In addition, melatonin has strong mitochondria-protective effects (Kim and Lee, 2008). Melatonin treatment increases the mRNA expression and the activity of both Cu–Zn SOD and Mn SOD; thus, their activity enables a prolonged antioxidant capability under high-glucose conditions. It also reduces the apoptosis of the retinal neurons and caspase 3 mRNA expression. Melatonin first reduces the oxidative damage to mitochondria, therefore reducing the release of cytochrome *c* (Li et al., 2013a). Melatonin has double solubility, a high lipophilic and partial hydrophilic property, which means that it has a high degree of dispersion; it can easily pass through the cell and mitochondrial membrane to exert antioxidant activity. Melatonin's effect on retinal cell apoptosis is not due to the hypoglycemic effect itself. Additionally, its antioxidative and neuroprotective effects also depend on the intervention time (Kanter et al., 2006; Sudnikovich et al., 2007). Melatonin prevents the decrease in the ERG a and b waves and OP amplitude and the increase in retinal lipid peroxidation, retinal catalase activity, NOS activity, and TNF α , GFAP, and VEGF levels in DR associated with type 2 diabetes

(Salido et al., 2013). No toxicity has been reported after long-term use. Its safe administration makes this molecule an attractive agent for the treatment of early DR.

4.4.2 Exendin-4 and its analogs

Exendin-4 (E4), a 39-amino acid peptide, is a cognate receptor (GLP-1R) agonist. It was found in the saliva of the *Gila monster*. E4 has been shown to bind to GLP-1R in pancreatic cells and to promote the proliferation of β cells in the pancreas (Lupi et al., 2008). It was reported that both GLP-1 and E4 have neuroprotective properties. Since GLP-1R is also expressed in the rat retina (Zhang et al., 2009b), primarily in the Müller cells, a study was carried out to test the effects of E4a in retinal protection when delivered intravitreally. It has been shown that intravitreal injection of E4a could protect the rat retinas from diabetic insults, but the effect was transient (Zhang et al., 2011). The amplitudes of both b waves and OPs were reduced in diabetic rats, which were greatly improved at 1 month after E4a treatment. However, such protective effects disappeared at 3 months after the treatment. Such action might have occurred through a local effect of E4a by its receptor GLP-1R because intravitreal injection of E4a had no effect on body weight or blood glucose level. Morphological examination also confirmed the protective effects of E4a. The maintenance of retinal neuronal cells by E4a might be achieved by its antiapoptotic function, but the pathways involved merit further exploration. E4a might exert its protective function by upregulating its GLP-1R and GLAST expression in the Müller cells (Zhang et al., 2009b, 2011).

Exenatide is a synthetic version of E4. The appropriate concentration of exenatide protects RGC-5 cells from high- or low-glucose-induced RGC impairment and mitochondrial changes. Exenatide improved the survival rate of the cells and suppressed changes in the mitochondrial morphology. Treatment with exenatide significantly inhibited cytochrome *c* release and decreased the intracellular expression levels of Bax and caspase 3, whereas Bcl-2 was increased. It is concluded that a mitochondrial mechanism might play a key role in the protective effect of exenatide on the RGC-5 cells, and exenatide might be beneficial for patients with DR (Fu et al., 2012; Hao et al., 2012).

4.5. Neurotrophic factors

Neurotrophic factors are thought to play a major role in the development and maintenance of the neural connections. The possibility of their induction in the tissues and extrinsic application in degenerative disorders was coined long

time ago (Semkova and Kriegstein, 1999). Here, we describe some of the research regarding the use of neurotrophic factors in DR.

4.5.1 Brain-derived neurotrophic factor

In low quantities, BDNF is expressed in RGCs and Müller glia (Seki et al., 2003), and it is important for the survival of RGCs and amacrine cells (Cusato et al., 2002). Under normal conditions, it acts as a synaptic modulator in the retinal dopaminergic system (Cellerino et al., 1998). Seki et al. (2004) reported that the degeneration of dopaminergic amacrine and RGCs is accompanied by a reduction in BDNF levels in the retina of rats with STZ-induced diabetes and demonstrated the therapeutic potential of BDNF for treating neurodegeneration of dopaminergic amacrine cells in the diabetic retina by intraocular administration. BDNF protects the neurons through (i) TrkB receptors, (ii) insulin-responsive pathways, and (iii) reduction of systemic glucose level locally in the retina.

4.5.2 Nerve growth factor

NGF is the first discovered and best-characterized member of a neurotrophin family and is produced by a number of different cells, including the cells of the visual system (Levi-Montalcini, 1987). However, there is no report on retinal production. Intracocularly injected NGF can reach the posterior portion of the eye and also the brain through the optic nerve. The level of NGF in the retina of rats with diabetes decreased significantly compared with the level of NGF of normal animals. The expression of NGF receptor, TrkA, is markedly reduced in RGCs. NGF treatment prevents both diabetes-induced programmed cell death in the neuroretina and diabetes-specific pathology in the vascular retina in experimental diabetic models (Colafrancesco et al., 2011; Hammes et al., 1995b).

Topical NGF administration (delivered as eye drops) can prevent the deleterious events that can lead the progressive RGC death in diabetes. The protective action of NGF on RGCs suggests a potential benefit of NGF-based therapy for ocular disorders such as DR (Colafrancesco et al., 2011). This observation also suggests that NGF in cooperation with other factors can be part of a permanently active endogenous retinoprotective mechanism.

4.5.3 Pigment epithelium-derived factor

PEDF was first purified from the conditioned media of human RPE cells with neuronal differentiating activity (Barnstable and Tombran-Tink, 2004). PEDF is a potent angiogenesis inhibitor in the mammalian eye. Its

amount decreases in the retina and vitreous in DR, suggesting that loss of PEDF activity in the eye may contribute to development of proliferative DR (Boehm et al., 2003). Intravitreal injection of PEDF can suppress BRB breakdown and vascular permeability induced by VEGF (Liu et al., 2004), which implies that PEDF takes part in regulating vascular permeability. Single intravitreal injection of PEDF mRNA delivered with the help of a viral vector relieved BRB breakdown in STZ-induced diabetic rats for 6 months. The effect was associated with the downregulation of retinal VEGF mRNA and ICAM-1 expression and concomitant upregulation of occludin expression (Yu et al., 2010). PEDF inhibits retinal leukostasis in diabetic rats by reducing ICAM-1 expression via suppressing oxidative stress generation (Yamagishi et al., 2006b).

Diabetes decreases PEDF and GS levels in the retina. PEDF increases expression of GS against the effect of IL-1 β in early DR acting as an anti-inflammatory factor in retinal Müller cells (Shen et al., 2011). PEDF not only inhibits AGE-induced endothelial cell damage but also prevents AGE-elicited retinal vascular hyperpermeability in rats by suppressing NADPH oxidase activity (Inagaki et al., 2003; Yamagishi et al., 2006a). The inhibition of NADPH oxidase is a molecular target for the antioxidative and protective properties of PEDF in early DR (Yoshida et al., 2009). PEDF application may offer a promising strategy for halting the development of DR through its antioxidative properties and by blocking the harmful action of AGE on diabetic retinas (Yoshida et al., 2009). Besides vascular responses, PEDF administration restored the decrease in amplitudes of a and b waves of ERG, which was associated with suppression of GFAP expression (Li et al., 2002). Thus, PEDF application may be useful for long-term preventive or adjunctive therapy for DR (Yu et al., 2010).

4.6. Others

A plethora of studies offer miscellaneous approaches to treating DR. These range from chemical inhibition of certain metabolic processes through using natural compounds to photomodulation. Here, we provide a short summary of these less systematic approaches.

4.6.1 Inhibition of metabolic changes

RAGE is a signal-transducing receptor for AGEs. The engagement of RAGE or AGEs elicits the diabetic complications; thus, the inhibition of RAGE expression might represent a potential target for DR treatment.

Various inhibitors of AGE–RAGE system have therapeutic utility for DR (Chen et al., 2013).

One pharmacological strategy for the treatment of DR is to utilize the small nucleophilic hydrazine compound, aminoguanidine (AG). This drug is a selective inhibitor of AGEs and has been shown to prevent a range of diabetic vascular complications, including DR (Hammes et al., 1995a; Kern and Engerman, 2001; Kern et al., 2000). AG effectively prevents capillary closure, microaneurysm formation, and the depletion of NOS-containing neurons in the diabetic retina (Gardiner et al., 2003). The beneficial effect of AG has been underlined by the fact that AG is a non-AGE-specific inhibitor with antioxidant or inducible NOS properties (Sakata et al., 1999). AG inhibited the development of retinal lesions in diabetic rats (Kern and Engerman, 2001). AG delays the development of experimental DR in early stages by reducing AGE deposition in retinal blood vessels and ameliorating the ultrastructural pathological changes in retinal capillaries in diabetic rats. AG treatment prevented pericyte loss, endothelial cell proliferation, capillary occlusion, and acellular capillary formation. AG exerts protective effects through specific inhibition of AGEs (Luo et al., 2012). AG inhibits VEGF through inhibition of AGEs and VEGF-induced endothelial cell proliferation and migration (Luo et al., 2012). However, as we mentioned earlier, although AG is a useful inhibitor of protein glycation, it may be toxic from the viewpoints of vitamin B6 metabolism and also by inhibiting pyridoxal phosphate-dependent enzymes (Okada and Ayabe, 1995). AG is known to inhibit diamine oxidase that catalyzes determination of diamines such as histamine. This effect on histamine could be a problem if high-histamine-generating food is consumed, but again only if one is taking insufficient vitamin B6 (Taylor, 1986). For the aforementioned reason, AG is not used in human therapy because of it has serious toxicity issues, which are undetectable except by specific tests that are not part of standard clinical blood tests.

Similar to AG, pyridoxamine and purearin are also AGE inhibitors. These compounds have been evaluated for treatment of diabetic complications (Chen et al., 2012a; Luo et al., 2012). Both substances downregulate the expression levels of RAGE and VEGF in diabetic rat retina providing the opportunity for management of DR.

4.6.2 Compounds and extracts from plants

Using nonsynthetic compounds of natural origin is a popular approach these days in treating any diseases. In the PubMed database alone, there are more

than 500,000 entries found for the isolation and use of such substances. Applications include everything from antimicrobial use to cancer research. Some have been tried in DR too; we found more than 200 entries for that alone. Here is a selection of those entries, which met at least some success.

4.6.2.1 Flavonoids

Flavonoids acting as antioxidants may function as terminators of free radical chains and as chelators of redox-active metal ions that are capable of catalyzing lipid peroxidation. Flavonoids have the ability to act as a scavenger of reactive radical species and prevent the Fenton reaction.

Baicalein (5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one) is a type of flavonoid, originally isolated from the roots of *Scutellaria baicalensis*. It has been used for centuries as a folk medicine in China and Japan, among others, for the treatment of inflammatory disease. Given that inflammatory processes play an important role in the pathogenesis of DR, baicalein with its anti-inflammatory properties blocks high-glucose-induced microglial and astroglial activation, thereby preventing the secretion of inflammatory and/or cytotoxic factors. Baicalein treatment inhibited high-glucose-induced Müller cell dysfunction and VEGF overexpression and consequently protected neurons and vasculature from damage in DR (Yang et al., 2009).

Phlorizin is a 2'-glucoside of phloretin. It belongs to the group of dihydrochalcones, a group of flavonoids. Phlorizin is effective in treating diabetic complications. It significantly reduces fasting blood glucose concentrations and levels of AGE and remarkably inhibits apoptosis and the expression of GFAP in the retinas of db/db mice. From the 1636 proteins that were identified from the retina tissue, in total, 348 proteins were differentially expressed in db/db mice compared with the controls. Only 60 proteins in the retinas of db/db mice were found to be differentially changed following phlorizin treatment, including 33 proteins that were downregulated and 27 proteins that were upregulated (Zhang et al., 2013).

Eriodictyol is a strong antioxidative flavonoid extracted from *Eriodictyon californicum*. Eriodictyol has effects on retinal TNF α , VEGF, ICAM-1, and eNOS formation as well as the plasma lipid peroxidation and BRB integrity in STZ-induced diabetic rats. Treatment with eriodictyol reduces TNF α , VEGF, ICAM-1, and eNOS in the diabetic rat retina and suppresses diabetes-related BRB breakdown. It does not have hypoglycemic effects but is rather protective due to an unknown molecular mechanism (Bucolo et al., 2012).

Hesperidin is a flavanone glycoside found abundantly in citrus fruits. It significantly suppresses BRB breakdown and increases the retina thickness, reduces blood glucose and aldose reductase activity, and downregulates retinal TNF α , ICAM-1, VEGF, IL-1 β , and AGEs levels. Furthermore, treatment with hesperidin significantly reduces plasma malondialdehyde content and increases SOD activity in diabetic rats. Hesperidin therefore attenuates retinal abnormalities via antiangiogenic, anti-inflammatory, and antioxidative effects and through an inhibitory effect on polyol pathway and AGE accumulation (Shi et al., 2012).

The total flavonoids extracted from Flos Puerariae (TFF; *Pueraria* sp.) have potential protective effects in DR of diabetic mice. The thickness of the retina was significantly increased and the retinal capillary basement membrane thickness was reduced in the TFF-treated diabetic mice. It also attenuated the diabetes-induced apoptosis of retinal neurons with decreased Bax and increased ratio of Bcl-2 to Bax (Li et al., 2013b).

4.6.2.2 Polyphenols

Green tea (GT), widely studied for its beneficial properties protecting against brain ischemia, is a rich source of polyphenols. Diabetic rats received GT orally for 12 weeks, which fully restored GFAP, oxidative markers (ROS), glutamine synthetase, occludin, glutamate transporter, and receptor levels in the retina. The protective effects of GT were also evaluated *in vitro* in Müller and ARPE-19 cell lines. GT treatment decreased the level of ROS and restored the glutamate transporter levels in both cell lines. GT protects the retina against diabetes-induced glutamate toxicity via an antioxidant mechanism (Silva et al., 2013). GT also acts against retinal oxidative stress and proinflammatory parameters in diabetic rats. Both SOD and CAT enzymatic activities were restored close to normal in GT-treated STZ-induced diabetic animals. Expression of proinflammatory parameters (TNF α and VEGF) was significantly inhibited in GT-treated diabetic retinas, and GT prevented retinal capillary basement membrane thickening as well (Kumar et al., 2012).

Chlorogenic acid (CGA) is a polyphenol found in various agricultural products such as coffee, beans, potatoes, and apples and is formed by esterification of caffeic and quinic acid. It has antibacterial, anti-inflammatory, antioxidant, and anticarcinogenic activities and beneficial effects on glucose metabolism (Dos Santos et al., 2006; Kim et al., 2010). Treatment with CGA has a dose-dependent protective effect on the vascular system in DR; it reduces VEGF expression, restores occludin, and decreases BRB

breakdown. Partial restoration of claudin-5 level was also observed, but this effect was not significant. At the same time, ZO-1 level was not affected by CGA treatment (Shin et al., 2013).

Additionally, 4-methyl-2,6-diisobornylphenol facilitated reductions in the diabetes-induced degradation of photoreceptors and RGCs (Logvinov et al., 2010), which is probably associated mainly with the antioxidant properties of this agent.

4.6.2.3 Other plant extracts

Arctiin, a bioactive compound isolated from dry seeds of *Arctium lappa* L. (Fructus Arctii), has been reported to have antidiabetic activity. At week 16 in STZ-induced diabetes, the glycosylated hemoglobin level was significantly decreased in all of arctiin-treated groups, and the serum glucose level was also decreased in the rats treated with a high dose. Treatment with arctiin ameliorated retinal edema, detachment of the retina, and VEGF expression in the retina. Arctiin decreases the severity of diabetic complications, demonstrating the importance of this compound as an inhibitor of DR (Lu et al., 2012).

The total triterpenic acid mixture, isolated from *Cornus mas* (Fructus Corni), could reverse the abnormalities of the diabetic retina, such as the upregulation of mRNA levels of endothelin receptor and iNOS. It markedly reduced vasodilatation mediated by acetylcholine and NO (Su et al., 2007).

Tinospora cordifolia (TC), commonly known as Guduchi from the Menispermaceae family, has a long history of use in Ayurvedic medicine. TC contains many pharmacologically active ingredients, such as alkaloids, glycosides, and steroids. TC plays a role in the prevention and management of DR due to its antihyperglycemic, antiangiogenic, anti-inflammatory, and antioxidant properties and prevents cataract and vascular changes (Agrawal et al., 2012; Rathi et al., 2002). The possible mechanism of its hypoglycemic action is that TC may potentiate insulin effect (Stanely et al., 2000). Apart from its antihyperglycemic actions, TC has also shown some promising effects in preventing diabetic complications. TC reduced VEGF levels in rats (Agrawal et al., 2012). This reduction might be due to the presence of an active ingredient, octacosanol, which is reported to downregulate VEGF gene expression by inhibiting matrix metalloproteinases and nuclear translocation of NF- κ B and reduce its DNA binding activity (Agrawal et al., 2012). The expression of VEGF in diabetic retina can be regulated by PKC, whose activation is related to many vascular abnormalities. TC significantly reduced PKC activation and level.

There was also an effect on the retinal cytokine elevation (Agrawal et al., 2012). The inhibition of $\text{TNF}\alpha$ - and $\text{IL-1}\beta$ -induced inflammation by TC in DR may be attributed to the action of one or more of these compounds blocking the binding of cytokines to their specific surface receptors. Both glutathione and catalase levels increased in TC-treated group as compared with diabetics (Agrawal et al., 2012).

Treatment with curcumin showed significant hypoglycemic activity compared with the diabetic group. Curcumin positively modulated the anti-oxidant system; the elevation of proinflammatory cytokines and oxidative stress were prevented by curcumin. Curcumin prevented the structural degeneration of endothelial cell organelles and the increase in capillary basement membrane thickness and retinal Müller glial cells (prevents upregulation of GFAP and downregulation of GS) in the diabetic rat retina. Curcumin may have potential benefits in the prevention of DR (Gupta et al., 2011; Zuo et al., 2013).

Several studies demonstrated that dietary fiber can significantly reduce the risk of cardiovascular diseases and type 2 diabetes (Wursch and Pi-Sunyer, 1997). Oat is a paradigm natural food supplement with a broad spectrum of beneficial biochemical and cell biological effects, based on its ability to reduce hyperglycemia-induced ROS overproduction. The interference in the overproduction of ROS by oat in diabetic rats normalizes parameters of oxidative stress in the retina and prevents the activation of major pathways involved in hyperglycemia-induced vascular damage. Oat could have potential benefits in the prevention of the onset and progression of DR. Oat reduced and even normalized downstream effectors of vascular response to injury. Besides reduction of ROS overproduction, it also has an indirect AGE-inhibiting effect; it disrupts the detrimental AGE–RAGE–NF- κ B pathways. Oat treatment also attenuated the increased VEGF and $\text{TNF}\alpha$ levels (Al-Malki, 2013).

Grape seed proanthocyanidin extracts (GSPEs) have been reported to possess a variety of potent properties including antioxidant, anti-inflammation, radical scavenging, and antitumor activities (Houde et al., 2006; Shao et al., 2003; Vayalil et al., 2004). GSPE-treated diabetic rats showed significant reductions in AGE levels and an antinonenzymatic glycation effect compared with untreated diabetic rats. GSPE had protective effects on the retinal tissue of diabetic rats. Some affected proteins returned to normal levels accompanying AGE recovery after GSPE therapy in DR. These proteins are most likely to participate in the regulation of small heat-shock proteins (Li et al., 2008b).

Wolfberry (*Lycium barbarum*) is a fruit, which contains large amounts of polysaccharides, phenolics, lutein, and zeaxanthin in ester forms, which are neuroprotective in the progression of macular degeneration (Faitova et al., 2006; Inbaraj et al., 2008). Dietary wolfberry and its bioactive constituents, zeaxanthin and lutein, functioned as modulators of cell survival/death signaling pathways through targeting pathways in cAMP-activated protein kinase and forkhead O transcription factor 3 α signaling, resulting in normalization of cellular ROS and subsequent attenuation of endoplasmic reticulum stress. This could lead to prevention of apoptosis and restoration of retinal structure in type 2 diabetic db/db mouse. RPE integrity and retinal structure were altered at early stages of diabetes in the db/db mouse model, and this abnormality could be attenuated by dietary wolfberry, which prevented or delayed the onset of the disease of DR (Tang et al., 2011).

Ginkgo biloba leaf extract (GBE) contains many different flavone glycosides and terpenoids, which have antioxidant action and anti-inflammatory effects, suppressing the production of reactive oxygen and nitrogen species (Ilieva et al., 2004). GBE inhibits the increase of low-density lipoprotein (LDL) and prevents oxidative damage to mitochondria, suggesting that its beneficial effects on neurodegenerative diseases are related to prevention of chronic oxidative damage (Yoshikawa et al., 1999). GBE blunts some of negative effects due to hyperglycemia, such as oxidation, inflammation, and VEGF expression, which are the main causes of DR (Bucolo et al., 2013).

Zeaxanthin is one of the dietary carotenoids that are specifically concentrated in the retina, especially in the macular region. Zeaxanthin significantly inhibits diabetes-induced retinal oxidative damage and elevation of VEGF and ICAM-1, abnormalities that are associated with the pathogenesis of DR. These results suggest that zeaxanthin supplementation has the potential to inhibit the development of retinopathy in diabetes (Kowluru et al., 2008). Lutein also prevents diabetes-induced visual dysfunction. It inhibits oxidative stress, thereby preserving the integrity of the neuroprotective pathways in early diabetic retina (Sasaki et al., 2010).

Astaxanthin is a carotenoid with powerful antioxidant properties that exists naturally in various plants, algae, and seafood (salmon, trout, krill, crayfish, shrimp, and other crustaceans), yeast, and the feathers of some birds. Astaxanthin was shown to reduce the apoptosis of RGCs and improve the levels of oxidative stress markers, including superoxide anion, malondialdehyde (a marker of lipid peroxidation), 8-hydroxy-2-deoxyguanosine (indicator of oxidative DNA damage), and Mn SOD activity in the retinal tissue of db/db mouse. Therefore, astaxanthin

may be further developed as an antioxidant drug to treat DR (Dong et al., 2013).

Cilostazol is a quinolinone-derivative medication used in the alleviation of the symptoms of intermittent claudication in individuals with peripheral vascular disease. In the retinas of OLETF rats, cilostazol treatment reduced GFAP and VEGF expression and the number of TUNEL-positive cells (Jung et al., 2013).

Berberine and rosiglitazone significantly decreased PPAR γ expression in diabetic retina, while berberine and fenofibrate obviously increased PPAR α and PPAR δ expressions in diabetic retina. Berberine modulates PPAR $\alpha/\gamma/\delta$ protein levels in diabetic retina, which may help to ameliorate retinopathy complication induced by STZ-induced diabetes and a high-carbohydrate/high-fat diet. Berberine might be a more beneficial drug to treat diabetic retinal complication compared with fenofibrate and rosiglitazone (Zhou and Zhou, 2007).

Cannabinoids are known to possess therapeutic properties including antioxidant, anti-inflammatory, and NMDA receptor activation-blocking activity (Hampson et al., 1998; Marsicano et al., 2002). Nonpsychotropic CBD demonstrated its neuroprotective effects via anti-inflammatory and BRB-preserving effects in diabetic rats along with a prominent Müller glial cell activation (El-Remessy et al., 2010). Exposure of retinal Müller glial cells to high glucose levels stimulates oxidative stress and peroxynitrite formation (Shelton et al., 2007). Diabetes-induced oxidative and nitrative stresses alter the function of Müller cells by impairing GS activity, leading to glutamate neurotoxicity and sustaining retinal neuronal cell death. Treating diabetic animals with CBD blocked the increases in oxidative and nitrative stresses and significantly reduced the number of apoptotic cells. CBD restores GS activity by reducing its tyrosine nitration in diabetic animals. This effect was associated with a significant reduction in Müller glial cell activation, which confirms the preservation of its morphology and function in diabetic animals (El-Remessy et al., 2010). CBD represents a novel therapeutic agent in the treatment of diabetes and stress-mediated retinal damage.

Resveratrol, a natural plant-derived phytoalexin, treatment effectively blocked the diabetes-induced increase of vessel leakage, pericyte loss, and VEGF protein levels in the mouse retinas. Resveratrol is effective in decreasing vascular lesions and VEGF induction in mouse retinas of early diabetes (Kim et al., 2012).

Astragalgin, a 3-O-glucoside extracted from *Astragalus membranaceus* and *Astragalus propinquus*, has a history of use as a herbal medicine in systems

of traditional Chinese medicine and has many pharmacological properties. It has been shown that astragalin decreases the overexpression of VEGF in cultured Müller glial cells after 3 days of treatment and alleviates the effects of high glucose. Astragalin has promising applications in preventing and treating DR (Ke et al., 2012).

Ginseng plants (Araliaceae), including North American ginseng (*Panax quinquefolius*) root extracts has multiple pharmacological actions because of their diverse phytochemical constituents. Ginsenosides are its major bioactive factors. Ginsenosides or panaxosides are the derivatives of protopanaxatriol, a class of steroid glycosides, and triterpene saponins (Wang et al., 2008). Bioactives of ginseng possess antioxidant properties, quenching free radicals, protecting LDLs from oxidation, and inhibiting lipid peroxidation (Kim and Park, 2003). Alcoholic ginseng root extract (per os daily) was administered for models of both type 1 (C57BL/6 mice with STZ-induced diabetes) and type 2 diabetes (db/db mice) for 2 or 4 months. In both the heart and retina of diabetic animals, ginseng treatment significantly prevented oxidative stress and diabetes-induced upregulation of extracellular matrix proteins and vasoactive factors. These data indicate that North American ginseng prevents the diabetes-induced retinal and cardiac biochemical and functional changes probably through inhibition of oxidative stress (Sen et al., 2013).

The extract, named fenugreek, of *Trigonella foenum-graecum* L. contains simple alkaloids (trigonelline, choline, gentianine, and carpaine) and other pyridines and pyrroles. Treatment with fenugreek resulted in marked inhibition in the expression of inflammatory (TNF α and IL-1 β) and angiogenic molecular biomarkers (VEGF and PKC β) and positive modulatory effects on retinal oxidative stress. The fenugreek-treated retinas did not show vascular leakage with and had relatively reduced thickening of capillary basement membrane. Fenugreek has great potential in preventing diabetes-induced retinal degeneration in humans with regular consumption in the specified dosage (Gupta et al., 2014).

4.6.3 Miscellaneous compounds

Several compounds of miscellaneous origin have been tested to counteract experimental DR symptoms. An incomplete list of these follows.

4.6.3.1 Retinoic acids

Retinoic acid (RA) and its derivatives are essential signaling molecules throughout life and may play an important role in the induction of glial cell

line-derived neurotrophic factor (GDNF; Nishikiori et al., 2007a; Thang et al., 2000), which is a differentiation factor in the retina (Thang et al., 2000; Wu et al., 2004a). RA inhibits the apoptosis; a marked decrease in apoptotic cell number could be observed during the development of DR after treatment with RA (Nishikiori et al., 2008). A possible protective mechanism might be that RA protects retinal cells by inducing GDNF production in glial cells in the retina (Nishikiori et al., 2007a,b). RAs are promising drugs for diabetic ophthalmic disease because they have anticataractogenic effects for diabetic cataracts (Nishikiori et al., 2007b), BRB-protective effects for vascular integrity (Miyajima et al., 2005; Nishikiori et al., 2007a), and neuroprotective effects against apoptosis in DR (Nishikiori et al., 2008).

4.6.3.2 Calpain inhibitors

Calpains are a family of 14 calcium-regulated, intracellular cysteine proteases, which modulate cellular functions by limited specific proteolysis (Huang and Wang, 2001). Calpains are activated by locally increased Ca^{2+} levels through calcium channels and intracellular stores (Camins et al., 2006). Inhibition of calpain signaling is a therapeutic target for several pathological conditions, including DR. Calpain plays a crucial role in metabolically induced RGC degeneration caused by DR and oxidative stress. The combination of antioxidants and calpain inhibition offers important opportunities for future neuroprotective treatment against RGC death. Levels of calpain are regulated by an endogenous specific inhibitor, calpastatin. An exogenous calpain inhibitor, SNJ-1945, has shown strong ability to penetrate BRB after oral administration (Shirasaki et al., 2005). Four weeks after the induction of diabetes, degeneration of RGCs and their axons was successfully prevented by SNJ-1945. This compound was able to suppress mRNA overexpression of calpain-1, and it has a significant preventive effect against diabetic changes to RGC synapses in DR. Proapoptotic end products of cleaved α -fodrin were significantly reduced in DR by oral administration of SNJ-1945 (Shanab et al., 2012).

4.6.3.3 Sitagliptin

Sitagliptin is an oral antihyperglycemic drug of the dipeptidyl peptidase-4 inhibitor class. Treatment with sitagliptin prevented the changes in the endothelial subcellular distribution of the tight junction proteins (occludin and claudin-5) induced by diabetes. Sitagliptin decreased the nitrosative stress, the inflammatory state, and cell death in diabetic retinas. It allowed

the recovery of a number of CD34+ cells present in the bloodstream with levels similar to their number in controls and increased the adhesion ability of endothelial progenitor cells to the retinal vessels and exerted beneficial effects on the BRB integrity in ZDF rat retinas (Gonçalves et al., 2012).

4.6.3.4 Carnosine

Carnosine (beta-alanyl-L-histidine) is an antiglycating dipeptide of the amino acids beta-alanine and histidine. It is highly concentrated in muscle and brain tissues. Carnosine has a number of antioxidant properties and has been proven to scavenge ROS and alpha-beta-unsaturated aldehydes formed from peroxidation of cell membrane fatty acids during oxidative stress. Oral carnosine treatment prevented retinal vascular damage after 6 months of experimental hyperglycemia. The protection was associated with a significant induction of Hsp27 in activated glial cells and normalization of increased angiotensin-2 levels in diabetic retinas. Oral carnosine treatment protects retinal capillary cells in experimental DR, independently of its other biochemical functions (Pfister et al., 2011).

4.6.3.5 Memantine

Vitreoretinal glutamate levels are elevated in experimental diabetes (Lieth et al., 1998). The expression of the NMDA glutamate receptors is upregulated in the diabetic retina suggesting a role of glutamate excitotoxicity. Memantine is an NMDA receptor blocker and acts as an uncompetitive antagonist (Smith, 2002). Long-term treatment with memantine significantly improves retinal function and prevents RGC loss in STZ-induced diabetic rats. Memantine also significantly reduces elevated VEGF protein levels in the retina and vitreous fluid and BRB breakdown in the retinas of diabetic animals. Memantine significantly improved amplitudes of ERG a and b waves. Another possible mechanism of the neuroprotective effect of memantine in the retinas of STZ-induced diabetic rats could be the inhibition of retinal type 3 serotonin and nicotinic Ach receptors, although elevated serotonin and acetylcholine levels have not been demonstrated in diabetic retina (Kusari et al., 2007).

4.6.3.6 Nepafenac

Nepafenac is a prodrug of amfenac, a nonsteroidal anti-inflammatory drug that inhibits COX-1 and COX-2 and the synthesis of proinflammatory prostaglandins (Kapin et al., 2003). The administration of nepafenac inhibits functional and morphological lesions characteristic to the early stages of DR.

It also inhibited the diabetes-induced apoptosis of endothelial cells and pericytes and the degeneration of retinal capillaries. In the retina, nepafenac partially inhibits the diabetes-induced activation of executioner caspases, such as caspase 3 and caspase 6. It may also be a direct scavenger of superoxide. Topically applied nepafenac decreased the OP latency despite persistent hyperglycemia. Nepafenac administered via eye drops reaches the retina of rats in sufficient concentration to inhibit multiple biochemical and morphological abnormalities in diabetes (Kern et al., 2007).

4.6.3.7 Vitamin D

Many studies provide evidence that lack of vitamin D has a role in pathogenesis of both type 1 and type 2 diabetes (Takiishi et al., 2010), including their accompanied syndromes such as DR (Aksoy et al., 2000; Albert et al., 2007; Kaur et al., 2011). Vitamin D is a potent inhibitor of retinal neovascularization in DR (Albert et al., 2007) through decreasing the level of VEGF (Ren et al., 2012).

4.6.3.8 Hydrogen sulfide

Hydrogen sulfide (H_2S) is the most recent addition to endogenous gas-transmitter family. It played beneficial roles in several diseases, and a lower level of H_2S was observed in the blood of diabetic patients and STZ-treated rats (Jain et al., 2010). Treatment with exogenous H_2S prevented diabetic neurodegeneration and enhanced expressions of synaptophysin and BDNF in retinas. Reduction was seen in BRB permeability and the number of acellular capillaries following treatment with exogenous H_2S in retinas of STZ-induced diabetic rats. This could be explained by the concomitant reduction in vitreous VEGF content and gene expression of HIF-1 α and VEGF-R2 and increased expression of occludin. Treatment with H_2S not only functioned as a direct scavenger of ROS but also influenced some important enzymes associated with oxidative stress. Treatment with H_2S or NaHS, a donor of H_2S , attenuated STZ-induced retinopathy, possibly through abating oxidative stress and suppressing inflammation (Si et al., 2013).

4.6.3.9 Hydrogen saline

If hydrogen is dissolved in physiological saline under high pressure to a supersaturated level, it becomes hydrogen saline ($H(2)$ saline or hydrogen-rich saline; Sun et al., 2011). It has considerable antioxidant and anti-inflammatory properties, and it also suppresses oxidative stress-induced injury. In the retina, it has protective effects against glutamate-induced

toxicity (Wei et al., 2012). H(2)saline treatment could depress caspase 3 activity, reduce retinal apoptosis and vascular permeability, and prominently attenuate the retinal parenchyma thickening that resulted from DR (Xiao et al., 2012b). In H(2)saline-treated STZ-induced diabetic rats, the diabetes-induced reduction of b wave amplitudes and OPs was restored, and the BRB breakdown and histological changes in the inner retina were reversed. Furthermore, H(2)saline reduced oxidative stress, increased anti-oxidant enzyme activities, and preserved synaptophysin and BDNF levels in diabetic rat retina (Feng et al., 2013).

4.6.3.10 N-acetylcysteine

Macrophage/microglia activation, pericyte loss, and endothelial/perivascular cell changes occur early in the pathogenesis of DR. These changes are associated with an increase in plasma markers of oxidative stress and inflammation and are minimized by treatment with a well-known free radical scavenger, N-acetylcysteine (Tsai et al., 2009).

4.6.3.11 Fasudil

Fasudil protects the vascular endothelium by inhibiting neutrophil adhesion and reducing neutrophil-induced endothelial injury. Intravitreal administration significantly increased eNOS phosphorylation, whereas it reduced ICAM-1 expression, leukocyte adhesion, and the number of damaged endothelial cells. Neutrophils from DR patients showed significantly higher adhesion to cultured endothelium and caused endothelial apoptosis, which was significantly reduced by fasudil. Fasudil may alter the Rho/Rho kinase pathway, which plays a critical role in diabetic retinal microvasculopathy; therefore, Rho kinase inhibition may become a new strategy in the management of DR, especially in its early stages (Arita et al., 2009).

4.6.3.12 Photobiomodulation

Daily 670 nm photobiomodulation treatment resulted in a significant inhibition in diabetes-induced RGC death and a 50% improvement of the ERG amplitude, especially photopic b wave responses. Photobiomodulation did not alter cytochrome oxidase activity in the retina or in the cultured retinal cells, but inhibited diabetes-induced superoxide production and preserved Mn SOD expression *in vivo*. It essentially prevented the diabetes-induced increase in leukostasis and expression of ICAM-1. It is a noninvasive, inexpensive, and easily administered simple adjunct therapy to attenuate the development of DR (Tang et al., 2013).



5. CONCLUDING REMARKS

As it has been shown in the preceding text, there are numerous open options to research DR and its treatment possibilities. Perhaps, it is conspicuous for the reader that symptoms of DR are identical regardless of which type of diabetes causes this syndrome. Therefore, both type 1 and type 2 diabetes models can be used to study treatment options. However, not all the aforementioned diabetes models are equally efficient in producing all the symptoms observed in human patients. In our opinion, there can be two main causes of this mismatch: (i) in humans, diabetes in most cases is controlled (i.e., patients use medications to lower their blood glucose levels to acceptable concentrations). Early good glycemic control and prevention of the abnormalities of apoptosis are important for diabetic patients to prevent the development and progression of sight-threatening DR (Gao et al., 2009b; Hammes, 2013). Also, (ii) the relatively short life span of the most often used animal models may be a problem. Rats and mice (especially those inflicted with diabetes) do not live long enough to produce all the alterations in circulation and develop compensatory mechanisms for the diabetic damage, and experimenters very often use young adult specimens for their studies. Therefore, efforts should be made to develop chronic models with controlled blood sugar levels and to achieve long-term survival (more than a year after diabetes induction) of experimental animals to produce all the symptoms (or as many of them as possible). These constraints may also explain why mice have been used less frequently as models in studying DR. Genetically modified transgenic or knockout mice are widely used in other areas of biomedical research. Several inbred strains are also used for studying diabetes and DR, but they could be more extensively studied if models were chosen carefully. Thus, considering all the requirements, long-term STZ-induced diabetes in rats with carefully controlled blood glucose level could be one of the best options to study diabetic complications such as DR. Among the genetically diabetic strains, spontaneously type 2 diabetic rats could be the best for this purpose; our bias is clearly toward the OLETF and SDT rats, because they produce the symptoms that are closest to the human disease. *Ex vivo* and *in vitro* models may be useful in studying certain aspects of pathogenesis of DR and test drug candidates but may not be sufficiently sophisticated to study all aspects, especially not those related to real functional and electrophysiological properties.

As for the protective compounds, besides the strict blood glucose control, two major lines of research should be pursued in the future: (i) to find

extremely selective blockers of specific antiapoptotic pathways or generate a mixture of neurotrophic factors to keep nerve cells alive under damaging metabolic conditions and (ii) to provide general protection against ischemia, which facilitates the formation of ROS, AGE, and ALE. For the first approach, compounds acting at different levels of the signal transduction pathway have been tried. Among inhibitors of neuron loss, octreotide, a peptide analog of SST acting at sst2A receptors, has been found the most effective. If a nonpeptide analog could be developed for this receptor, chances for routine medicinal use would definitely increase. A similar notion is true for PACAP. Currently, only shortened or modified versions of the peptide are available for experimentation (Bourgault et al., 2008); non-peptide PACAP agonists do not exist. Experiments are in progress to show if PACAP is protective in the form of artificial tears (D. Reglodi, unpublished results). Likewise, if an optimal mixture of trophic factors (IGF, NGF, BDNF, and PEDF) could be created, it could be applied topically. PEDF is a particularly promising candidate, and since its production is restricted to the retina, unwanted side effects can be minimal. Hormones and hormone-like materials (angiotensin and EPO) present in several tissues of the body carry less hope because of their potentially strong side effects. A more promising way is to fight angiogenesis with anti-VEGF compounds or antibodies. In fact, the latter form of treatment gains momentum in clinical trials either alone or in combination with surgical treatments in the clinical practice. A similarly promising approach is to fight ROS, AGE, and ALE formation. GT, ginseng and *Ginkgo* extracts, curcumin, and astragaline are particularly potent. Their administration is simple, but it should be noted that these compounds have to be administered over a long period of time. This can be done in the form of nutritional supplements. Adverse action or side effects are improbable.

As the final point, a desirable experimental design will be described here, which may lead to results that can be successfully translated to humans. Aged OLETF and/or SDT rats or, alternatively, animals with STZ-induced controlled long-term diabetes (at least 1-year duration) should be used. Metabolic parameters should be monitored every day, blood sugar level should be kept around 10–15 mmol/l. Control ERG measurements should be performed before DR develops. When early symptoms of DR (e.g., vascular leakage, retinal edema, and deteriorating ERG) appear, protective treatment regimes should be initiated. Besides keeping an untreated diabetic group alive as long as possible, one experimental group could receive specific neuroprotective/antiapoptotic treatment and the other nonselective anti-ROS

compound(s). The possible protective agents should be administered either topically (e.g., in case of neuropeptide analogs or growth factors) in a pre-determined manner (e.g., monthly intravitreal injection) or per os daily (anti-ROS compounds). This way, the efficacy of specific and generic treatment strategies and their long-term manageability could be determined. The animals that die during the experimental period should be taken to autopsy to assess the severity of diabetes and DR. There are obviously several difficulties in executing such a wide-scope and long-term experiment with enough animals to draw valid conclusions. However, at this stage of DR research, one or more research groups have to take the risk to produce translatable results.

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Stereological and Allometric Studies on Neurons and Axo-Dendritic Synapses in Superior Cervical Ganglia

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Abstract

The superior cervical ganglion (SCG) plays an important role in neuropathies including Horner's syndrome, stroke, and epilepsy. While mammalian SCGs seem to share certain organizational features, they display natural differences related to the animal size and side and the complexity and synaptic coverage of their dendritic arborizations. However, apart from the rat SCG, there is little information concerning the number of SCG neurons and synapses, and the nature of relationships between body weight and the numbers and sizes of neurons and synapses remain uncertain. In the recognition of this gap in the literature, in this chapter, we reviewed the current knowledge on the SCG structure and its remodeling during postnatal development across a plethora of

large mammalian species, focusing on exotic rodents and domestic animals. Instrumentally, we present stereology as a state-of-the-art 3D technology to assess the SCG 3D structure unbiasedly and suggest future research directions on this topic.



1. INTRODUCTION

While mammalian superior cervical ganglion (SCG) neurons and their axo-dendritic synapses seem to share certain organizational features, they display natural differences related to such factors as animal size, animal side, and the complexity and synaptic coverage of their dendritic arborizations (Forehand, 1985; Loesch et al., 2010; Purves and Lichtman, 1985; Purves et al., 1986; Smolen and Beaston-Wimmer, 1986). Larger species possess SCG neurons which exhibit greater convergence, more complex arborizations and a greater proportion of axo-dendritic compared to axo-somatic synapses (Forehand, 1985; Purves and Lichtman, 1985; Purves et al., 1986). However, apart from the rat SCG, there is still little information concerning numbers of SCG neurons and axo-dendritic synapses, and the nature of the relationships between body weight and the numbers and sizes of neurons and synapses remain uncertain.

Recognizing this gap in the literature, over the past 10 years, we have investigated the quantitative structure of SCG from a broad range of mammals, including both small laboratory and exotic rodents such as rats and capybaras (Loesch et al., 2010; Ribeiro et al., 2004), pacas (Abrahão et al., 2009; Melo et al., 2009), guinea pigs (Toscano et al., 2009), and preás (Ladd et al., 2012) and also domestic large mammals such as dogs and cats (Fioretto et al., 2007), sheep (Fioretto et al., 2011), and horses (Fioretto et al., 2007; Loesch et al., 2010; Ribeiro et al., 2004). In our own published work, the quantitative assessment of SCG structure—neurons, synapses, neuropil, vessels, and connective tissue among others—was conducted using 3D technology employing the design-based stereology.

By pursuing our investigations in various mammals, we have found a suitable animal model for studying allometric relationships between SCG volume and neuron number and between SCG volume and synapse number. The model provides a structural foundation, which is of paramount importance for understanding “the aging sympathetic nervous system” and for clinical research, since SCG plays an important role in grave neuropathies including Horner’s syndrome (Bell et al., 2001; Boydell, 1995; Kisch, 1991), stroke (Campbell et al., 2000; Palmer, 2007; Shaibani et al., 2006), and epilepsy (Kokaia et al., 1994).



2. STRUCTURAL ORGANIZATION OF SCG

2.1. Macrostructure

Due to the SCG's role in some neuropathies, it is useful to know SCG's macrostructure including its dimensions and accurate localization, especially when pursuing operative procedures in the neck of animals, for example in cervical tumors, Horner's syndrome (Boydell, 1995; Panciera et al., 2002), Wobbler's syndrome (Nanai et al., 2006), or during the treatment of guttural pouch mycoses in horses (Freeman, 2006; Millar, 2006).

2.1.1 Localization

The cervical part of the autonomic nervous system in mammals consists of a vast array of nerves and ganglia connected to the central nervous system on one side and to the viscera on the other side. The sympathetic cervical chain lies dorsally to the vagus nerve and ventrally to transverse processes of vertebrae and prevertebral muscles. Two paravertebral ganglia are present in the neck: SCG or cranial cervical ganglion and the caudal cervical ganglion or stellate ganglion (when the latter includes between one and three uppermost thoracic sympathetic ganglia). In addition, a small intermediate ganglion is sometimes found, that is, the middle cervical ganglion (Baljet and Drukker, 1979; Gabella, 2004; Hedger and Webber, 1976).

The SCG of rats, guinea pigs, preás, cutias, pacas, capybaras, sheep, dogs, cats, and horses is located near the base of the skull and deeply into the bifurcation of the common carotid artery, in the origin of the external and internal carotid arteries close to the distal ganglion of the vagus nerve (Abrahão et al., 2009; Coppi and Loesch, 2011; Fioretto et al., 2007, 2011; Gabella, 2004; Hedger and Webber, 1976; Ladd et al., 2012; Loesch et al., 2010; Melo et al., 2009; Ribeiro, 2006; Ribeiro et al., 2004; Toscano et al., 2009) (Fig. 2.1). In camels, SCG was located on the rostro-lateral aspect of m. longus capitis, ventral to m. sternomastoideus and covered by the mandibular gland. The caudo-dorsal border of the ganglion was parallel to the vagus nerve, while the rostro-ventral margin ran along the carotid sinus branch of the glossopharyngeal nerve. The rostro-dorsal end of the ganglion pointed toward the jugular foramen and the caudo-ventral apex encompassed the rostral extremity of the cervical sympathetic trunk (Sheng et al., 1998). By the same token, sympathetic and parasympathetic nerve structures of young pigs, SCG, and distal vagus ganglion (DVG), respectively, were just close to each other without any connection and

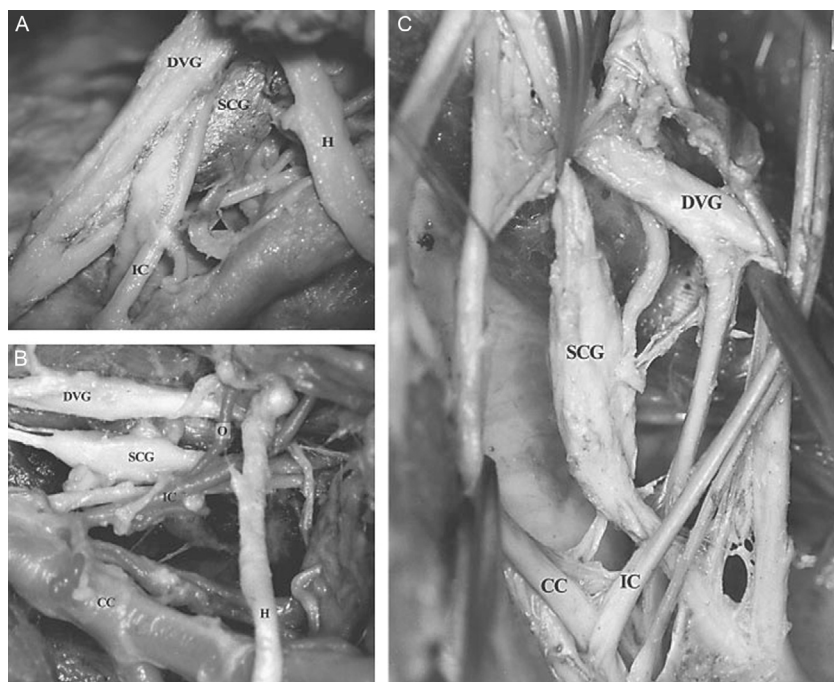


Figure 2.1 Macrostructure of the SCG of an adult dog (A), cat (B), and horse (C). The SCG is located near the distal vagus ganglion (DVG) and related to internal carotid (IC) artery, hypoglossal nerve (H), common carotid artery (CC), and occipital artery (O) in the cat. Authorization will be required once the review process is accomplished. *Image and legend extracted from Fioretto et al. (2007).*

located near the base of the skull and could descend to the first cervical vertebra level (Kabak et al., 2005; Pospieszny and Bruzewicz, 1998).

Topographically speaking, the SCG is located dorso-cranially to the larynx, cranio-medially to the thyroid gland, and ventro-caudally to the tympanic bulla. This ganglion is also very closely adjacent to the hypoglossal nerve. In all species studied, the SCG was white in color and roughly spindle shaped, independently of the animal age (Abrahão et al., 2009; Coppi and Loesch, 2011; Fioretto et al., 2007, 2011; Ladd et al., 2012; Loesch et al., 2010; Melo et al., 2009; Ribeiro, 2006; Ribeiro et al., 2004; Toscano et al., 2009).

2.1.2 Macromorphometry

The macromorphometric aspects of the SCG—length, width, and thickness—vary greatly according to the animal species and animal age



Figure 2.2 The complete catalog of mammalian species. (A) (from left to right): rats, guinea pigs, preás, cutias, pacas, capybaras and (B) (from left to right): cats, dogs, sheep, horses—from which superior cervical ganglia (SCGs) were harvested and investigated in our laboratory over the past 10 years. Scale bars: 15 mm (A) and 30 mm (B).

investigated, that is, rats, guinea pigs, preás, rabbits, cutias, pacas, capybaras, cats, dogs, sheep, horses, and camels (Abrahão et al., 2009; Coppi and Loesch, 2011; Fioretto et al., 2007, 2011; Kabak, 2007; Ladd et al., 2012; Loesch et al., 2010; Melo et al., 2009; Najafi and Nejati, 2009; Ribeiro, 2006; Ribeiro et al., 2004; Sheng et al., 1998; Toscano et al., 2009) (Fig. 2.2A and B). Data concerning SCG's dimensions in different animal species and ages have been collated in form of a table (Table 2.1).

2.1.3 Target organs

In mammals, the SCG provides sympathetic innervation to the head and neck as well as to the mandible, submandibular and pineal glands, cephalic blood vessels, choroid plexus, eye, carotid body, salivary, and thyroid glands (Arbab et al., 1986; Bowers et al., 1984; Cardinali et al., 1981; Gibbins, 1991; Gibbins et al., 1996; Morris et al., 1999; Reuss and Moore, 1989; Uddman et al., 1989; Voyvodic, 1989). Removal of SCG brings about several neuroendocrine dysfunctions in mammals, including the disruption of water balance in pituitary stalk-sectioned rats and the alteration of the

Table 2.1 Mean values of macromorphometric parameters: length (mm), width (mm), thickness (mm) of superior cervical ganglia (SCG) from a plethora of mammalian species: rats, preás, guinea pigs, cutias, pacas, capybaras, cats, dogs, pigs, sheep, horses, and camels—at different time points (newborn, young, adult, and elderly) during postnatal development

Species	Age	Length (mm)	Width (mm)	Thickness (mm)	References
Camels	Adult	17.50	5.00	3.00	Sheng et al. (1998)
Pigs	Young	3.16	1.49	1.11	Kabak (2007)
Sheep	Young	8.52	2.31	2.00	Najafi and Nejati (2009)
		7.10	4.40	2.40	Fioretto et al. (2011)
Rats	Adult	3.3	0.90	–	Ribeiro et al. (2004)
Horses		19.7	7.70	–	
Capybaras		15.9	5.70	–	
	Young	13.27	5.35	–	Ribeiro (2006)
Dogs	Young	3.50	1.80	–	Fioretto et al. (2007)
	Adult	4.40	2.34	–	
Cats	Young	3.30	0.80	–	
	Adult	3.30	1.60	–	
Horses	Young	19.6	5.90	–	
	Adult	26.3	6.40	–	
Guinea pigs	Newborn	2.25	1.11	0.63	Toscano et al. (2009)
	Young	2.70	0.99	0.67	
	Adult	3.70	1.58	0.79	
	Elderly	3.83	1.45	1.05	
Pacas	Newborn	4.50	2.21	1.26	Melo et al. (2009)
	Young	5.37	1.98	1.31	
	Adult	7.36	3.17	1.59	
	Elderly	7.66	2.86	2.20	
Pacas	Newborn	3.74	1.66	1.23	Abrahão et al. (2009)
	Young	5.60	1.34	1.32	
	Adult	7.40	1.69	1.53	
	Elderly	7.70	2.00	2.19	

Table 2.1 Mean values of macromorphometric parameters: length (mm), width (mm), thickness (mm) of superior cervical ganglia (SCG) from a plethora of mammalian species: rats, preás, guinea pigs, cutias, pacas, capybaras, cats, dogs, pigs, sheep, horses, and camels—at different time points (newborn, young, adult, and elderly) during postnatal development—cont'd

Species	Age	Length (mm)	Width (mm)	Thickness (mm)	References
Rats	Adult	3.20	0.8	0.63	Loesch et al. (2010)
Capybaras	Adult	16.5	5.4	4.67	
Horses	Adult	6.80	20.6	5.79	
Preás	Newborn	1.29	0.84	0.82	Ladd et al. (2012)
	Young	2.08	1.03	0.78	
	Adult	2.30	1.06	0.80	
	Elderly	2.08	1.17	0.86	
Cutia	Newborn	2.68	2.16	1.14	Ladd (2007), unpublished data from a Master thesis
	Young	3.11	1.96	1.14	
	Adult	4.55	2.36	1.28	

Data were extracted from the references provided on the rightmost column.

normal photoperiodic control of reproduction in hamsters, ferrets, voles, rams, and goats (Cardinali et al., 1981).

2.2. Microstructure

The SCG is the most studied sympathetic ganglia and many of its structural features are reproducible in the other sympathetic ganglia, although important differences are being found with more detailed studies, especially between paravertebral and prevertebral ganglia. In addition to ganglion nerve cells (principal ganglion neurons), sympathetic ganglia contain several other cell types. These include small granular cells (or small intensely fluorescent (SIF) cells), vascular cells (mainly endothelial cells), mast cells, and fibroblasts (in thin septa of connective tissue and in the capsule) (Gabella, 2004; Miolan and Niel, 1996).

2.2.1 Ganglion capsule

The SCG's capsule (Fig. 2.3) is conspicuous and composed of layers of collagen fibers and flattened fibroblasts. Therefore, each ganglion consists of an



Figure 2.3 Macrostructure of the left superior cervical ganglion (SCG) from an adult preá depicting the conspicuous SCG connective capsule (*) surrounding SCG parenchymal compartments (arrow). Scale bar: 100 μm .

agglomeration of clusters of neurons separated by nerve fibers, capillaries, and prominent septa of collagen fibers (Fioretto et al., 2007)

The SCG is divided into distinct compartments by capsular septa of connective tissue as reported for mammalian sympathetic ganglia (Fioretto et al., 2007; Gabella et al., 1988; Miolan and Niel, 1996; Ribeiro et al., 2004; Schmidt, 1996; Szurszewski and King, 1989; Szurszewski and Miller, 1994). In addition, Gabella (1976) postulated that the capsule of connective tissue which surrounds the ganglia of the sympathetic trunk is continuous with the epineurium of the associated nerves. Shanthaveerappa and Bourne (1964) described the “perineurial epithelium” of sympathetic ganglia as being continuous with that of the attached nerve trunks. By the same token, Arvidson (1979) and Al-Khafajit et al. (1983) described epineurial and perineurial components of the SCG’s capsule in mice and rats.

The presence of a perineurial layer in the capsule and its continuity with the perineurium of attached nerves is of paramount importance, since the perineurium of peripheral nerve trunks acts as a diffusion barrier, which is not readily penetrated by macromolecules. Nonetheless, the permeability of sympathetic ganglia capsule has yet to be elucidated (Al-Khafajit et al., 1983).

Fioretto et al. (2011) reported that the sheep SCG is composed of several ganglion units, each one being constituted of clusters of neurons and glial cells. Each ganglion unit is separated by SCG capsular septa of connective tissue. Apart from ganglion neurons and glial cells, other cell types have been found within SCG, such as SIF cells, Schwann cells, fibroblasts, mast cells apart from capillaries harboring endothelial cells. The aforementioned authors have suggested that there are variations in the SCG structure in large mammals when compared to that in small laboratory rodents, such as rats (Ribeiro et al., 2004) and mice (Purves et al., 1986).

With regards to the thickness of SCG capsule during animal maturation (from young to adult specimens), there was a 32.7% increase in that of dogs, a 25.8% increase in that of cats, and a 33.2% increase in that of horses. By performing an interspecies comparison of SCG capsule thickness, a 0.14-fold increase was observed from cats to dogs, a 2.2-fold increase was seen from dogs to horses and, finally a 2.6-fold increase between cats and horses (Fioretto et al., 2007). In adult animals, Ribeiro et al. (2004) reported that SCG capsule thickness ranged from 15 to 30 μm in rats, from 30 to 70 μm in capybaras, and from 60 to 80 μm in horses. Additionally, Ribeiro (2006) described a range of 30–50 μm for SCG capsule thickness in young capybaras, which represents a 28.5% reduction in relation to adult capybaras.

2.2.2 SCG neurons

SCG neurons are generally spindly shaped—or circular—and readily distinguishable due to their large size, clear nucleus, and the evident nucleolus. Ganglion neuron profiles are normally surrounded by one to two intraganglionic capillaries (in this case they are named intraunit capillaries) as well as by one to three glial cell nuclei (satellite cells) whose processes formed the ganglion neuron glial capsule (Abrahão et al., 2009; Coppi and Loesch, 2011; Fioretto et al., 2007, 2011; Ladd et al., 2012; Loesch et al., 2010; Melo et al., 2009; Toscano et al., 2009) (Fig. 2.4).

In horses, the neuron perikarya occupy a larger proportion of the sectional area than in dogs and cats and the neuropil is therefore proportionally less extensive. All neurons are uninucleate and some nuclei were located in the center of the perikaryon, while the majority is eccentric, but none resided at the periphery of the neuron profile. Between 1 and 2 nucleoli were observed in each neuron nucleus (Fioretto et al., 2007; Loesch et al., 2010; Ribeiro et al., 2004).

In pacas—a South American wild rodent—SCG neuron profiles are circular or, more commonly, ovally shaped. As with guinea pigs and other

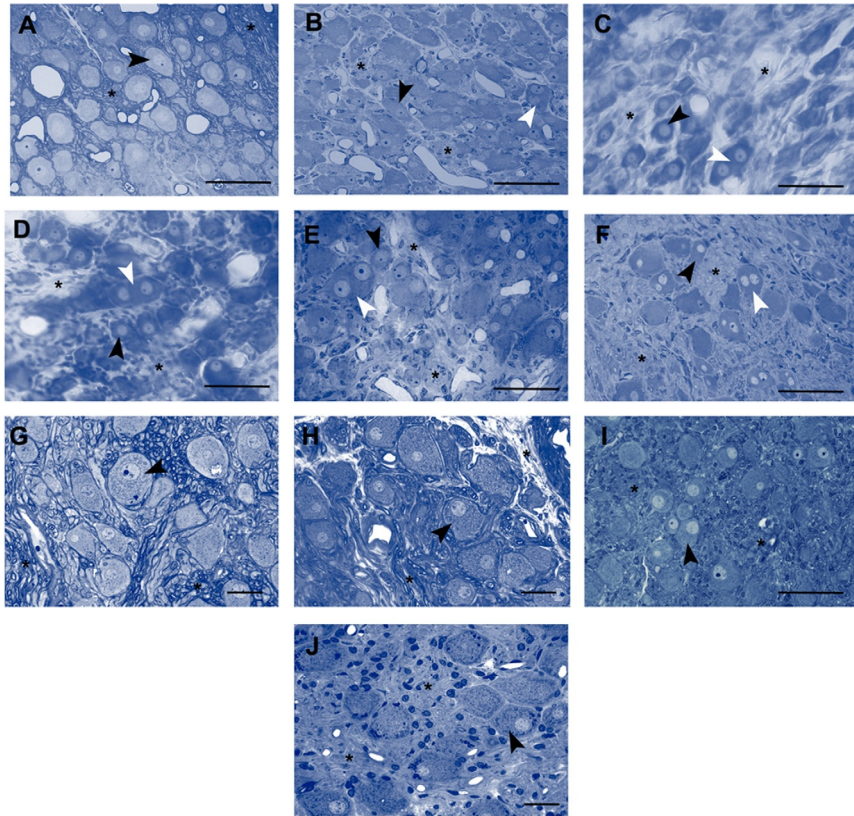


Figure 2.4 Light microscopic images of Toluidine Blue-stained physical or optical sections of superior cervical ganglia (SCGs) from a rat (A), guinea pig (B), preá (C), cutia (D), paca (E), capybara (F), cat (G), dog (H), sheep (I), and from a horse (J), depicting details of the ganglion microstructure: uninucleate neurons (black arrowheads) and binucleate neurons (white arrowheads)—the latter is seen only in the SCGs of exotic rodents (B, C, D, E, F). SCG neurons are separated by spaces mainly occupied by the neuropil (*). Scale bars: 75 μ m.

South American wild rodents—preás, cutias, capybaras—ganglion neurons are either uni- or binucleate. In uninucleate neurons, some nuclei are located in the center of the perikaryon, while the majority is eccentric, but none of them are placed at the periphery of the neuron profile. On the contrary, in binucleate neurons, the nuclei occupy the two poles of the cell having a very distinct and defined position within the perikaryon (Abrahão et al., 2009; Melo et al., 2009).

More recently, Ladd et al. (2012) described the SCG neurons' structure in preás—another wild rodent found in South America. According to the

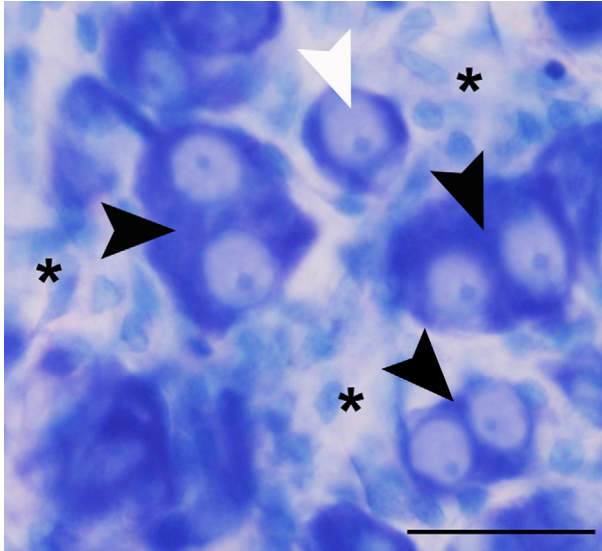


Figure 2.5 Light microscopic deconvoluted image at a maximal focal plane of a Giemsa-stained, 40- μ m-thick, optical section of a left superior cervical ganglion (SCG) from an adult preá depicting three large binucleate neurons (black arrowheads) surrounding one uninucleate neuron (white arrowhead). Note that in binucleate neurons, the nuclei are very distinct and typically occupy the two poles of the cell. SCG uni- and binucleate neurons are separated by spaces mainly occupied by the neuropil (*). Scale bar: 30 μ m.

authors, SCG neuron profiles are roughly circular or spindly shaped. SCG neurons are densely distributed and packed in newborn and young specimens. However, in adult and elderly preás, neurons are further apart and separated by spaces mainly occupied by neuropil (neurites and glial processes) and by blood vessels and connective tissue. Regardless of animal ages, nuclei are located either centrally or eccentrically in the perikarya of uninucleate neurons, though they occupy the two cell poles in binucleate neurons (Fig. 2.5).

2.2.2.1 Binucleate neurons

Mammalian sympathetic ganglion neurons are commonly uninucleate, though cells with two nuclei are also found (Dalsgaard and Elfvin, 1982; Filichkina, 1981; Huber, 1899; Macrae et al., 1986; Purves et al., 1986; Smith, 1970) and the frequency in which they appear changes considerably from one species to another and during the postnatal development. In the heart, binucleate cells appear at the stage in the development of the

innervation when the immature neuroblasts seen in early stages are becoming fewer in number and when their migration along nerve bundles passing from the cardiac plexus has almost ceased. On this basis, binucleate nerve cells are found when cell division is occurring and it may represent a stage in a mechanism to increase the number of ganglion cells, by a process of active division, at a time when migration of neuroblasts into the heart has virtually ceased (Smith, 1970).

One explanation for the occurrence of two nuclei in the SCG neurons of guinea pigs, preás, cutias, pacas, and capybaras is that a mitotic arrest late in the differentiation process can occur. There is no known reason for this, but it has been suggested that it might be due to a response to nerve growth factor (NGF) stimulation of the ganglion cells in their final differentiation to adrenergic cells. It is, however, unclear why only these cells respond in such a manner and no other adrenergic cell populations (Levi-Montalcini and Aloe, 1983; Purves et al., 1988) and why binucleate SCG neurons only occur in rodents—excluding mice and rats—and are not present in even larger mammals such as cats, dogs, sheep, and horses.

The functional significance of binucleate cells is yet to be clarified. It is possible that the occurrence of the two nuclei is an adaptation to some specific demand imposed on the cells due to protein synthesis. It is unclear whether the binucleate cells' genome is broader, since there is no indication that one of the nuclei has a larger proportion of euchromatin than the other, nor that the nuclei are different from those of the uninucleate cells in their heterochromatin/euchromatin ratio (Ribeiro, 2006; Smith, 1970). These speculations are, however, contradicted by Filichkina (1981), where binucleate SCG neurons in rabbits did not show an increase in the total protein synthesis or axonal protein transport when compared to uninucleate SCG cells.

The number of uni- versus binucleate neurons was found to vary with the stage of development with about 40% of the cells being binucleate in adult guinea pigs and 50% in the late prenatal stage (60-day-old fetuses) (Forsman et al., 1989). On the contrary, our recent study in preás (Ladd et al., 2012) showed that the number-derived proportion of uninucleate versus binucleate neurons was higher in newborn animals when compared to all remaining age groups, that is, in newborn preás, there were about two uninucleate neurons for each binucleate nerve cell and the proportion decreases and ultimately equates to 1:1 from young to old animals. Mechanistically speaking, we hypothesized that those uninucleate neurons were undergoing cell death or cell repair leading to a ratio equality between uni- and

binucleate neurons during postnatal development: maturation and aging. A second hypothesis would be that uninucleate neurons might be turning into binucleate neurons during postnatal development (Ladd et al., 2012).

On the whole, there is still a dearth of knowledge of the origin of binucleate neurons and their functional meaning, since there is no plausible explanation for the large occurrence of binucleate SCG neurons, especially in rabbits and wild rodents as previous publications have demonstrated (Abrahão et al., 2009; Filichkina, 1981; Ladd et al., 2012; Loesch et al., 2010; Melo et al., 2009; Ribeiro, 2006; Ribeiro et al., 2004; Toscano et al., 2009).

2.2.3 Glial cells

Two types of glial cells are found in most peripheral sensory and autonomic ganglia and nerves: (i) Schwann cells, which ensheath axons in peripheral nerves and may produce myelin (Bunge et al., 1986; Webster, 1975) and (ii) satellite cells, which are associated with neuron cell bodies within the peripheral ganglia (Pannese, 1981; Pomeroy and Purves, 1988). In peripheral ganglia, most glia are satellite cells, although Schwann cells may also be found associated with intraganglionic tracts. While Schwann cells in myelinated nerves have been proposed to regulate the ionic environment of the nerve and to provide trophic factors after injury, the role of satellite cells in peripheral ganglia remains less clear (Hall and Landis, 1991; Pannese, 1981) and warrant further systematic investigations (Fig. 2.6).

2.2.4 SIF cells

Despite somewhat confused terminology: SIF cells or small granule-containing cells (SGC) or interneurons, these particular cells have been found in all mammalian sympathetic ganglia studied hitherto (Chiba and Williams, 1975; Gabella, 2004).

SIF cells in the rat SCG have been studied extensively and, in this species, some or all of them receive preganglionic innervation and at least some of them synapse with principal ganglion cells (Matthews and Raisman, 1969; Williams, 1967; Williams and Palay, 1969; Yokota, 1973). Most rat SIF cells occur in clusters and are in close relation to fenestrated capillaries (Siegrist et al., 1968) (Fig. 2.6).

Yokota (1973) performed a three-dimensional analysis of a cluster of four SIF cells within the rat SCG. All four of these cells received afferent synapses but only two of them made efferent somatodendritic synapses with principal ganglion neurons. The other two cells were naked, that is, without Schwann

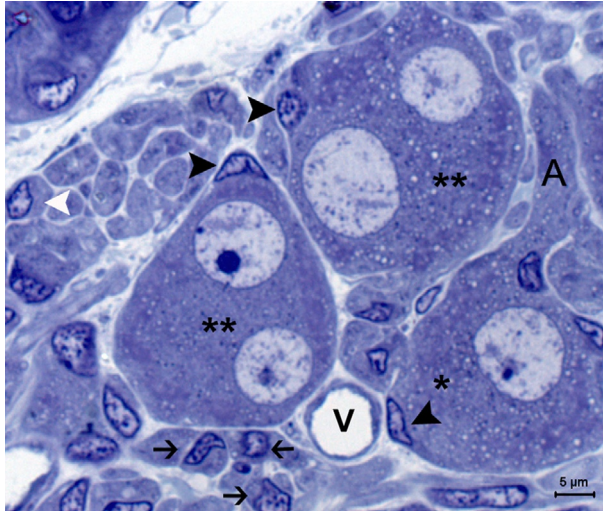


Figure 2.6 Light microscopic image of a Toluidine Blue-stained 0.5- μm -thick semithin section of a superior cervical ganglion (SCG) from a young capybara. Some details of SCG microstructure are shown: one uninucleate (*) and two binucleate (**) neurons surrounded by three glial cell nuclei (black arrowheads) and separated by a blood vessel (v). It can also be noticed a Schwann cell (white arrowhead), a cluster of three vascular small intensely fluorescent (SIF) cells (arrows) and one axon (A) emerging from the uninucleate neuron (*). Scale bar: 5 μm . Image extracted from [Ribeiro \(2006\)](#). However, the present figure legend has been modified to show additional structures, not shown in [Ribeiro \(2006\)](#).

cell's sheath over a greater area than those which possessed efferent synapses. However, it should be mentioned that since Yokota's careful study was confined to the immediate neighborhood of these four SIF cells, the possibility that there may have been additional (more distant) efferent synapses cannot be ruled out ([Chiba and Williams, 1975](#)).

A direct diffusion mechanism of the secretory products within the rat SCG was suggested by [Taxi et al. \(1969\)](#) and Yokota's findings supported the view that there may be two functional pathways for SIF cells in the SCG. Some SIF cells may operate as interneurons and others through a neurosecretory mechanism. We should not however exclude the possibility that the same cell may harbor these two mechanisms of action ([Chiba and Williams, 1975](#)).

Although interneurons are presumed to exist in the SCG of species other than rats, morphological proof for the existence of efferent synapses in these other species has not been presented. In most species studied, it was possible

to distinguish, on the basis of morphological characteristics, two categories of SIF cell in the SCG. Type I SIF cells were those which have long varicose processes extending among ganglionic neurons. Type II SIF cells, on the other hand, are intimately related to subcapsular or stromal blood vessels: an arrangement reminiscent of some endocrine glands. Generally speaking, type I SIF cells are solitary, whereas type II SIF cells are found in clusters (Chiba and Williams, 1975).

Rats and guinea pigs break this rule, however, since in both species fairly long varicose fluorescent processes can be observed in the clustered SIF cells. In both these rodents, small clusters of SIF cells are located among ganglion neurons. Therefore, it is speculated that type I and type II SIF cells form intermingled clusters in some species, specifically in the rat and guinea pig SCG. Given the reasons above Chiba and Williams (1975) made no attempt to differentiate between type I and type II SIF cells in either of these species. By the same token, SIF cells were seen placed in two different locations, namely close to neurons or encompassing tight clusters comprising two to five cells in the proximity of blood vessels within the SCG of cats, dogs, and horses (Fioretto et al., 2007).

Matthews and Raisman (1969) and Jew (1985) reported the presence of SGC in the SCG, which are distinct to sympathetic neurons and other satellite cells. These SGC were also identified as SIF cells and formed tight clusters associated with small capillaries and, as stated by Miolan and Niel (1996), may play a neuroendocrine role. SIF cells were classified into two types: type I with granular vesicles ranging from 80 to 100 nm and type II with granular vesicles varying from 150 to 300 nm (Gabella, 2004). This author also described that SIF cells presented synapses to preganglionic fibers and few SIF cells had synaptic contacts to ganglion neurons. Furthermore, SIF cells, which had afferent and efferent synapses, were named interneurons (Fioretto et al., 2007).

Black et al. (1974) summarized the differences observed between the SCGs of hamsters, guinea pigs, rats, cows, rabbits, cats, and monkeys. There are significant differences in the numbers and anatomic distribution of SIF cells in these species. Whereas the numbers of SIF cells vary with the size of the ganglia, the number of SIF cells per milligram of SCG tissue is very similar in cows, rabbits, cats, and monkeys. By contrast, rodent SCG contains from 20 to 100 times as many SIF cells per milligram of SCG tissue as do nonrodent species.

The biologic significance of such relatively huge numbers of SIF cells in the rodent SCG is unknown. Among nonrodents, the percentage of type I SIF

cells varies from a high of 74% in rabbits to a low of 0.48% in cats, though SIF cells classification is ambiguous in those species. On the contrary, all rodents exhibit mixed clusters in which both types of SIF cells are present. Thus, the number of SIF cells per milligram of SCG tissue and the anatomic arrangement of these cells within the SCG differ clearly between rodents and nonrodents (Black et al., 1974).

2.2.5 Nerve fibers

Rami communicantes are short nerve trunks connecting the ganglia of the sympathetic chain—including SCG—to the spinal nerves. The rami are particularly short in the rat and even when they are multiple and separated into two or more bundles they cannot be distinguished as white and gray rami (Gabella, 2004). Mixed within each of the above-mentioned rami there are preganglionic and postganglionic fibers and, since they have no distinctive structural features to indicate their orientation and nature and their identification can be made only after experiments of selective nerve sections (Gabella, 2004).

Depending on the level of origin, preganglionic fibers travel some distance up or down the sympathetic chain, forming synaptic contacts with SCG neurons. The length of preganglionic fibers can therefore be considerable. In the upper thoracic chain segment, they are mainly ascending (cranially directed) and in the cervical sympathetic trunk—where SCG is inserted—all the preganglionic fibers are directed cranially. In this trunk, however, there are also caudally directed postganglionic fibers, originating in the SCG, and cranially directed postganglionic fibers, originating in the middle and lower cervical ganglia (Bowers and Zigmond, 1981). In the rat, unlike other species, such as humans and cats, the great majority of preganglionic fibers are unmyelinated. For example, less than 1% of the axons in the cervical sympathetic trunk are myelinated (Brooks-Fournjer and Coggeshall, 1981; Dyck and Hopkins, 1972; Hedger and Webber, 1976).

2.2.6 Synapses

The chemical organization of the sympathetic nervous system is somewhat more complex. Generally speaking, there are at least three types of ganglion neurons in the paravertebral portion of the sympathetic nervous system: those that contain norepinephrine alone, those that contain norepinephrine along with neuropeptide Y (NPY), and those that contain acetylcholine and vasoactive intestinal peptide. The first type provides innervation to the parenchyma of the target organs, while the second mainly innervates blood

vessels. The third type innervates the sweat glands (Gibbins and Morris, 2006; Smolen, 1988).

The SCG is the site of synapses between preganglionic sympathetic nerve fibers and postganglionic sympathetic neurons (Guth and Bernstein, 1961; Loesch et al., 2010) (Figs. 2.7 and 2.8). The postnatal biochemical maturation of SCG has been described in rodents (Black et al., 1971a; Thoenen et al., 1972a). Both choline acetyltransferase—which is contained chiefly in the preganglionic terminals (Hebb and Waites, 1956)—and tyrosine hydroxylase (TH)—which is localized to adrenergic neurons—have been described in the SCG (Black et al., 1971b; Smolen and Raisman, 1980). In the SCG of rodents, it has been shown that TH—the rate-limiting enzyme in catecholamine biosynthesis—undergoes a normal postnatal increase in activity (Black et al., 1971a; Thoenen et al., 1972a). In addition, it has been depicted that when the afferent input to SCG neurons is removed at the time of birth, TH activity fails to undergo its normal postnatal increase and remains at neonatal levels (Black and Mytilineou, 1976; Black et al., 1972; Thoenen et al., 1972b).



3. STEREOLOGICAL ASSESSMENT OF THE SCG DURING POSTNATAL DEVELOPMENT: EFFECTS OF AGING AND ALLOMETRY

3.1. Ganglion volume

In order to estimate the volume of the SCG the most accurate and modern method of choice is design-based stereology using Cavalieri's principle (Abrahão et al., 2009; Ladd et al., 2012; Melo et al., 2009; Ribeiro, 2006). There are several advantages in applying this method: it is possible to estimate the coefficient of error and the estimations are not dependent upon the shape of SCG (Gundersen et al., 1999; Howard and Reed, 2010).

The basic principle is very straightforward. The organ is cut into slabs of equal thickness beginning with a random start. An estimate of the total volume of the organ can then be calculated by multiplying the slab mean thickness by the sum of the areas of each slab. Then,

$$V_{\text{SCG}} = T \sum A_{\text{SCG}},$$

where T is the between-section distance (in μm) and $\sum A_{\text{SCG}}$ is the sum of the delineated profile areas of the chosen set of SCG sections. Profile areas are estimated from the numbers of randomly positioned test points (at least

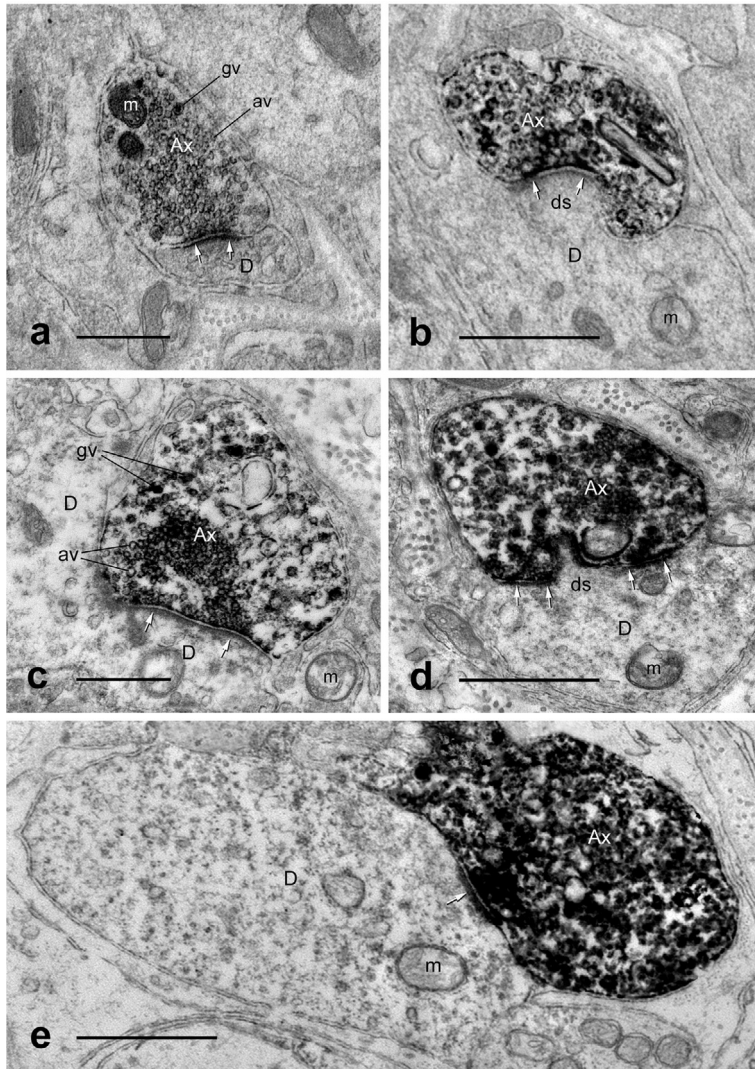


Figure 2.7 Transmission electron microscopic images of SCG in rat (a, b), capybara (c, d), and horse (e) immunolabeled for SYP (black immunoprecipitate). (a) Note SYP-positive axon profile (Ax) asymmetrically synapsing on a dendrite profile (D); postsynaptic densities (white arrows) are clearly visible. Note also that the axon profile is rich in small agranular vesicles (av); a few granular vesicles (gv) can also be seen (m mitochondrion). (b) SYP-positive axon profile synapsing with dendritic spine (ds). (c) SYP-positive axon terminal asymmetrically synapsing on a dendrite profile; the axon terminal contains a number of small agranular vesicles. (d) SYP-positive axon profile synapsing on dendritic spine. A few granular vesicles can be seen in addition to small agranular vesicles. (e) Asymmetric axo-dendritic synapse with heavily labeled axonal profile. Bars 0.5 μm. Image and legend extracted from [Loesch et al. \(2010\)](#).

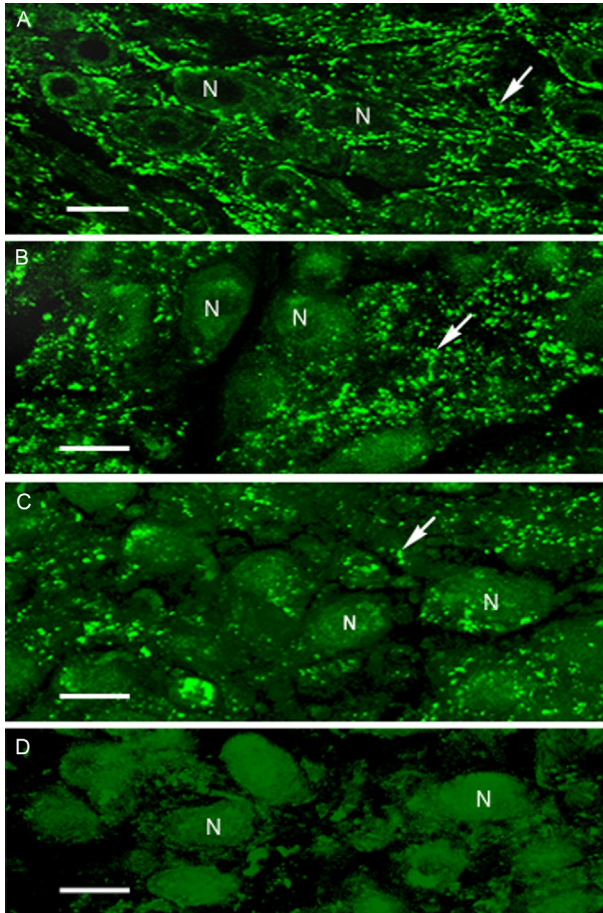


Figure 2.8 Confocal immunofluorescence microscopy of SCGs (here, 30 consecutive 1- μ m images were merged as maximal projections) immunolabeled for SYP (light green in original images). (A) Rat, (B) capybara, and (C) horse. In (A–C), SYP-positive labeling (arrows) was located in the neuropil among the ganglion neurons (N). Note that labeling is more densely packed in the rat SCG compared with that in capybara and horse. (D) Immunohistochemical control of horse SCG; no immunolabeling for SYP was observed when the primary antibody was replaced by nonimmune normal goat serum. Bars 50 μ m. *Image and legend extracted from Loesch et al. (2010).*

~ 100 per SCG) hitting the whole reference space and the areal equivalent of a test point (for more details, see [Howard and Reed, 2010](#); [Ladd et al., 2012](#)). Ganglion volume is an important parameter which may reflect global alterations in the organ. This parameter is affected by several factors like species-related differences ([Loesch et al., 2010](#); [Ribeiro et al., 2004](#)), age ([Abrahão](#)

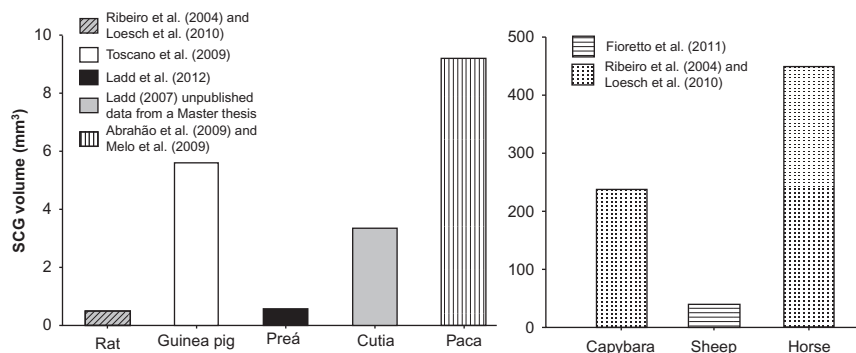


Figure 2.9 Simple histogram (bar chart) showing the superior cervical ganglion (SCG) volume across a plethora of different mammalian species: rats, guinea pigs, preás, cutias, pacas, capybaras, sheep, and horses. Every bar uppermost edge indicates the mean value for each species. Data were extracted from the references identified by specific and corresponding drawing patterns.

et al., 2009; Ladd et al., 2012; Melo et al., 2009; Toscano et al., 2009) and side (Abrahão et al., 2009) (Fig. 2.9).

In addition, hypertrophy is a widely frequent adaptive mechanism during either SCG postnatal development from medium-sized to large mammals (Abrahão et al., 2009; Ladd et al., 2012; Melo et al., 2009; Miolan and Niel, 1996; Ribeiro, 2006; Ribeiro et al., 2004; Toscano et al., 2009) or after unilateral ganglionectomy (Fioretto et al., 2011). There has also been noticed an allometric association between SCG volume and body weight (Loesch et al., 2010; Ribeiro et al., 2004).

It should be stressed that the larger proportion of the SCG volume is occupied by the nonneuronal tissue compartment, that is, neuropil, connective tissue, and blood vessels, and that differences in ganglion volume may reflect a variation in the total volume of the aforementioned tissue compartments (Abrahão et al., 2009; Ladd et al., 2012; Toscano et al., 2009).

3.2. Total number of neurons

Cutting-edge 3D technology—employed by using design-based stereology—provides us with a much more accurate and unbiased method for quantifying aspects of the structure of the SCG neurons in healthy and diseased mammals from a plethora of species (Abrahão et al., 2009; Ladd et al., 2012; Melo et al., 2009; Ribeiro et al., 2004; Toscano et al., 2009). The most powerful stereological tool for estimating the total number of a given particle in 3D is the disector method, described for the first time by

Sterio (1984). The disector probe can be applied either physically or optically (Howard and Reed, 2010).

Recently, it is considered state-of-the-art to associate the optical (Fig. 2.10) (Ladd et al., 2012) or the physical disector (Abrahão et al., 2009; Melo et al., 2009) with the fractionator sampling regime (Gundersen, 2002; Howard and Reed, 2010)—which uses several sampling fractions—to assess remodeling-induced changes in the number of SCG neurons during postnatal development (maturation and aging) or as a consequence of both allometric and disease-related adaptive responses.

In a recent study by Ladd et al. (2012), the total number of SCG neurons in preás was estimated by multiplying the counted number of particles—uninucleate or binucleate neurons—by the reciprocal of sampling fractions:

$$N_{\text{uni- or biSCG}} := \text{ssf}^{-1} \text{hsf}^{-1} \text{asf}^{-1} \Sigma Q^{-}$$

where $N_{\text{uni- or biSCG}}$ is the total number of uninucleate or binucleate neurons, ssf is the mean section sampling fraction, hsf is the mean height sampling fraction, asf is the mean area sampling fraction, and ΣQ^{-} is the total number of particles (neurons) counted using optical disectors (for more details, revisit Howard and Reed, 2010; Ladd et al., 2012). The several effects exerted by species, maturation (or aging), body weight, and disease on the quantitative structure of SCG may be translated as decrease (Santer, 1991; Toscano et al., 2009), increase (Abrahão et al., 2009; Melo et al., 2009), or stability (Ladd et al., 2012) in the total neuron number. Figure 2.11 summarizes the differences in the total number of SCG neurons in different species of mammals.

As reported earlier, a catalogue of publications describing the existence of binucleate neurons—which may imply that some cell division and resulting neurogenesis are still in place—in the SCG of adult wild rodents such as capybaras (Ribeiro et al., 2004), pacas (Abrahão et al., 2009; Melo et al., 2009), and yellow-toothed cavies (preás) (Ladd et al., 2012) in conjunction with studies depicting substantial increases in the total number of SCG neurons in aged pacas (Abrahão et al., 2009; Melo et al., 2009) add further fuel and strongly advocate that a postnatal neurogenesis may indeed occur in sympathetic ganglia.

A robust strategy for tackling neurogenesis in the mammalian SCG is coupling design-based stereology with immunohistochemistry. The latter is attained by using commercially available cell-cycle phase or proliferation markers such as thymidine-analog 5-bromo-2-deoxyuridine (BrdU) and Ki-67, though every marker has advantages and pitfalls and the conclusions they lead to have to be drawn in the overall context of the experiment.

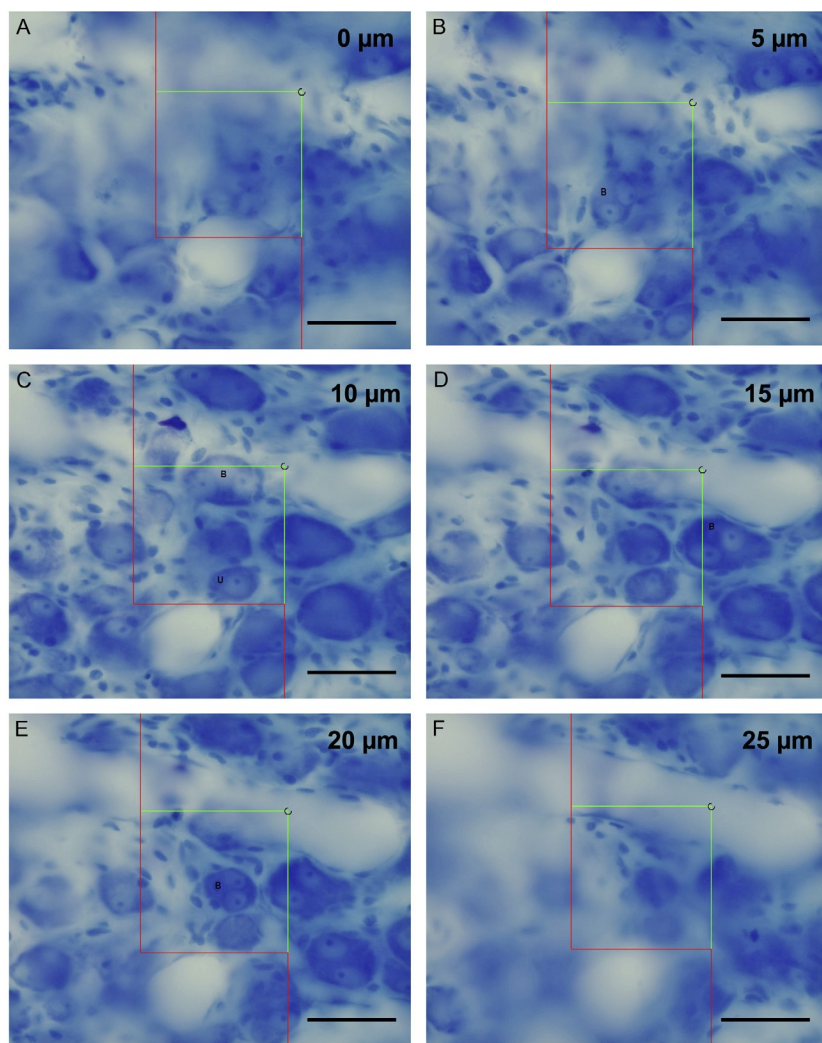


Figure 2.10 Images of successive focal planes throughout a Giemsa-stained optical section of a superior cervical ganglion (SCG) from an adult preá, illustrating the application of the optical disector. The distance between each focal plane is 5 μm . On plane (A) (uppermost surface of the section) a field of view—selected using an unbiased counting frame—is followed along the whole section thickness (planes B, C, D, E, and F) and neurons are sampled and counted as they come into focus on each focal plane. For instance, on plane (C) (10 μm apart from plane A) one uninucleate (U) and one binucleate (B) neuron are sampled. The lowermost focal plane (F) (bottom surface of the section) is 25 μm apart from plane (A) and no particles are sampled on it. Scale bars: 30 μm .

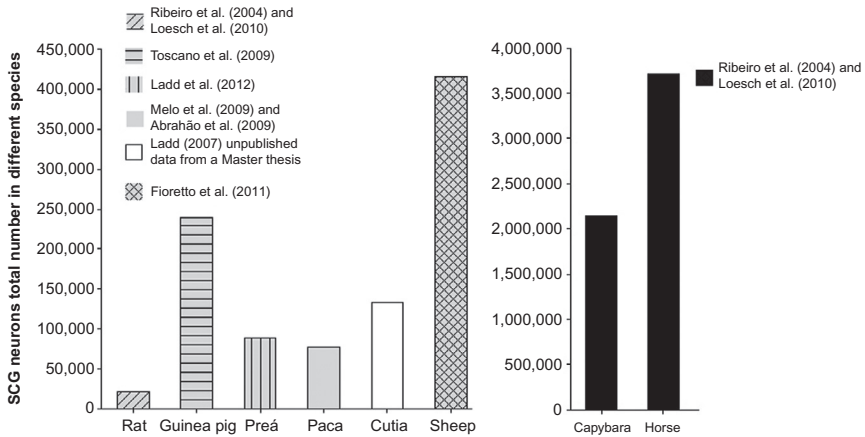


Figure 2.11 Simple histogram (bar chart) showing the total number of superior cervical ganglion (SCG) neurons across a catalogue of different mammalian species: rats, guinea pigs, preás, pacas, cutias, sheep, capybaras, and horses. Every bar uppermost edge indicates the mean value for each species. *Data were extracted from the references identified by specific and corresponding drawing patterns.*

In light of that, in our recent study (Ladd et al., 2012), the total number of BrdU-positive neurons was stereologically estimated and “always” related to the total number of Giemsa-stained neurons in order to see whether the BrdU subpopulation of neurons would exert an effect on the whole neuronal population, viz, the Giemsa-stained nerve cells. Therefore, by plotting the BrdU chemical-coded subpopulation of SCG neurons against the whole Giemsa-stained SCG neuronal population, we have found that the total number of neurons is surprisingly constant in the SCG of preás during the entire postnatal development, viz, maturation and aging. The aforementioned stereological-plotting approach is novel in the SCG-specialized literature and leads to a more mechanistic explanation for the events underpinning the adaptation and remodeling of SCG neurons during postnatal development.

On the other hand, the increase in neuron number is not always attributable to neurogenesis, but may also be the outcome of late maturation or incomplete differentiation of nerve cells (Ciaronia et al., 2000; Farel, 2003; Miolan and Niel, 1996). In juvenile bullfrogs, dorsal root ganglion (DRG)—a sensory ganglion—houses a population of immature, incompletely differentiated neurons that can be induced to differentiate into a mature form in response to injury (Farel and Boyer, 1999). Indeed, new DRG neurons derive mostly from differentiation rather than from

proliferation of precursor cells (Meeker and Farel, 1997). By the same token, pelvic ganglion neurons more than double between birth and adulthood, probably due to continuous maturation of p75-positive undifferentiated neuronal precursors rather than to cell division (Yan Hui Keast, 2008).

3.3. Neuron volume

Cell volume estimated by design-based stereology is considered as a state-of-the-art, assumption-free, and unbiased procedure for estimating neuron size, when compared to 2D or 3D particle shape-dependent methods (Ladd et al., 2012; Ribeiro et al., 2004). Two main stereological methods are currently used for estimating neuron volume: nucleator (Gundersen, 1988) and rotator (Vedel Jensen and Gundersen, 1993). Both methods require isotropic and uniform random or vertical and uniform random sections and the usage of either physical or optical disector (Howard and Reed, 2010).

The nucleator employs the following formula to estimate the neuron volume ($\bar{v}_N$):

$$\bar{v}_{N\text{mye/sub}} := \Sigma(4p/3)\bar{I}_n^3$$

where $\bar{I}_n^3$ is the mean of all cubed distances from a central point (nucleolus) within the perikaryon to its cell boundaries (Gundersen, 1988).

The rotator is based upon the Pappus–Guldinus theorem. Technically speaking, the method requires a uniquely defined subspace of the particle, for example, the nucleus or the nucleolus of a cell and the optical disector is used as a probe to sample particles. The following formula is used to estimate neuron volume ($\bar{v}_N$):

$$\bar{v}_N = \pi t \sum_i l_i^2$$

where t is the distance between six parallel half-lines (which constitute a grid) placed perpendicular to the vertical axis of the section and l_i^2 represents the squared distance between cell profile boundary and the vertical axis measured in the focal plane where the nucleolus was identified (Vedel Jensen and Gundersen, 1993). Figure 2.12 collates estimates of SCG neuron volume obtained from quite a few species. Generally speaking, binucleate SCG neurons are twice as big as uninucleate ones.

Sympathetic neuron hypertrophy seems to be a predominant event during postnatal development in order to compensate for a neuron loss in the same period (Cabello et al., 2002) or as an adaptive mechanism for coupling with

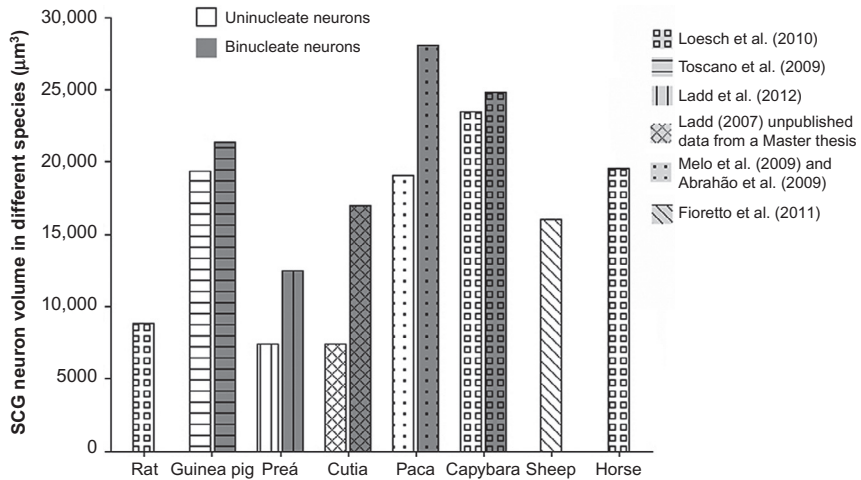


Figure 2.12 Simple histogram (bar chart) showing data on the volume of superior cervical ganglion (SCG) uni- and binucleate neurons across a surfeit of different mammalian species: rats, guinea pigs, preás, cutias, pacas, capybaras, sheep, and horses. Every bar uppermost edge indicates the mean value for each species. Data were extracted from the references identified by specific and corresponding drawing patterns.

aging or with body size increase (Abrahão et al., 2009; Ladd et al., 2012; Melo et al., 2009; Sanchez et al., 2008). Along the same lines, neuron volume increased by 217% in dog's caudal mesenteric ganglion—a prevertebral ganglion (Gagliardo et al., 2005) and in the human SCG neurons (Liutkiene et al., 2007) during postnatal development. In contrast, Ribeiro (2006) reported no changes in the volume of SCG neurons in capybaras during postnatal development, whereas Toscano et al. (2009) have recently communicated 27.5% and 40% decreases (atrophy) in the volume of uni- and binucleate SCG neurons in guinea pigs during aging, respectively, which may be attributable to a selective age-related atrophy and neurodegeneration mediated by an increase in the sortilin expression in SCG neurons, compounded with elevated expression of pro-NGF levels in some targets (Al-Shawi et al., 2008). A third hypothesis, which would account for the increase in the size of uninucleate neurons during aging, might be that uninucleate neurons are on the brink of becoming binucleate neurons (which are considerably larger). Given the paradoxical outcome measures reported above, the chances are that alternative neuron-size-regulating mechanisms, apart from hypertrophy, may be acting or coacting in the autonomic nervous system during postnatal development in order to guarantee functional homeostasis, especially in the total amount of neurotransmitters produced (Cabello et al., 2002; Ladd et al., 2012).

3.4. Synapses

Estimates using model-based (particle shape-assumption dependent) and design-based stereology (disector method) suggest that the SCG of an adult rat harbors $4\text{--}12 \times 10^6$ synapses (Field and Raisman, 1985; Siklós et al., 1990; Smolen and Raisman, 1980) and $19\text{--}36 \times 10^3$ neurons (Ostberg et al., 1976; Purves et al., 1986; Ribeiro et al., 2004). This gives an average synapse:neuron ratio of about 290:1 and the overwhelming majority of these, 90% or more, are axo-dendritic (Forehand, 1985). Larger species possess SCG neurons which exhibit greater convergence, more complex arborizations and a greater proportion of axo-dendritic compared to axo-somatic synapses (Forehand, 1985; Purves and Lichtman, 1985; Purves et al., 1986). Therefore, in autonomic ganglia—including the SCG—a decision should be made to confine counts to axo-dendritic synapses rather than axo-somatic ones, since the former occur more frequently (Forehand, 1985; Gibbins and Morris, 2006; Loesch et al., 2010; Matthews, 1983).

Quantitative data on synapses are undoubtedly obtained using transmission electron microscopy because this is the only technique which reveals whether or not a preganglionic bouton makes contact with the surface of a neuron and shows the morphological features conventionally associated with synaptic specializations, that is, clusters of small vesicles directed toward a well-defined density on a presynaptic membrane. However, even this appearance may not imply a functional synapse and the same is true for light microscopy (Gibbins and Morris, 2006). Ideally, a range of chemical markers needs to be localized and correlated with electrophysiological data for the same populations of neurons (Gibbins and Morris, 2006).

The immunochemical labeling of SYP, a synaptic vesicle membrane glycoprotein, has been used to detect functioning synaptic terminals (Calhoun et al., 1996; Loesch et al., 2010; Mochida et al., 1994; Navone et al., 1986; Tarsa and Goda, 2002; Weidenmann and Franke, 1985). SYP is a member of the physin family, which also comprises synaptophysin 1, synaptoporin, pantophysin, mitsugumin, and synaptogyrin (Janz et al., 1999; Leube, 1994). Given the variety of potential labels of synapses and their vesicles, it is possible that the synapse labeling achieved in a particular study is subtotal and that the quantitation based upon synaptophysin staining may underestimate total synapse number.

Loesch et al. (2010) have recently found that rat SCG harbors 10.3 million SYP-labeled axo-dendritic synapses and this estimate is within the range of previously published values for the same species and based upon both

design-based (Siklós et al., 1990) and model-based (Field and Raisman, 1985; Ostberg et al., 1976; Smolen and Raisman, 1980) estimates. The numbers equate to about 430 axo-dendritic synapses per neuron and this estimate is somewhat greater than earlier values of 250–330 per neuron (Forehand, 1985; Ostberg et al., 1976). In the same study, Loesch et al. (2010) reported that the number of SYP-labeled axo-dendritic synapses was found to be 45 million and 56 million in horses and capybaras, respectively. Numbers per neuron were roughly 48-fold larger in capybaras and 25-fold higher in horses (Fig. 2.13).

Due to the fact that the target tissue of the SCG increases in volume and surface area, it is reasonable to hypothesize that the size and number of ganglion neurons and the number of synapses would vary as a function of the quantity of target tissue innervated by them (Loesch et al., 2010). However, having a heterogeneous population of nerve cells (e.g., secretomotor, pilomotor, vasomotor), SCG neurons, and their synapses may be subject to a variety of structural changes depending upon the organs they target. For instance, neurons innervating the iris have the largest cell bodies and most extensive dendritic arborizations, whereas vasomotor neurons are the smallest. Neurons innervating the middle cerebral artery have dendritic

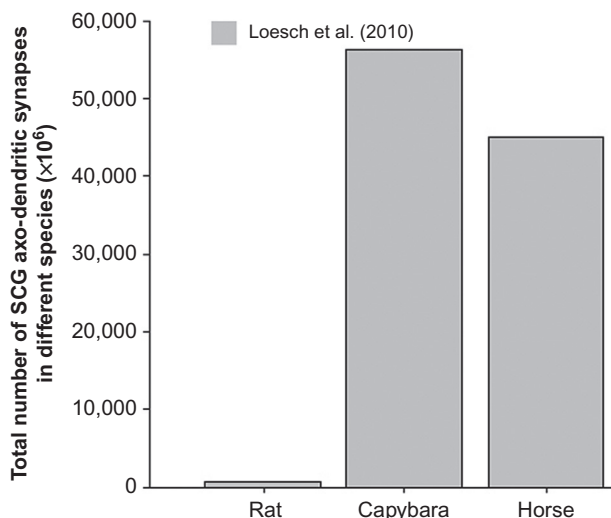


Figure 2.13 Simple histogram (bar chart) showing data on total number of superior cervical ganglion (SCG) axo-dendritic synapses across three different mammalian species: rats, capybaras, and horses. Every bar uppermost edge indicates the mean value for each species. Data were extracted from Loesch et al. (2010).

growth early in postnatal development, whereas the dendritic complexity of neurons supplying the submandibular gland increases well into adulthood (Andrews, 1996).



4. CONCLUDING REMARKS AND FUTURE RESEARCH DIRECTIONS

We have reviewed processes underlying SCG adaptation and remodeling during postnatal development: maturation and aging. Further investigations are warranted—using a plethora of cell-cycle phase markers such as: BrdU, BrdU/PCNA, thymidine, and Ki-67 and combined with cell differentiation (p75) and apoptosis markers (caspase 3)—to shed light on a portfolio of other mechanisms—cell repair, cell division, maturation, differentiation, and death—that we hypothesize to be intertwined and implicated in the qualitative and quantitative structural changes in SCG, its neurons and synapses during postnatal development.

Another interesting line of investigation would be whether age-related changes preferentially target a neuron population and the interaction between ganglion neurons, their innervation targets, and the signaling neurotrophic factors produced by the latter. This could be achieved by labeling neurons with specific markers, for example, NPY coupled with the usage of neurotracers. Would the differences in the sizes of the innervation targets and in the quality of the signals raised by the target organs explain the changes in SCG, its neurons and synapses during maturation and aging?

Finally, we hope that the data yielded by previous and forthcoming studies will bridge the gap between basic and translational clinical research, since the role SCG plays is of paramount importance in the understanding and treatment of some important neuropathies including Horner's syndrome, stroke, and epilepsy.

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New Insight into the Regulation of Floral Morphogenesis

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Abstract

The beauty and complexity of flowers have held the fascination of scientists for centuries, from Linnaeus, to Goethe, to Darwin, through to the present. During the past decade, enormous progress has been made in understanding the molecular regulation of flower morphogenesis. It seems likely that there are both highly conserved aspects to flower development in addition to significant differences in developmental patterning

that can contribute to the unique morphologies of different species. Furthermore, floral development is attractive in that several key genes regulating fundamental processes have been identified. Crucial functional studies of floral organ identity genes in diverse taxa are allowing the real insight into the conservation of gene function, while findings on the genetic control of organ elaboration open up new avenues for investigation. These fundamentals of floral organ differentiation and growth are therefore an ideal subject for comparative analyses of flower development, which will lead to a better understanding of molecular mechanisms that control flower morphogenesis.



1. INTRODUCTION

The evolution of seeds and flowers was major events in land plant evolution and probably the most important factors responsible for the dominance of gymnosperms and angiosperms on land floras for the past 250 million years (Myr). Little is known regarding genetic mechanisms that guided the evolution of seeds in the Middle Devonian, but evolution of flowers has received considerable attention by developmental and evolutionary biologists.

With more than 270,000 known species, angiosperms are by far the most diverse and widespread group of plants. The ancestry of angiosperm is still uncertain. The fossil records showed that the angiosperms appeared at about 130 Myr, the early Cretaceous period. According to different authors, the first angiosperms appeared during the early Cretaceous, from 125 to 130 Myr (Sun et al., 2011). During this period, basal angiosperms, magnollids, early monocots, and early eudicots appeared almost simultaneously (Friis et al., 2006). By the end of the Cretaceous, 65 Myr, the angiosperms had radiated and become the dominant plants on Earth, as they are today. The origin and diversification of angiosperm—what Charles Darwin considered it a “perplexing phenomenon” and even characterized as an “abominable mystery”—have puzzled scientists for more than a century (Cronquist, 1988; Darwin, 1903; Doyle, 1994a). The rapid explosion in diversity that followed their origin in the early Cretaceous may be linked to modularity within their new structure, the flower (Carroll, 2001). The flower is the defining reproductive adaptation of angiosperms and is the predominant source of characters for angiosperm taxonomy and phylogeny reconstruction (Doyle, 1994b).



2. EVOLUTION AND VERSATILITY OF ANGIOSPERM FLOWERS

Over the past decades, the advance of rigorous methods of phylogenetic analysis, the emergence of molecular techniques, and a renewed interest in the developmental pathways followed during the growth of plant organs have improved the understanding of angiosperm relationships (Chaw et al., 2004; Crane et al., 1995; Davies et al., 2004; Soltis et al., 2002, 2005). The angiosperms consist of some small relic basal clades (basal angiosperms), magnollids, and two main clades monocots and eudicots (The Angiosperm Phylogeny Group, 2009; Fig. 3.1). The basal angiosperms and magnollids share with some primitive traits, such as a typical spiral rather than whorled arrangement of flower organs (Albert et al., 1998; Endress, 2001a). The monocots show extreme variation in floral form, including bilaterally symmetric flowers with elaborately modified perianth parts. The organization of flower parts is a bit less variable in the core eudicots.

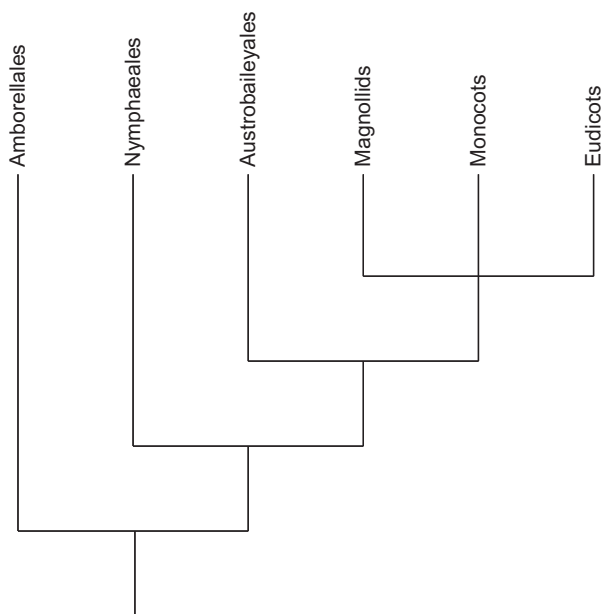


Figure 3.1 Phylogeny of angiosperms.

The determination of seed plant phylogeny has been immeasurably useful in defining the likely scenario for how the angiosperm flower evolved. Based on this rooting of the angiosperms, it seems likely that the primitive angiosperm flower was small, with few floral organs and lacking a well-developed perianth, similar to that of *Amborella* (Endress, 2001a), together with Nymphaeals (water lilies) represent the most basal angiosperms. The flowers at the base of crown group angiosperms can be reconstructed by consideration of floral structures within this grade. These ancestral flowers were probably already hermaphroditic and had an undifferentiated perianth, in which organs were arranged in more than two cycles or a spiral (Frohlich, 2006). The acquisition of distinct petals and sepals occurred during the angiosperm radiation (Crane et al., 1995; Soltis et al., 2005). In fact, based on the morphological evidence, petals appear to have evolved multiple times independently (Irish, 2009). Petals are thought to have evolved either as modifications of stamens or as modifications of bracts or other leaf-like structures (Kramer and Irish, 2000). The elaboration of these perianth organs resulted in larger, showier flowers, which has been proposed to be an evolutionary novelty that facilitated outcrossing by attracting pollinators (Dilcher, 2000).

With the accumulating knowledge about the molecular genetic basis of floral development in some model plants including *Arabidopsis thaliana*, snapdragon (*Antirrhinum majus*), petunia (*Petunia hybrida*), rice (*Oryza sativa*), and others, we now obtained insights into the molecular mechanisms underlying angiosperm floral morphogenesis. Floral homeotic genes are central to the specification of flower organ identities and were probably a major driver of flower evolution. Here, we focus on the mechanisms of the regulation of floral morphogenesis.



3. ABC MODEL OF FLORAL ORGAN IDENTITY GENES

3.1. Emergence of ABC model

Over the last couple of decades, a great number of flower abnormalities that could be traced to specific genes were reported by genetics. Such reports were scattered in the botanical, horticultural, and genetic literature or for considerations that the respective genes could be useful for the breeding of ornamental flowers. Mutations that affect flower differentiation were mainly reported in *A. thaliana* and *A. majus* (snapdragon).

The floral organ identity genes were originally identified in *A. thaliana* and *A. majus* on the basis of their phenotypes, which featured the homeotic transformation of one floral organ type into another. Flowers of *A. thaliana*

apetala 2 (*ap2*) mutant plants contain carpels in the positions that are normally occupied by sepals, and stamens in the positions that are normally occupied by petals. *apetala 3* (*ap3*) and *pistillata* (*pi*) flowers show homeotic transformations of petals into sepals and stamens into carpels, whereas *agamous* (*ag*) flowers form petals in place of stamens and sepals in place of carpels. In addition, the floral meristem of *ag* flowers fails to terminate in the production of the fourth-whorl organs. Instead, *ag* flowers are indeterminate and continue to produce new cells that are incorporated into many extra whorls of sepals and petals.

Genetic analysis of these *A. thaliana* floral homeotic mutants and their counterparts in *A. majus* led to the formulation of the classic ABC model for the specification of floral organ identity in 1991 (Coen and Meyerowitz, 1991). The ABC model proposes that organ identity in each whorl is determined by a unique combination of three activities by organ identity genes, called A, B, and C (Weigel and Meyerowitz, 1994). A function, conferred by the class A homeotic genes AP1 and AP2 in *A. thaliana* and the redundant AP2-like genes LIPLESS 1 and 2 (LIP1 and LIP2) in *A. majus*, specifies sepal identity in whorl 1 (Keck et al., 2003). A function combined with B, conferred by the class B genes AP3 and PI in *A. thaliana* and DEFICIENS (DEF) and GLOBOSA (GLO) in *A. majus*, specifies petal identity in whorl 2. B-function combined with C, conferred by the class C gene AG in *A. thaliana* and PLENA (PLE) and FARINELLI (FAR) in *A. majus*, specifies stamen identity in whorl 3. C-function alone determines carpel identity in whorl 4 and also confers floral determinacy. A second important aspect of the ABC model is that functions of A and C are mutually repressive, such that class C activity expands in A mutant flowers and vice versa. Although the details differ, the basic developmental program for floral organ patterning that is encapsulated by the ABC model seems to be widely conserved among plant species that have been extensively studied, including petunia (Angenent et al., 1992), tulip (Kanno et al., 2003), orchid (Tsai and Chen, 2006), and even plants with less showy flowers such as rice (Nagasawa et al., 2003), maize (Whipple et al., 2004, 2007), and wheat (Hama et al., 2004).

All the ABC genes belong to MIKC-type MADS-box genes except AP2. The MADS-box genes, encoding the MADS-box domain family of transcription factors, are involved in controlling all major aspects of the life of land plants. MIKC-type protein has a characteristic domain structure, including a MADS (M-), intervening (I-), keratin-like (K-), and C-terminal (C-) domain (Münster et al., 1997). The MADS-domain is about 58 amino acids long and is encoded by a DNA sequence termed the MADS-box. The term MADS-box gene was derived from *Saccharomyces*

cerevisiae MINICHROMOSOME MAINTENANCE 1 (MCM1), *A. thaliana* AGAMOUS, *A. majus* DEFICIENS, and *Homo sapiens* SERUM RESPONSE FACTOR (SRF) (Schwarz-Sommer et al., 1990). The MADS-domain is the major determinant of DNA binding, but it also performs functions in dimerization of MADS-box proteins and in the binding of accessory factors. Dimers of MADS-box domain transcription factors bind to CArG-boxes, stretches of DNA with the consensus sequence 5'-CC[A/T]₆GG-3' (Kaufmann et al., 2005). The I-domain, located directly downstream of the MADS-domain, is relatively variable in length and not strongly conserved. The K-domain is, after the MADS-box domain, the best conserved domain of MIKC-type MADS-box protein. This region form amphipathic α -helices that are predicted to form coiled coils and thereby mediate protein-protein interaction of MADS-domain proteins (Yang et al., 2003). The most variable region is the C-domain at the C-terminal end of MIKC-type proteins. C-domain is important for the activation of transcription of target genes (Honma and Goto, 2001) and may be also important for the formation of multimeric complexes.

3.2. Updated ABC model and higher order MADS-box protein complex

By 2001, the basic ABC model for the *Arabidopsis* floral organ specification was established (Theissen and Saedler, 2001). According to this model, the identities of each floral organs—sepals, petals, stamens, and carpels—are determined by combinations of MADS-box proteins that are bound to the promoters of the downstream targets. The expression of the target proteins is thus activated or repressed for the differentiation of each type of floral organ. Two dimers of each tetramer recognize two DNA sites (CArG-boxes) on the same DNA that is brought into close proximity due to the binding of the DNA.

Studies of genetic redundancy have led to the identification of the class E floral homeotic genes—originally known as *APL2*-like genes (Theissen et al., 1996), but later renamed into *SEPALLATA* (*SEP*)-like genes (Pelaz et al., 2000). *Arabidopsis* has four different *SEP*-like genes, termed *SEP1*–*SEP4*. *sep1 sep2* mutants have only subtle phenotypes (Pelaz et al., 2000), *sep1 sep2 sep3/+* mutant flowers show a reduction in stamen number (Ditta et al., 2004), whereas the *sep1 sep2 sep3* mutant has all floral organs converted into sepals, and flower development becomes indeterminate (Pelaz et al., 2000). In *sep1 sep2 sep3 sep4*, quadruple mutants vegetative rather than sepals develop in all whorls of indeterminate flowers (Ditta

et al., 2004). Thus, only the quadruple mutant exhibits a complete loss of floral organ identity. These dosage effects suggest that the involvement of multiple proteins interacting in complexes can buffer the effects of the loss of single components of the complex.

Ovules are initiated before the anthesis of flowers, thus ovule can also be considered floral organs. The determination of ovule differentiation was termed to D-function genes conferring ovule identity on tissues that develop within the carpels (Colombo et al., 1995). Cosuppression of the petunia *FBP7* and *FBP11* genes causes the replacement of ovules with carpel-like structures, whereas constitutive expression of *FBP11* is sufficient to induce ectopic ovule formation on sepals and petals. In *A. thaliana*, ovule identity is conferred by four closely related genes: *AG*, *SEEDSTICK (STK)*, and *SHATTERPROOF 1* and *2 (SHP1* and *SHP2)* (Pinyopich et al., 2003). In *shp1 shp2 stk* triple mutants, ovules are converted into leaf-like or carpel-like structures, whereas ectopic expression of either *STK* or *SHP* genes is sufficient to induce the homeotic transformation of sepals into carpaloid organs (Favaro et al., 2003). The *SEP* genes are also involved in ovule formation, because *sep1*^{-/+} *sep2 sep3* flowers have severely compromised ovule development and resemble *stk shp1 shp2* mutant flowers (Favaro et al., 2003). The accumulated knowledge then led to a modified ABC+DE model. According to the ABCDE model, class A and class E genes are required to specify sepals, A, B, and E control petals; B, C, and E determine stamens; C and E specify carpels; and D and E is necessary for ovule development (Ditta et al., 2004; Zahn et al., 2005b).

The molecular mechanism revealing higher order MADS-box protein complex formation was supported when Egea-Cortines et al. (1999) reported that DEF, GLO, and SQUAMOSA (SQUA) from *A. majus* have ability to form multimeric complexes with a strong interaction to DNA in electrophoretic mobility shift assays. The authors hypothesized that the protein complex is actually a protein tetramer, composed of a DEF-GLO heterodimer and a SQUA-SQUA homodimer, in which the DEF-GLO and SQUA-SQUA dimers recognize different CArG-boxes (Egea-Cortines et al., 1999). This observation was later generalized by Theissen and Saedler (2001) in the “floral quartet model.” At present, it is widely accepted that MADS-domain transcription factors regulate specific sets of target genes through the formation of dimers and multimeric protein complexes that bind to DNA and activate or repress their targets. The “floral quartet model” suggests that MADS-domain proteins form whorl-specific tetrameric complexes during floral organ determination. Two protein

dimers of each tetramer recognize two different CArG-boxes, which might be brought into close vicinity by bending the DNA between the CArG-boxes enabling the MADS dimers to act cooperatively in a tetrameric complex to regulate the target gene expression (Honma and Goto, 2001).

Several dimers and potential tetramers have been reported in the complete *Arabidopsis* MADS-box type II family protein–protein interactomes, using yeast-two-hybrid system (Y2H; deFolter et al., 2005). Furthermore, the protein interaction between SEP3, AP3, and PI and the formation of “quartets” has been corroborated *in vitro* (Melzer and Theissen, 2009), while, to date, approximately 20 potential higher order complexes involving *Arabidopsis* MADS-box proteins have been documented in a heterologous *in vivo* system (Immink et al., 2009). In the case of SEP3, it has been shown that this protein functions not only as a regulator of floral meristem but can also form ternary complexes with AP3 and PI in planta and colocalizes these proteins to the cell nucleus (Immink et al., 2009). These characters, plus the fact that the SEP3 protein contains four different motifs in the carboxyl-terminal which are known to function as transcription activation domains, have led some authors to suggest that SEP3 can function as the “glue” in the MADS-box protein interactions and regulation (Immink et al., 2009).



4. DOWNSTREAM TARGETS OF FLORAL ORGAN IDENTITY GENES

Floral organ identity genes specify the destiny of organ types in individual floral whorls. Except for *AP2*, most of the floral organ identity genes encode transcriptional factor containing MADS-domain. In the ABCE model, The determination of each floral organs are regulated by the protein complexes compositing of A-, B-, C-, and E-class MADS-box proteins in sole or combination behaviors. Products of these genes bind to *cis*-elements assembling to the consensus CArG-box sequences (de Folter and Angenent, 2006). In the early studies, various strategies have been used to identify the downstream genes targeted by floral regulators by screening differential expression profiles. Research advances are established on methods including contributions of genetic mutations and application of inducible gene expression system in combination with microarray technology and chromatin immunoprecipitation (ChIP) experiment. Recently, ChIP followed by or whole-genome microarray analysis (ChIP-CHIP) and ultrahigh-throughput Solexa sequencing (ChIP-SEQ) have been used as a powerful tool to obtain global direct downstream genes of plant transcription factors

(Kaufmann et al., 2010a). Although the ChIP assay has been successfully applied on many plant species in both dicot and monocot, comprehensive studies of downstream targets of floral organ identity genes mainly focusing on the flower development of *Arabidopsis* by using ChIP-CHIP or ChIP-SEQ.

4.1. Target genes of B-class transcription factors

B-function regulators APETALA3 (AP3) and PISTILLATA (PI) control the formation of petals and stamens during *Arabidopsis* flower development. Researches about the molecular network for petal speciation have been concentrated on the underlying control of AP3 and PI since the early years. The direct target for the AP3 was firstly identified and targeted to the CArG-boxes on its own promoter via a positive feedback regulatory loop (Goto and Meyerowitz, 1994; Honma and Goto, 2000, 2001). However, the autoregulation of PI was probably not accomplished directly by AP3/PI heterodimer due to lacking of CArG-boxes on the promoter of *PI* gene (Honma and Goto, 2000). Other than MADS-box genes, *NAC-LIKE*, *ACTIVATED BY AP3/PI (NAP)* which was induced by AP3 showed to involve in the transiting process of cell division to cell expansion at the late stages of floral development (Sablowski and Meyerowitz, 1998). B-class genes' transcripts were maintained not expanding to the fourth floral whorl by a boundary contributed by *SUPERMAN (SUP)* between the third and fourth whorls (Sakai et al., 2000). SUP which belongs to a C2H2-type zinc finger transcriptional factor family was directly regulated by AP3/PI and required for spatial restriction of *AP3* and *PI* (Sakai et al., 2000). Identification of downstream candidates of AP3 and PI using a microarray-based strategy recovered that targets of AP3/PI may play roles in transcriptional regulation, stress-related processes, cellular signaling, and cell wall modification (Zik and Irish, 2003). Both previously identified target, such as NAP, and newly recovered genes can be as potential direct targets of AP3/PI in petal and stamen development, considering the presence of CArG-boxes sequences in the regulatory region of the candidate genes (Zik and Irish, 2003). However, it remains unclear whether the candidate genes identified in array-based analysis are direct targets or not. Using ChIP, regulatory regions of candidate targets bound by AP3/PI proteins can suggest a direct regulatory interaction *in vivo*. Direct regulation of the A-class floral homeotic gene, APETALA1 (AP1) by AP3/PI heterodimer suggests that the genes involved in organ specification regulate the transcriptional activity of each

other (Sundstrom et al., 2006). Furthermore, genes encode two GATA transcription factors, GNC (GATA, nitrate-inducible, carbon-metabolism-involved) and GNL (GNC-like), which shared redundant roles in promoting chlorophyll biosynthesis were negatively regulated by AP3 and PI (Mara and Irish, 2008). In addition, AP3/PI directly bound to promoter elements of the three *BANQUO* genes (*BNQ1*, *BNQ2*, and *BNQ3*) indicated an inhibiting role in photomorphogenic and developmental signaling pathways (Mara et al., 2010). The genomewide binding patterns for AP3 and PI showed highly correlated due to that AP3/PI act as heterodimer. Binding sites were founded of several floral regulators including *UNUSUAL FLO- RAL ORGANS* (*UFO*), *RABBIT EARS* (*RBE*), *LEAFY* (*LFY*), *AP1*, *AP2*, *SEPALLATA3* (*SEP3*), *SUP*, and *SPOROCTELESS* (*SPL*) (Wuest et al., 2012). AP3 and PI activity through the flower development were accomplished by controlling different sets of genes at distinct phase in order to fine-tune flower formation progressively (Wuest et al., 2012). In addition to activate genes involved to in the control of numerous developmental processes required for organogenesis, AP3/PI act an opposing regulatory role to repress key regulators of carpel formation (e.g., *CRABS CLAW*, *CRC*) (Wuest et al., 2012).

4.2. Target genes of A-class transcription factors

Floral development is orchestrated through a trio that includes initiation of floral meristem, formation of floral primordia and floral organ identities, and hormonal pathway which was headed by *APETALLATA1* (*AP1*). It can establish floral meristem identity by repressing genes that are part of the shoot developmental program (*FD*, *SPL9*, and *SPL15*), inhibiting floral repressor (e.g., *TARGET OF EAT1*, *TOE1*, *TOE3* and *SCHNARCHZAPFEN*, *SNZ*), or its antagonist *TFL1* (Kaufmann et al., 2010b). At the part of facilitating floral promordia and organ specification, *SEP3* promoter was targeted by *AP1* to activate *SEP3* expression during floral development which further leading to the formation of *AP1/SEP3* heterodimer (Kaufmann et al., 2010b). *AP1* regulated cell proliferation directly controls the expression of genes involved in the metabolism of and response to the hormone gibberellin (GA), such as *GA3ox1*, *GA2ox1*, and *RGA-LIKE2*. Several growth-regulatory proteins controlling cell proliferation and cell expansion were also targets of *AP1* (Krizek and Anderson, 2013).

Bifunctional transcription factor *AP2* involved in a wide range variety of flower development by direct regulating flowering time and floral organ

development genes. Its dual functions in repressing flowering and floral development were revealed by acting as both transcriptional activator and repressor, inducing expression of floral repressor *AGAMOUS-LIKE15* (*AGL15*) and miR156e, while repressing transcript of floral activator *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* and miR172b (Yant et al., 2010). The C-class gene, *AGAMOUS* (*AG*) as the well-known target of AP2 was directly repressed by AP2 by recruiting the cosuppressor TOPLESS (TPL) and the HISTONE DEACETYLASE 19 (HDA19) (Krogan et al., 2012; Yant et al., 2010). Additionally, cooperative repressive complex of AP2 can restrict petal fate by either binding to the promoters of *AP3* and *SEP3* to restrict their expressions from the first floral whorl or indirectly controlling the expression domain of *PI* (Krogan et al., 2012).

4.3. Target genes of C- and E-class transcription factors

The SEP3 protein facilitated the formation of dimeric MADS-box protein complexes suggested the widespread involvement of SEP3 as the multifunction protein in various plant developmental processes (Immink et al., 2009). Due to this reason, the direct target genes of E-class transcription factors in regulating floral organ identity were only studied in SEP3 (Kaufmann et al., 2009). In addition to binding to the MADS-box genes for floral organ identity, *AP1*, *AP3*, *AG*, *SEP1*, *SEP2*, *SEP4*, and itself, binding sites on the ovule identity genes (e.g., *SHP1* and *SHP2*) were recognized by SEP3 which suggested the ability of SEP3 to activate other floral homeotic box genes (Kaufmann et al., 2009). Many SEP3 downstream targets were also non-MADS-box genes and genes in the hormonal pathways, in particular the auxin signaling (Kaufmann et al., 2009). The Auxin-responsive *OsMGH3* served a common downstream target of rice SEP and AGL6-like proteins of *OsMADS1* and *OsMADS6*, respectively (Yadav et al., 2011). Besides, SEP3 and AP1 coordinately targeted many floral organ size regulators, such as *Growth-Regulating Factor1-5* (*GRF1-5*), *GRF-interacting factor1* (*GIF1*), *TCP4*, and *ARGOS-LIKE* (*ARL*) to promoting organ growth (Krizek and Anderson, 2013).

C-function gene *AG* controlled gynoecium development by directly regulating several essential genes, including *CRC* gene which was repressed by AP3/PI (Gomez-Mena et al., 2005; Wuest et al., 2012) and *DELAYED ANTHHER DEHISCENCE* (*DAD1*) which played a key role in coordinating jasmonic acid-induced pollen maturation (Ito et al., 2007). Even C-function gene *AG* and E-function *SEP3* played overall

nonoverlapping roles in regulating floral organ speciation; various target genes of AG and SEP3 were identified in carpel and ovule development, especial the redundant *SHP1*, *SHP2* and *STYLEISH* (*STY1*) (Kaufmann et al., 2009; Sablowski, 2010). Moreover, AG/SEP3 directly targeted the BELL-like *Arabidopsis* homeodomain protein *ATH1* and *NUBBIN* (*NUB*) and *JAGGED* (*JAG*) that function in cell proliferation and differentiation (Gomez-Mena et al., 2005; Ito, 2010; Kaufmann et al., 2009).



5. NOVEL GENES INVOLVED IN FLORAL MORPHOGENESIS

After floral organ identity genes specify the development of each whorl of floral organs, regulation of cell proliferation, and cell expansion cause the diversity of angiosperm flowers. Divergent selection on floral traits such as flower size and/or shape imposed by variable abiotic and/or biotic conditions can drive population differentiation (Brunet, 2009) and could potentially contribute to reproductive isolation (Schiestl and Schluter, 2009; Venail et al., 2010).

In *Arabidopsis*, constitutively expression the auxin-inducible gene *AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE* (*ARGOS*) or the gene encoding the AP2/ERF type transcription factor *AINTEGUMENTA* (*ANT*) can result in floral organs because of extending the period in which cells are competent to undergo cell division (Hu et al., 2003; Krizek, 1999; Mizukami and Fischer, 2000). In contrast, floral organs reach a smaller final size in plants lacking *ARGOS* or *ANT* function (Hu et al., 2003; Krizek, 1999; Mizukami and Fischer, 2000). *ANT* may act by regulating the expression of cell-cycle genes such as *CYCLIND3;1* (*CYCD3;1*) (Mizukami and Fischer, 2000). Another two proteins that share a small motif and endoplasmic reticulum-localization with *ARGOS* are *ARGOS-LIKE* (*ARL*) and *ORGAN SIZE RELATED1* (*OSR1*) (Feng et al., 2011). *ARL* promotes organ growth through effects on cell expansion (Hu et al., 2006) *OSR1* primarily affects cell proliferation via maintenance of *ANT* expression in maturing lateral organs, and it also promotes cell expansion indirectly (Feng et al., 2011). Although *ARGOS*, *ARL*, and *OSR1* have overlapping functions in organ growth, they are regulated by different hormones, suggesting that these genes may integrate distinct signals during organ morphogenesis (Feng et al., 2011; Hu et al., 2003, 2006). In *AINTEGUMENTA-LIKE/PLETHORA* (*AIL/PLT*) protein family, at least two transcription factors

AINTEGUMENTA-LIKE 5 (AIL5) and AIL6 sharing high sequence similarity within the DNA-binding AP2 repeat region of ANT could act redundantly with ANT to regulate floral organ growth and patterning. *ant ail6* double mutants produce smaller sepals (Krizek, 2009). In contrast, misexpression of AIL5 and AIL6 can result in the production of larger floral organs (Krizek and Eaddy, 2012).

Arabidopsis GROWTH-REGULATING FACTORS (GRFs) and GRF-INTERACTING FACTORS, which function as transcription factors and coactivators, could physically interact to promote cell division in floral organs (Kim and Kende, 2004; Kim et al., 2003). Loss-of-function of these genes results in smaller petals owing to reduced numbers of cells (Horiguchi et al., 2005; Kim and Kende, 2004; Lee et al., 2009). These proteins appear to have partially overlapping functions in floral organ growth as higher order mutants generally show more severe defects.

One of the conspicuous patterns in the angiosperms is the large number of shifts between radial and bilateral floral symmetry (Endress, 1999). Phylogenetic and systematic studies confirm that bilateral symmetric flowers have evolved several times from radially symmetric ancestors (Endress, 2001b). Transitions to bilateral flower symmetry have been associated with the evolution of specialized pollinators and have been crucial in the diversification of flowering plants. Over the past decade, the developmental genetic pathway of bilateral flower symmetry has become well established for the model species *A. majus*. The genes identified are *CYCLOIDEA* (*CYC*)-like genes, which belong to the class II TCP (TEOSINTE BRANCHED1/*CYCLOIDEA*/PCF) family of transcription factors. These genes have been recruited multiple times to pattern dorsal flower identity in core eudicot lineages that have independently evolved bilateral symmetric flowers. Until recently, *CYC*-like genes had been mostly thought to be related to the control of dorsal and lateral floral organ development (Hileman and Cubas, 2009). It is now understood that groups of class II TCP genes are transiently expressed in different developing tissues, such as flower and shoot meristems and leaf and floral organ primordial, where they help give shape to these structures. Indeed, these genes not only control floral organ number, petal shape and stamen abortion (*CYC*-like genes) but also have strong effects in leaf shape, size, and curvature (*CINCINNATA* genes) and prevent branch outgrowth (*TEOSINTE BRANCHED1* genes) (Martin-Trillo and Cubas, 2010). Given that they control basic developmental processes related to tissue proliferation and differentiation, it means that

the recruitment of TCP genes to new functions could be constrained by the ancestral function of those genes.



6. APPLICABILITY OF ABC MODEL FOR OTHER ANGIOSPERMS

6.1. Foundation of ABC and ABCE models for establishing floral organ identity

Flowers exhibit enormous variation in shape, size, and structure. Even though extreme variation of flower morphologies is found through angiosperms, relative simplicity of four types of floral organ identities can be distinguished. Understanding of regulation in floral development about ABC model in model plant species has been well studied and expendably applied to a wide range of plant species (Bowman, 1997; Litt and Kramer, 2010; Soltis et al., 2007). Studies on the E-function MADS-box genes assembled the missing puzzle cut to the ABC model and the later proposed ABCE model can be seen as a framework for regulating floral development (Honma and Goto, 2001; Pelaz et al., 2000). Since its proposed, formation of tetrameric protein complexes composed by floral organ identity proteins in the floral quartet model provides supporting evidence for the specificity of distinct floral organs by A-, B-, C-, and E-function homeotic proteins coordinately (Theissen and Saedler, 2001). Multiple SEP homologs are present through the angiosperm lineages (Zahn et al., 2005a), with important relevance of ABCE model generally supporting the conservation of this model during the evolution of angiosperms.

6.2. “Fading border” model for basal angiosperms

The basal lineages of angiosperms represent only a few percent of the flowering plant species, but it is here where the greatest diversity in floral structure and form is found. The flowers of basal angiosperms vary considerably in size, the number of floral parts, and the arrangement of floral organs in spiral and whorls. In some basal angiosperms (*Amborella* and *Illicium*), floral organs are spiral arranged with a transition from bracts to outer and inner tepals, from tepals to stamens, and finally to carpels.

The expression patterns of putative floral homeotic genes were determined in several basally diverging angiosperms lineages, including *Amborella*, *Nuphar*, *Illicium*, *Magnolia*, and *Asimia* (Kim et al., 2005). In general, orthologues of class B genes were found to be expressed in stamens and perianth organs, orthologues of class C genes in stamens and carpels, and class

E genes in all floral organs (Kim et al., 2005). The expression patterns obtained for ABCE genes from basal angiosperms are generally consistent with the morphology of the flowers as predicted by ABC model. For example, floral organs in *Amborella*, *Nuphar*, and *Illicium* expression class B gene orthologues are always more or less “petaloid” (or stamens). Together with floral developmental studies, the “fading borders model” was proposed. This model indicates that the gradual transitions in floral organ morphology result from a gradient in the level of expression of floral organ identity genes across the developing floral meristem. Relatively overlapping expression level of ABC genes at the margin of floral whorls would result in the formation of morphologically intermediate floral organs rather than organs that are clearly distinct (Soltis et al., 2006).

6.3. “Sliding boundary” model correlating with flower diversity

Shifting boundaries of ABC gene expression may have contributed to the diversification of the angiosperm flower many times independently (Theissen and Melzer, 2007). In contrast to the flowers of higher eudicots, many nongrass monocot and some basal eudicot flowers have similar appearance of first whorl floral organs to those of second whorl. To explain this floral morphology, the modified ABC model was proposed (van Tunen et al., 1993). This model proposed that the expression of class B genes was extended to whorl 1, with the result that the floral organs of the first and second whorls have almost the same petaloid structure. When putative class B genes, *DEF*-like and *GLO*-like genes, were investigated in lily and tulip, they were found to be expressed in the organs of the first three whorls of the flower, as predicted by the modified ABC model (Kanno et al., 2003). A similar phenomenon is also discovered in orchids. Orchid flowers have unique floral organs including three types of perianth organs and a reproductive gynostemium fused by androecia and gynoecia. The three types of perianth organs are three outer tepals in the first whorl, and two lateral inner tepals as well as a medium inner tepal called the labellum. All these tepals are usually of petaloid appearance and express class B floral homeotic genes (Tsai et al., 2004, 2005). Moreover, evolution of development of petaloid organs is addressed with B-function genes’ expressions shifting to petaloid stamen of *Canna* (Almeida et al., 2013) and transferring to petaloid bracts of *Cornus* (Feng et al., 2012). In contrast to the standard flower structure, *Lacandonia* renders an ABC model by having B-function genes displaced to the center of the floral meristem (Alvarez-Buylla et al., 2010).

The heterotopic arrangement of stamen and carpel in *Lacandonia* not only represents unique evolution of floral homeotic arrangement but also proves the plasticity of ABCE model.

Many flowers of the Ranunculaceae (basal eudicot) have distinctly different petaloid organs in the first two whorls. Again, expression studies suggested that petaloidy of organs in the first floral whorl is due to a shift of class B gene expression toward the first floral whorl. In addition, differential expression of particular lineage of DEF/AP3-like genes has contributed to the distinction of the petaloid organs in the first and second floral whorl (Kramer et al., 2003). Shifts in class A and class C gene expression have been less frequently considered. Recently, in *Phalaenopsis* orchid, a *gynostemium-like petal* (*gylp*) mutant was discovered. In this mutant, the petals bear ectopic pollinia and their epidermal cells are morphologically intermediate between those of wild-type petals and those of the gynostemium (Chen et al., 2012). Expression analysis showed that transcripts of a class C gene (*PeMADS1*) were detected in the gynostemium-like inner lateral tepals of the *gylp* mutant. Thus, shifting expression of class C genes could also contribute the diversification of floral morphology.

6.4. Complexity of orchid floral morphology and ABC model

Containing more than 20,000 species, the Orchidaceae classified in class Liliopsida, order Asparagales is one of the largest angiosperm families. According to the classic view, the orchid flower is composed of five whorls of three segments each including two perianth whorls, two staminal whorls, and one carpel whorl (Fig. 3.2). Orchidaceae represent an unusually coherent group among monocots, possessing several reliable floral morphological synapomorphies, including the presence of a gynostemium, or column, fused by the style and at least part of the androecium, a highly evolved petal called labellum, and resupination caused by 180° torsion of the pedicel (Rudall and Bateman, 2002). All expected whorls in the flowers are present in orchids, and the highly sophisticated flower organization offers an opportunity to discover new variant genes and different levels of complexity within morphogenetic networks. Thus, the Orchidaceae can be used to testify the validity of the “ABC model” in the monocots and to study how MADS-box genes are involved in defining the different highly specialized structures in orchid flowers.

In the last 10 years, isolation and characterization of individual MADS-box genes from orchids occurred at a faster pace. However, because of their

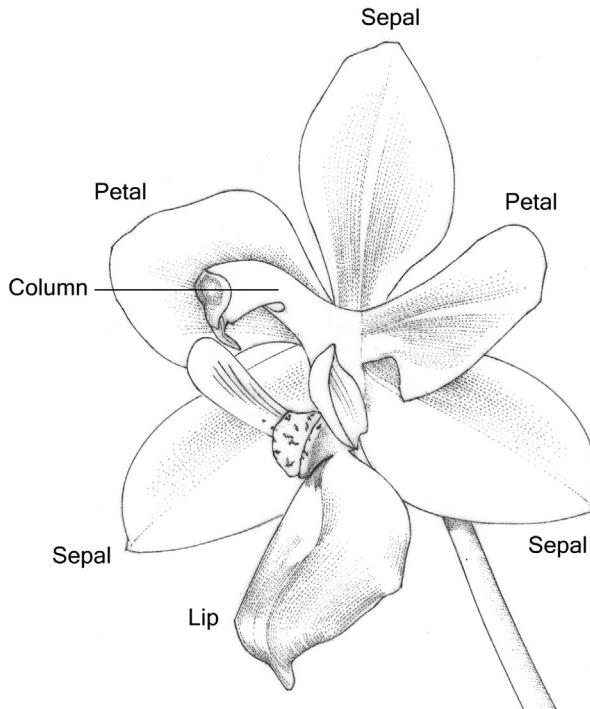


Figure 3.2 The flower of *Phalaenopsis equestris*. Orchid flowers have three sepals and three petals. One of the petals is morphologically different in structure and is known as the labellum or lip. The male and female reproductive parts are fused in a structure, the gynostemium or column, in the center of the flower. The pollen grains stick together to form the pollinia located at the upper tip of the column under the anther cap.

role in perianth and gynostemium specification, nearly all efforts focused on class B and C genes from species in Epidendroideae, the largest orchid subfamily containing most varieties of horticultural importance like *Phalaenopsis*, *Dendrobium*, and *Oncidium* (Hsiao et al., 2011; Mondragón-Palomino, 2013).

All class B genes *AP3*-like genes identified in orchids are members of the paleo*AP3* lineage. The paleo*AP3* genes identified in orchids were subdivided into four subclades including *AP3B1* (the same as clade 1 proposed by Mondragón-Palomino and Theissen, 2008), *AP3B2* (the same as clade 2), *AP3A1* (the same as clade 3), and *AP3A2* (the same as clade 4) (Mondragón-Palomino and Theissen, 2008; Pan et al., 2011;

Tsai et al., 2004). Expression and protein–protein interaction studies suggest that the specific combination of duplicate gene expression and protein–protein interactions is responsible for the development of different floral organs (Mondragón-Palomino and Theissen, 2011; Pan et al., 2011; Tsai et al., 2004, 2005, 2008a,b). Based on these studies, “orchid code” and “Homeotic Orchid Tepal (HOT) model” were, respectively, proposed to illustrate the regulation of perianth morphogenesis in orchids (Mondragón-Palomino and Theissen, 2008; Pan et al., 2011). In the HOT model, *PI* and *AP3B* clades determine the formation of sepals. The combination of *PI* and both *AP3A1* and *AP3B* clades controls the lateral petal formation at the inflorescence stage, whereas *PI* and *AP3A1*, *AP3A2* clade genes, and *AGL6*-like genes contribute to lip morphogenesis in the floral bud stage (Pan et al., 2011). The HOT model is somewhat similar to the “orchid code” (Mondragón-Palomino and Theissen, 2008) but more precisely points out the effect of *PeMADS4* (*AP3A2* clade) in determining lip morphogenesis at a relevant later floral development stage.

More recently, the study of floral terata from *Cymbidium ensifolium* and *Phalaenopsis equestris* advanced our understanding of *AGAMOUS*-like genes in orchid flower development. The phenotype of the *multitepal* mutant of *C. ensifolium* is analogous to *agamous* from *A. thaliana* in that the gynostemium is replaced by an ectopic flower which produces outer and inner tepal-like structures centripetally (Wang et al., 2011). The study showed that duplication of C-class genes may have occurred in Epidendroideae. After the duplication event, the two lineages underwent functional diversification to produce distinct functional repertoires. *CeMADS1* may initiate the development of fused male and female reproductive organs and be involved in floral meristem determinacy. However, *CeMADS2* may play a maintenance role to complete gynostemium morphogenesis and have a redundant function in floral meristem determinacy (Wang et al., 2011). In the *gryp* mutant of *Phalaenopsis* hyb. “CD1,” the inner lateral tepals bear ectopic pollinia and their epidermal cells are morphologically intermediate between those of wild-type tepals and those of the gynostemium (Chen et al., 2012). The expression of *PeMADS1* (class C gene) and *PeMADS7* (class D gene) was detected exclusively in the column of the wild-type flowers, while only *PeMADS1* was detected in the gynostemium-like inner lateral tepals of the *gryp* mutant. Thus, the class C genes play more important roles in orchid gynostemium development than that of class D genes.

6.5. Duplication and divergence of MADS-box genes

Duplication of floral organ identity genes has been evolved during the evolution history of angiosperms (Purugganan et al., 1995; Theissen et al., 2000). Many genes subsequently become divergent which causes subfunction or neo-function to respond for the fine-tune regulation of perianth development (Moore and Purugganan, 2005; Ng and Yanofsky, 2001). The occurrences of key evolutionary events in A-, B-, C-, and E-class genes seem to facilitate the complexity of regulating mechanism which affected the alternations of various floral organs (Hernandez-Hernandez et al., 2007; Irish and Litt, 2005; Kramer et al., 2004; Litt and Irish, 2003; Ng and Yanofsky, 2001). A major duplication event in the AP3 lineage has been cited as an important factor for the radiation of higher eudicot and may reflect the evolution of a petal-specific AP3 function (Kramer et al., 1998). Comparative studies between the gene products of the ancestral homologs and the raised copies can be analyzed through many genetic and molecular experiments, the biological functions of how these genes may have changed during the course of angiosperm diversification.



7. CONCLUSION AND PERSPECTIVES

We are already approaching an understanding of the underlying genetic basis for rapidly evolving aspects of the flower. The molecular mechanisms underlying the formation of flowers are considerable progress over the past two decades. At present, more and more complete plant genome sequences and large sets of expressed sequence tags were generated by next-generation sequencing technology and deposited in the available public database. For example, a floral genome project was established to extend knowledge of developmental genes known from model species across a selection of angiosperms (Albert et al., 2005; <http://fgp.bio.psu.edu/>). More specifically, comprehensive collection of Orchidaceae floral transcriptomes was deposited at OrchidBase (<http://orchidbase.itps.ncku.edu.tw>) for providing genetic information to study evolutionary trajectory of orchid flowers (Fu et al., 2011; Hsiao et al., 2013; Tsai et al., 2013). Thanks to the combination of comparative developmental biology and genomics, exciting new insights are being revealed in the evolution of development and the underlying regulatory mechanisms. We believe that only an integrative approach, combining classic genetics, functional genomics, bioinformatics, and comparative genomics, will be able to unravel the “abominable mystery” of evolution of angiosperm flowers.

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New Insight into Role of Myosin Motors for Activation of RNA Polymerases

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Abstract

In the eukaryotic cell nucleus, actin and myosin are emerging as essential regulators of nuclear function. At gene level, they regulate chromatin and modulate RNA polymerase transcription, and at the RNA level, they are involved in the metabolism of ribonucleo-protein complexes. Furthermore, actin and myosin are involved in maintaining the structure of cell nucleus by mediating chromatin movement and by interacting with components of the nuclear lamina. This plethora of functions is now supported by evidence that nuclear actin polymerizes just like the cytoplasmic actin fraction. Based on these considerations, we now hypothesize that the nuclear myosin forms function as actin-based motors. In this chapter, our goal is to start from the knowledge acquired in the cytoplasmic field to explore how nuclear myosin functions in gene transcription. One of the pressing issues discussed here is whether nuclear myosin produces local tension or functions as transporters. Based on two current models reported in the literature, we discuss the topology of the actin-based nuclear myosin 1 motor and how it is believed to facilitate propulsion of the RNA polymerase machinery while maintaining chromatin that is compatible with transcription. These mechanisms will be placed in the context of cell cycle progression.



1. INTRODUCTION

Actin and myosin synergize to comply with global cellular dynamics (Louvét and Percipalle, 2009; Pederson and Aebi, 2002, 2005). By moving unidirectionally on actin filaments, myosin drives a wide range of vital functions for the cell including cellular movement, contraction, cytokinesis, membrane trafficking, signal transduction, vesicle, and RNA transport (Baker and Titus, 1998; Coluccio, 2008a; Mermall et al., 1998). Both actin and myosin are abundant in the eukaryotic cell nucleus as shown by studies throughout the 1970s (Louvét and Percipalle, 2009). However, the direct involvement of actin and myosin in nuclear structure and function has been a matter of heated debate and even skepticism. In the last 15 years, however, several independent studies have delivered fundamentally new hypothesis on how gene expression can be regulated in the eukaryotic cell nucleus by actin and myosin, bookmarking a rapidly growing research area. These new ideas have also found fertile ground in the scientific community considering the highly dynamic nature of the cell nucleus and its ability to undergo reprogramming in response to environmental and developmental cues (Misteli and Spector, 2011). Many investigations have demonstrated a direct involvement of actin and certain myosin forms in chromosome dynamics, chromatin structure and function, gene transcription, and

RNP assembly, processing, and transport (Chuang et al., 2006; Dundr et al., 2007; Fomproix and Percipalle, 2004; Hofmann et al., 2001, 2004; Kukalev et al., 2005; Kumaran et al., 2008; McDonald et al., 2006; Olave et al., 2002; Percipalle et al., 2001, 2002, 2006; Philimonenko et al., 2004; Vreugde et al., 2006; Ye et al., 2008; Kapoor et al., 2013; Kapoor and Shen, 2014).

Although independent roles for actin and myosin cannot be ruled out, the main and most intriguing consequence of having actin and, so far, up to seven different myosin isoforms within the cell nucleus is that specialized myosin motors are likely to be implicated in different aspects of nuclear function. The etymology of the word “motor” comes from the Latin word “motor,” literally “mover,” agent noun from past participle stem of *movere* “to move.” Over time, the word motor was used to define an “agent or force that produces mechanical motion” and that of a “machine that supplies motive power.” Therefore, by suggesting that motors are present in the nucleus, we implicitly suggest that there are nuclear machines or factors whose movement must be controlled or regulated in order to be efficient. In the cytoplasm of eukaryotic cells, myosin generates energy by hydrolyzing ATP and releasing inorganic phosphate (Hwang and Lang, 2009). The chemical energy produced is then converted into mechanical energy that allows for movement. The mechanochemical cycle that originates from these concerted mechanisms is at the basis of myosin movement and comprises binding of myosin to actin filaments, conformational changes that occur on the myosin, release of the myosin from the filament, conformation reversal of the myosin, and rebinding of myosin to the actin filament. The outcome is local myosin translocations or steps that summed up together collectively lead to long-range movement on the actin filament. The resulting myosin-based propulsion stabilizes and/or moves the actin filaments (i.e., contractile stress fibers) and conducts myosin-bound cargoes such as vesicles, organelles, or other proteins to specific regions within the cell (Katoh et al., 1998). Myosin motors have directionality on actin filaments, with all myosin types moving toward the barbed end, except myosin VI that moves toward the pointed end. In the cytoplasm, the majority of actin filaments have the barbed end directed toward the plasma membrane and the pointed end toward the interior. This arrangement allows certain myosins to function primarily for cargo export (myosin V) and other myosins as major motors for import (myosin VI). This directionality is achieved by conformational changes that allow movement in only one direction.

In the nucleus, we have now evidence that actin and myosin are attached to multiprotein complexes such as chromatin, RNA polymerases, and

ribonucleoprotein complexes. Therefore, the obvious question here is whether there is a nuclear mechanochemical cycle that allows for myosin motor-based movement on actin filaments. If this is the case, we would expect that myosin motors are required for movement of nuclear complexes on actin filaments. An insightful study has recently demonstrated that nuclear actin can undergo regulated polymerization (Baarlink et al., 2013; Belin et al., 2013). This important discovery provides a starting platform to address how nuclear myosin could function as motor and its impact on gene expression.

The focus of this chapter is on emerging evidence that nuclear myosin isoforms dynamically interact with the RNA polymerase machinery and drive transcription across active genes. By integrating knowledge from the “cytoplasmic” myosin field, we will discuss possible experimentally testable models that may explain how these nuclear myosin isoforms can perform a motor function during co-transcriptional movement of the RNA polymerase machinery across active genes.



2. MYOSIN SUPERFAMILY

Since the discovery of ATP-dependent contractile activities in non-muscle cells (Hoffmann-Berling, 1954), 18 subtypes of unconventional myosin classes have been identified (see Table 4.1). A huge number of studies have quantitatively dissected their structural features, how they interact with actin to generate mechanochemical energy and how these mechanisms are regulated (Coluccio, 2008a). The aim of this chapter is to recapitulate some of this knowledge and highlight their significance and potential impact on the structure and function of the eukaryotic cell nucleus.

2.1. Myosin conservation, structural properties, and insights into selectivity of nuclear cargo binding

Myosin is a member of a large family of motor proteins (Foth et al., 2006), virtually present in all organisms. The primary structure of myosin is highly conserved among the different family members. Both N-terminus and C-terminus of the protein comprise evolutionary conserved domains referred to as “head” and “tail” domains, respectively. The motor function is located within the head domain. Its core is highly conserved across all the myosin classes both structurally and at the amino acid level. Structural analysis performed on the myosin II head domain showed evidence of a catalytic pocket containing an ATP-binding site and an actin-binding site

Table 4.1 Myosin has been classified into 18 different classes based on phylogenetic analysis of the conserved head/motor domain

Class	Isoform	Cellular localization	Cellular function	Cargo binding
I	8	Cytoplasm, myofibril, nucleus	Membrane trafficking, signal transduction	—
II	14	Cytoplasm, myofibril, nucleus	Muscle contraction, cytoskeletal organization	—
III	2	Cytoplasm, myofibril	Muscle contraction	—
V	3	Cytoplasm, nuclear	Outward protein transport	+
VI	1	Cytoplasm, myofibril, nuclear	Inward protein transport	+
VII	2	Cytoplasm, myofibril	Intracellular transport, muscle contraction	+
IX	2	Cytoplasm, cytoskeleton	Cytokinesis, cell shape	N/A
X	1	Cell projection, lamellipodium	Cytokinesis, cell shape	+
XV	2	Cytoplasmic, myofibril	Muscle contraction	+
XVI	1	Cytoplasm, nucleus	S-phase regulation, intracellular movement	N/A
XVIII	2	Cytoplasm, nucleus	Stromal cell differentiation and tumor suppression	N/A

The nuclear myosin forms are highlighted in bold.

(Dominguez et al., 1998; Houdusse et al., 1999; Rayment et al., 1993). The core head domain reveals structural similarities to kinesins (Vale and Milligan, 2000), suggesting that intracellular motion is possibly generated through conserved mechanisms. The myosin head domain is linked through a converter region to the “lever arm” or “neck region,” structurally organized into a long helix whose length depends on the number of IQ motifs (from none to six) with consensus sequence IQxxxRGxxxR. The IQ motifs are important regulatory elements for myosin as they are targeted by regulatory light chains. In the specific case of myosin 1, the IQ motifs are targeted by calmodulin (Coluccio, 2008b), whereas so far, the only class of myosin

that lacks such a neck region is the class XIV *Toxoplasma gondii* myosin A (Heintzelman and Schwartzman, 1997). The length of the lever arm also determines the number of light chains and has an impact on the way specific myosin types move (Uyeda et al., 1996). For instance, myosin V contains more light chains relative to myosin II, and myosin V therefore moves in larger steps along actin filaments after an equivalent ATP hydrolysis cycle (Ruppel and Spudich, 1996).

The C-terminal “tail” region considerably varies in sequence length, domain composition, and organization, which reflects the specialized functions of the domain in each myosin type. The tail, in fact, mediates filament assembly through dimerization or interactions with specific cargoes. This promiscuity in the spectrum of protein-to-protein interactions in which the tail domain is involved accounts for the involvement of different myosin types in distinct and apparently unrelated cellular activities. In contrast, the conserved head domain with ATP-binding pocket (Walker et al., 1982) and F actin-binding site is common to all myosins (it is the motor domain) (Cheney et al., 1993; Hwang and Lang, 2009) and allows for conserved propulsion mechanisms.

The primary structure of nuclear myosin isoforms shows that the head domain, including both ATP-binding pocket and actin-binding site, is also conserved (see Fig. 4.1). These considerations therefore altogether suggest that myosin-based motion is likely to be mechanistically conserved, regardless of whether it takes place in the cytoplasm or in the nucleus. In fact, the tail domain of nuclear-based myosin isoforms does not substantially differ from the cytoplasmic-based variants. An example is provided by the canonical myosin 1c and the two recently identified nuclear alternatively spliced isoforms (Ihnatovych et al., 2012; Pestic-Dragovich et al., 2000), which display extra amino acid sequences in the very N-terminus but exhibit conserved tails. So one of the most intriguing aspects is to find out what provides selectivity for cytoplasmic or nuclear cargoes given the degree of amino acid conservation among the three myosin 1c isoforms. An interesting hypothesis, still to be proven, is that it is precisely the regulated nuclear and subnuclear compartmentalization that guarantees selectivity in the cargo binding by each of the isoforms. Certain nuclear forms of myosin have been shown to accumulate to subnuclear compartments upon phosphorylation. It therefore seems possible that posttranslational modifications may also contribute to guide selectivity of individual nuclear myosin forms for their cargoes.

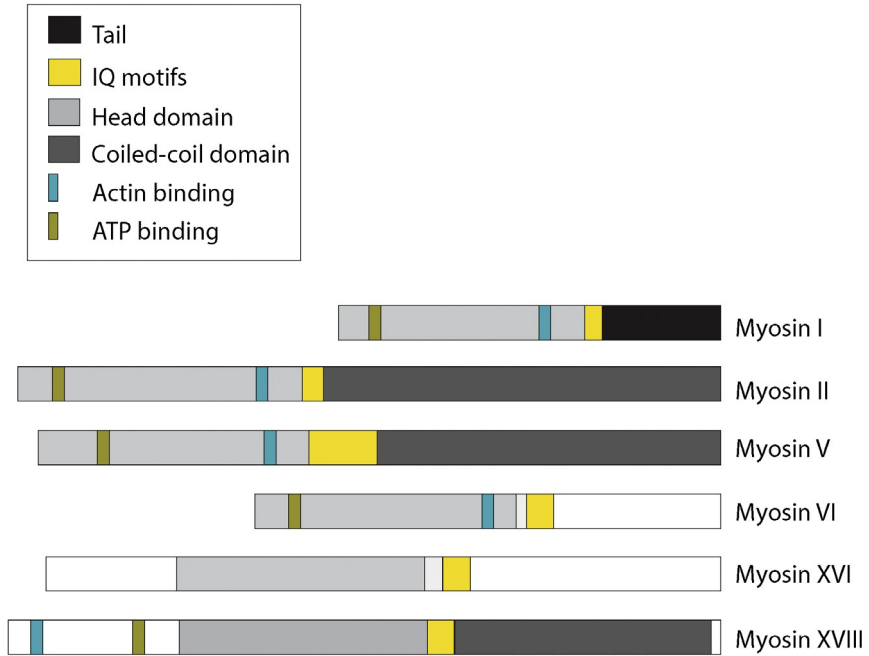


Figure 4.1 A cartoon illustrating the primary structures and domains organization of the six nuclear forms of myosin identified so far in the cell nucleus. This number is probably underestimated and many others are likely to be uncovered in the future. A comparison of their primary amino acid sequences and structural features does not anyway provide clues on the mechanisms and intracellular signaling pathways that modulate their nuclear import. Furthermore, except for the nuclear form of myosin 1, their precise roles in nuclear structure and function are yet to be determined.

2.2. Nuclear actomyosin complexes: Lessons from cytoplasm

During their motor function, all myosin isoforms undergo changes in conformations that are direct consequences of ATP binding and ATP hydrolysis. These conformational changes that occur in the myosin structure accompany binding to and movement along actin filaments (Hwang and Lang, 2009; Vale and Milligan, 2000; Volkmann and Hanein, 2000). The complete cycle including ATP binding, hydrolysis, and phosphate release is known as the “power stroke” cycle. A complete round of ATP hydrolysis produces a single “step” or movement of the myosin protein along the filament. The myosin ATPase cycle, partly regulated by intracellular free calcium (Ebashi and Endo, 1968), is therefore the fundamental unit of movement that generates local force (Tyska and Warshaw, 2002) and

explains the reiterated interactions with actin during active motion. The efficiency of myosin-based motors over a certain distance depends on the affinities of interaction between individual myosin molecules and actin. In order to progress from one cycle to the next, the myosin head lacks a bound ATP, and it is attached to the actin filament in a very short-lived conformation known as the “rigor conformation.” Following ATP binding, the myosin head domain is subjected to a conformational change that reduces its affinity for actin and causes the myosin head to release the actin filament. Importantly, ATP binding by the myosin induces a large conformational shift in the “lever arm” that tilts the head into a position further down along the filament. Upon ATP hydrolysis, when the resulting ADP and inorganic phosphate are still associated with myosin, a weak contact between myosin and the actin filament is established. This contact is further strengthened immediately following release of the inorganic phosphate. This finally triggers the power stroke and generates force step used by myosin motor proteins. The power stroke therefore occurs at the release of phosphate when the myosin is tightly bound to actin. The effect of this release is a conformational change in the molecule that pulls against the actin. As myosin regains its original conformation, the ADP is released, but the myosin head remains tightly bound to the filament at a new position from where it started, thereby bringing the cycle back to the beginning. Although the interaction between a myosin molecule and actin is conserved and dependent on the myosin ATPase activity (Coleman and Mooseker, 1985; Hwang and Lang, 2009), the topology of actomyosin complexes varies depending on the number of heavy chains that accompany an individual myosin. Those myosin isoforms comprising a single heavy chain contact actin filaments at only one site, in contrast to myosin isoforms containing two heavy chains, which bind to actin filaments at two sites. Myosin II is the only myosin isoform that assembles into minifilaments that are able to apply force to move actin filaments relative to each other (Clarke and Spudich, 1974; Hynes et al., 1987; Sheetz et al., 1992; Sheetz et al., 1986). Minifilament assembly is regulated by the phosphorylation state of the heavy chain tail (Egelhoff et al., 1993) and the motor activity is stimulated by phosphorylation of the regulatory light chains (Griffith et al., 1987).

Our aim here is not to dissect the aforementioned mechanisms in further details. For that, we refer to primary research and review articles cited in the preceding text and published throughout the years by experts in the field. However, due to the conserved features of actin and myosin, it is highly relevant to discuss these mechanisms in the light of nuclear function. The main

question is how myosin motors can be operated in the cell nucleus. Motor function and movement are based on the reiterated execution of power stroke cycles on actin filaments. In the cell nucleus, structures that resemble the canonical actin filaments have been described in the giant nucleus of *Xenopus* oocytes (Bohnsack et al., 2006; Samwer et al., 2013), even though with some concerns (Gall, 2006). More recently, several studies have underscored the presence of a highly dynamic nuclear actin population (Ho et al., 2013) and have finally visualized dynamic nuclear actin polymers that cross-react with phalloidin (Baarlink et al., 2013). In some cases, these nuclear actin polymers do not resemble canonical filaments (Belin et al., 2013). Immunoelectron microscopy studies to localize actin in the cell nucleus, however, have not yet revealed canonical nuclear actin filaments and even more so in the proximity of nuclear myosin isoforms in the absence of stimulation (Kiseleva et al., 2004; Obrdlik et al., 2010). Further analysis is therefore required to determine whether the filaments observed by Baarlink et al. (2013) occur under nonstimulated conditions and to what extent and whether these filaments display the same structural features and periodicity identified in the canonical cytoplasmic-based actin filaments.

The important point here is that the cell nucleus contains all ingredients for actin to be able to polymerize in a dynamic manner. Therefore, it becomes suddenly possible that myosin functions as motor. In fact, because of the very dynamic nature of these actin filaments, an interesting conceptual twist is that different nuclear myosin forms may function “traditionally” that is over long nuclear actin filaments to mediate long-distance intranuclear motion; alternatively, nuclear myosin forms could also function on short actin polymers to rapidly generate local tensions for specialized nuclear functions. Both scenarios are plausible, but they probably depend on the type of myosin under consideration.

2.3. Molecular motors and molecular switches

Myosin types have been classified based on functional parameters such as duty ratio, processivity, the amount of force and amplitude of step generated during one ATPase cycle, and the velocity and directionality of the movement on actin filaments (see for instance Coluccio, 2008a). These parameters altogether contribute to characterize the type of movement that can be generated by individual myosin isoforms, with the duty ratio being of particular importance as measure of the strength of the myosin-actin interaction. Myosin II, for instance, displays high duty ratio and high degree of processivity, but does not support cargo movement. Through its

polymerization properties and tight interaction with the actin filaments, myosin II generates a sliding mechanism that is utilized at the cellular level in contraction and force production in skeletal, cardiac, and smooth muscles (Reggiani and Botticelli, 2008). Critical to the cytoplasmic functions of these motors is the phosphorylation of the 20 kDa regulatory myosin light chain MLC(20) (Goeckeler and Wysolmerski, 1995). Further, these mechanisms are conserved in the case of non-muscle-type myosin II where the sliding function is important in cell–cell adhesion, cell migration, and cytokinesis and leads to the maintenance of cellular architecture.

Other myosin isoforms directly support movement of large molecular cargoes along actin filaments toward specialized cellular locations. Myosin V is a well-characterized actin-dependent processive motor that has been implicated in the intracellular motility of pigment granules in mammalian and amphibian oocytes and in the transport of vesicles and mRNA in yeast. One of the main biochemical properties that allow myosin V to be an efficient motor is that its biochemical cycle time is dominated by states with high affinity for actin (De La Cruz et al., 1999). Myosin VI is another example of high duty ratio, processive motor. This myosin isoform has been localized at plasma membrane and Golgi apparatus together with exocytic and endocytic vesicles (Aschenbrenner et al., 2003; Buss et al., 1998; Warner et al., 2003), which altogether suggest a role in vesicle transport.

Myosin I is the largest single-head class of myosin species so far identified. However, most of the cellular functions remain to be determined. In contrast to the myosin isoforms mentioned in the preceding text, myosin 1 spends most of its time in a loosely bound actin configuration, and it is therefore considered a low-duty ratio, nonprocessive motor (Coluccio, 2008a,b). For this reason, a molecule of myosin 1 does not seem to support long-range movement of a cargo along actin filaments. A primary consequence of these features is that myosin 1 could be used to rapidly generate local tension for short-range translocations. In nature, molecules interact with their local environment to accomplish complex functionalities. In many cases, biological specificity (as well as physical and chemical specificity) is achieved by the switching of molecules between different configurations or conformations, gained in the presence of allosteric regulators. For myosin, individual molecules acquire discrete configurations that depend on their ATP cycle. As consequence of ATP hydrolysis, these different states reversibly switch from one state to another. These switching mechanisms in response to chemical stimuli could, in turn, be used to modulate local interactions in a microenvironment (see Fig. 4.2). In the case of a low-duty myosin, such as myosin 1,

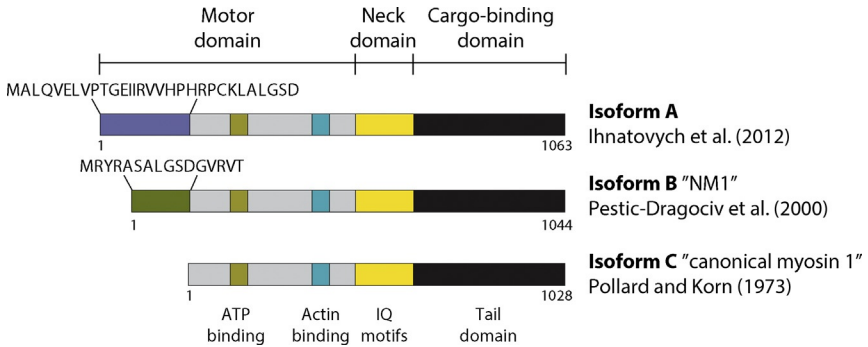


Figure 4.2 The myosin 1c isoforms. The canonical myosin 1 protein (isoform C) lacks a precise nuclear localization signal (NLS) and it is primarily found in the nucleus. Isoform B (Pestic-Dragovich et al., 2000), also called NM1, has a 16 amino acid long extension located in the very N-terminus of the protein. NM1 is entirely localized in the cell nucleus and it is implicated in different aspects of gene expression. The newly discovered isoform A (Ihnatovych et al., 2012) exhibits a 34 amino acid long N-terminal extension that is different in amino acid composition from the one found in NM1. Isoform A is found in the cell nucleus where it appears to localize to speckles, but its precise function is not known. Isoforms A and B are alternatively spliced forms and their N-terminal extensions not found in isoform C are generated from independent exons. The roles of these N-terminal extensions are not known but it seems that they are not involved in regulating their nuclear import but could be involved in mediating interactions with nuclear components.

the low-affinity actin-binding state may allow for transient interaction of the myosin with another factor or macromolecular assembly within the timeline imposed by the ATP cycle of the myosin itself.

The benefit of having a myosin-based molecular motor is that transport to specialized cellular locations can be efficiently achieved. On the other hand, by generating local tension, a myosin-based molecular switch that does not support movement could mediate self-assembly, self-organization, and controlled growth of macromolecular assemblies at specific locations. In the cell nucleus due to the constraints imposed by molecular crowding, it is especially relevant and meaningful to have molecular switches that, for instance, provide the local tension to reorganize chromosomes in well-defined geometries upon chemical stimulation or even facilitate assembly of chromatin and chromatin-modifying components on active genes. Taken in a wider perspective, individual nuclear myosin isoforms, for instance, with low-duty ratios, and their dynamic interactions with nuclear components may ultimately customize the coupling between molecules and substrate or between molecules, thus regulating nuclear structure and function at many different layers.



3. MYOSIN TYPES IN EUKARYOTIC CELL NUCLEUS

The number of myosin species that have been identified so far in the cell nucleus steadily increases. It is now far too large to be considered an artifact or as an improbable biochemical leakage from the cytoplasmic compartment that serves no function for the eukaryotic cell nucleus. Fortunately, this point is now accepted by the scientific community and the result is a huge recrudescence of interest for nuclear myosin, which is not surprising given the potential to redesign the way we think about gene expression.

In the light of what is known about myosin properties and their cytoplasmic functions, in the succeeding text, we summarize recent and past literature focusing on those myosin isoforms that have been identified in the cell nucleus (see [Fig. 4.1](#)).

3.1. Myosin II

As mentioned earlier, in the cytoplasm, myosin II forms high-order assemblies. This is achieved through interactions of the extended coiled-coil domain in the heavy chain with the corresponding domain in adjacent myosin II molecules. Further, additional tail-to-tail interactions with other myosin II assemblies lead to the formation of a bundle or thick filament that can contain several hundred myosin heads oriented in opposite directions at the two ends of the filament. Upon ATP hydrolysis, the myosin heads move on actin filaments producing a characteristic sliding motion ([Cai and Sheetz, 2009](#); [Even-Ram et al., 2007](#); [Shelden and Knecht, 1995](#); [Vicente-Manzanares et al., 2007](#)).

As myosin II is a component of stress fibers and the contractile ring that separates two cells during cell division, it is not surprising that the mechanisms in the preceding text are widely spread not only in muscle cells but also in non-muscle cells where contractile cellular functions are required. Intriguingly, there is also reason to believe that myosin II could function in the cell nucleus. A fraction of cellular myosin II has recently been shown to localize to the nucleus where it is believed to act as core transcription factor ([Li and Sarna, 2009](#)). Myosin II was found to be tethered to a specific promoter sequence of the intercellular adhesion molecule 1 (ICAM-1). Dephosphorylation and phosphorylation of MLC20 interestingly altered transcription levels of ICAM-1. This mechanism turned out to be relevant for colonic inflammation where suppression of the nuclear myosin light-chain kinase led to an increase in the levels of the unphosphorylated

MLC20 and enhanced transcription rates for ICAM-1. Since actin interacts with the RNA polymerase II machinery and is required for transcription (Percipalle, 2013), by extrapolation, the authors propose a mechanism whereby DNA-bound myosin II and actin molecules bound to the RNA polymerase II slide past each other, producing relative motion between DNA and polymerase. In this model, motion would be regulated through phosphorylation and dephosphorylation rounds modulated by MLC20.

The aforementioned model is speculative at this stage. It remains to be understood whether it is gene-specific or it can be applicable as such to the RNA polymerase II basal function. The main conclusion of the study however strongly argues that in colonic circular smooth muscle cells, nuclear myosin II is important for ICAM-1 gene regulation and may have an impact on inflammation.

3.2. Myosin 1

Myosin 1 is a single-headed myosin form that in contrast to the canonical myosin II cannot self-associate into bipolar filaments (Coluccio, 2008b). Myosin 1 is also the first non-canonical myosin to be discovered (Pollard and Korn, 1973). On the bases of phylogenetic analyses, myosin 1 has been grouped into subclasses 1–4. The difference among these isoforms is in the number of calmodulin-binding motifs (IQs) present in the neck region that vary from 1 to 6 (Barylko et al., 2000; Coluccio, 2008b). Based on the length of the C-terminal tail domain, myosin 1 has been classified in short-tailed and long-tailed isoforms, as determined by the number of tail homology domains (THDs). While short-tailed myosin 1 contains one THD (TH1), long-tailed myosin 1 has two additional THDs, TH2 and TH3 that provide an extra actin-binding site independent of ATP hydrolysis and a site that exhibits Scr homology, respectively (Pollard et al., 1991). Although very little is known about their regulation at the cellular level, myosin 1 isoforms are believed to be regulated by heavy chain phosphorylation by specific kinases or by calcium binding to calmodulin in the neck region or possibly by tail domain targeting to specific receptors (Barylko et al., 2000; Coluccio, 2008b).

Myosin 1c is encoded by the *MYO1C* gene (Gillespie et al., 2001). The cytoplasmic myosin 1c isoform appears to associate with membranes and to play a role in transport of vesicles containing membrane proteins such as the glucose transporter (Bose et al., 2004), in the regulation of the ion channels in the stereocilia of the hair cells of the inner ear (Cyr et al., 2002; Holt et al.,

2002), and in the formation of membrane extensions in neuronal growth cones (Wang et al., 2003).

In vertebrates the *MYOIC* gene encodes two extra isoforms that are mostly found in the cell nucleus (see Fig. 4.3). The presence of myosin 1c in the cell nucleus was initially suggested by Nowak et al. (1997). Using a pan myosin 1 antibody in electron microscopy experiments performed on COS1 cells, both nucleoplasmic and nucleolar labeling revealed that myosin 1 is present all over in the cell nucleus. Pestic-Dragovich et al. (2000) further showed evidence of a specific form of myosin 1c isoform B that is currently referred to as nuclear myosin 1 (NM1). The protein contains a 16 amino acid long extension of the N-terminus encoded by a single exon and results from alternative splicing of the *MYOIC* gene (Fig. 4.3). Although the majority of cellular NM1 is located in the cell nucleus, the N-terminal extension does not seem to serve as nuclear localization signal and its function is not yet determined. NM1 has been implicated in multiple nuclear functions that range from organization of chromatin structure and function (Percipalle and Farrants, 2006; Percipalle et al., 2006; Visa and Percipalle, 2010), transcriptional regulation in complex with eukaryotic RNA polymerases (Fomproix and Percipalle, 2004; Hofmann et al., 2006a,b; Pestic-Dragovich et al., 2000; Philimonenko et al., 2004; Sarshad et al., 2013;

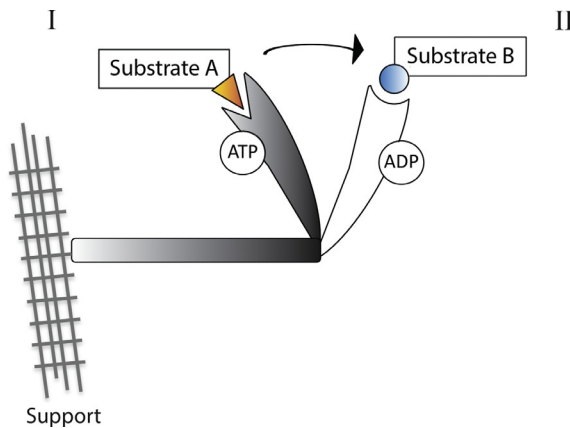


Figure 4.3 Schematic drawing of a general molecular switch, where two conformations of the same molecule (I) and (II) are generated for instance upon ATP hydrolysis into ADP. Swinging from one or the other conformation while bound to a support could generate specificity to interact with substrate A or substrate B, respectively. In the case of NM1, these substrates could be actin and a nuclear factor required for specialized function in gene expression such as the subunit of a chromatin remodeling complex (Percipalle et al., 2006; Sarshad et al., 2013).

Ye et al., 2008), and posttranscriptional control of gene expression as component of ribosomal subunits (Cisterna et al., 2006) and involved in their maturation (Obrdlik et al., 2010) (see Fig. 4.4). Emerging evidence also identifies the potential key roles in nuclear structure as part of filamentous cables revealed by field emission scanning electron microscopy that connect nuclear envelope with chromatin and nuclear pore complex and happen to be rich in actin (Kiseleva et al., 2004; Obrdlik et al., 2010). Although further characterization is required, these nuclear cables were identified in the *Xenopus* oocyte nuclei and were shown to be sensitive to actin-depolymerizing drugs (see Fig. 4.4). There is also evidence that motor proteins such as NM1 are implicated in the dynamic organization of chromatin and chromosomes, having a potential impact on genome maintenance (Kumaran et al., 2008; Lanctôt et al., 2007). NMI was also shown to be involved in transcription-dependent and transcription-independent intra-nuclear chromosome movement (Chuang et al., 2006; Hu et al., 2008; Mehta et al., 2008). Recently, a previously uncharacterized third *MYOIC* gene product was discovered by the laboratory of Wilma Hofmann (Ihnatovych et al., 2012). This new isoform has a unique N-terminal

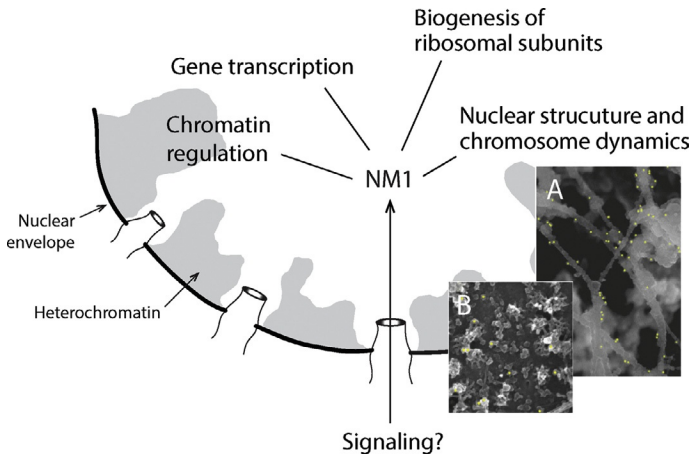


Figure 4.4 Nuclear functions of NM1. NM1 has been shown to have a role in chromatin regulation and interacts with components of the nuclear lamina; NM1 is directly involved in gene transcription by RNA polymerase I and RNA polymerase II; NM1 is incorporated into nascent ribosomal ribonucleoprotein complexes and contributes to maturation into ribosomal subunits; NM1 decorates actin-rich nuclear filaments (A) and the basket of the nuclear pore complex (B) (both images reproduced with permission from Obrdlik et al., 2010); NM1 is involved in chromatin dynamics. It is likely that all these nuclear functions are coordinated by intracellular signaling mechanisms that are yet to be identified.

extension of 30 amino acids and has been termed isoform A (Fig. 4.3) to distinguish it from NM1 (Pestic-Dragovich et al., 2000), now referred to as isoform B, and from the canonical form myosin 1c, now referred to as isoform C (Reizes et al., 1994; Zhu et al., 1996). Similar to NM1, the isoform A localizes to the cell nucleus and its distribution correlates with that of the RNA polymerase II. However, upon inhibition of RNA polymerase II transcription, this isoform, unlike NM1, relocates to nuclear speckles. In contrast to NM1, the isoform A is excluded from nucleoli and does not colocalize with the RNA polymerase I machinery.

The fact that several forms of myosin 1c are present in the cell nucleus indicates diversity and, at the same time, complexity. It strongly points to the possibility that individual isoforms of the same myosin have specialized nuclear roles.

3.3. Myosin Va

Myosin V is a double-headed motor protein and it is a cargo-carrying myosin isoform. Myosin V is a highly processive motor that spends most of its time strongly associated with actin. Myosin V contains more light chains and a longer “lever arm” relative to myosin II, which allows myosin V to move in larger steps along the actin filaments (Trybus, 2008). Since myosin V moves through a “hand-over-hand” lever arm mechanism (Sellers and Weisman, 2008), myosin V can perform intracellular transport of multiple cargoes over long distances within the cell. Based on these properties, myosin V is considered a universal motor protein and it is indeed involved in many cellular processes such as vesicle transport and anchorage, spindle pole alignment, and RNA translocation (Langford, 2002; Reck-Peterson et al., 2000; Tekotte and Davis, 2002).

In melanocytes and nerve cells, myosin Va synergizes with F-actin to promote transport of pigment granules (Rogers et al., 1999). Phosphorylation of the specific serine residue, Ser1650, in the tail domain of myosin Va by calmodulin-dependent protein kinase II (CaMKII) results in the release of myosin Va from pigment granules (Karcher et al., 2001). The phosphorylated Ser1650, conserved in all three known myosin V paralogs (a, b, c), was recently detected in the cell nucleus, and in particular within speckles (Pranchevicius et al., 2008). Although at this stage there are no mechanistic details available, this finding suggests that specific phosphorylation of myosin Va may be a requirement for nuclear compartmentalization in response to transcriptional activity. In view of its cytoplasmic role as transporter involved

in cargo export, a speculation is that myosin Va contributes to this function by facilitating actin-based transport of specific cargoes in and out of speckles.

Identifying nuclear cargoes that interact with phosphorylated and non-phosphorylated myosin Va is therefore a future challenge that will almost certainly allow us to understand how the architecture of the cell nucleus is shaped and dynamically changes in response to specific stimuli.

3.4. Myosin VI

Unlike all other myosin isoforms, the unconventional myosin VI moves backward toward the minus end of actin filaments (Buss et al., 2004; Wells et al., 1999), with a large and variable step size (Rock et al., 2001). *In vitro* motor function is performed as monomer or dimer (Buss and Kendrick-Jones, 2008), but whether this structural organization is maintained *in vivo* is a matter of speculation. Myosin VI is involved in intracellular cargo movement and, as an anchor, attaching tail-associated cargoes to actin. It has been implicated in several cellular processes including endocytosis, exocytosis, maintenance of Golgi morphology, and epithelial cell migration (Buss and Kendrick-Jones, 2008; Geisbrecht and Montell, 2002). Cargo binding occurs through two sites located in the tail domain that also contains a PIP₂-binding site that targets myosin VI to the plasma membrane (Buss and Kendrick-Jones, 2008).

Recent evidence has shown that myosin VI is found in the cell nucleus at high levels, colocalizes with newly transcribed mRNA, and associates with the RNA polymerase II complex (Vreugde et al., 2006). Furthermore, the interaction between myosin VI and the RNA polymerase machinery appears to be transcription-dependent. Myosin VI becomes recruited to the promoter and intragenic regions of active genes, encoding urokinase plasminogen activator, eukaryotic initiation factor 6 (p27/eIF6), and low-density lipoprotein receptor, but is not found at noncoding, nonregulatory intergenic regions, which collectively support an active role in transcription and potential for chromatin organization.

Whether myosin VI performs this function coupled to actin remains to be understood. However, similar considerations as for myosin Va are applicable in the case of myosin VI. In the cytoplasm, myosin V is a cargo transporter implicated in cargo export. An entirely speculative scenario is that a nuclear myosin VI motor may facilitate transcription by exporting repressive or inhibitory factors away from the local transcription site, concomitantly with passage of the polymerase across the active gene.

3.5. Myosin XVI and myosin XVIII

Myosin XVI is an unconventional myosin with a peculiar head domain containing a large N-terminal extension with several ankyrin repeats, mediating association with the protein phosphatase 1 (PP1) catalytic subunits PP1 α and PP1 γ (Patel et al., 2001). These properties suggest that myosin XVI serves as a serine/threonine phosphatase-1 targeting and/or regulatory subunit. In higher vertebrates, sequence analysis of the ATP-binding site predicts reduced ATPase activity, and therefore, myosin XVI may not function as motor protein (Cameron et al., 2007). The motor domain is followed by a single putative light-chain binding domain. The tail domains are short in both known alternatively spliced variants myosin XVIa and myosin XVIb.

Myosin XVI has been localized to the cell nucleus. In contrast to unconventional myosins such as myosin 1 and myosin V where nuclear localization depends on the IQ motifs (Dzijak et al., 2012; Lindsay and McCaffrey, 2009), nuclear localization of myosin XVI depends on the carboxy-terminal domain that for myosins often confers class-specific functions (Reck-Peterson et al., 1999). However, the import mechanism is unclear as the implicated targeting domain lacks canonical arginine-/lysine-rich nuclear localization signal motifs (Cameron et al., 2007). Furthermore, although growing evidence supports a regulatory role for myosin XVI in cell cycle progression (Cameron et al., 2013), it is still unclear whether and how myosin XVI contributes to nuclear function.

Myosin XVIIIb is found in the cytoplasm of cardiac and skeletal muscle (Salamon et al., 2003). Myosin XVIIIb is necessary for myofibrillar development (Ajima et al., 2008) and is a possible tumor suppressor gene (Ajima et al., 2007; Bleeker et al., 2009), involved in the suppression of anchorage-independent growth (Ajima et al., 2007). Upon differentiation of myoblasts into myotubes, a fraction of myosin XVIIIb however localizes in the cell nucleus (Salamon et al., 2003). Although nuclear myosin XVIIIb may have an important role in the differentiation process of myoblasts, its precise nuclear functions however remain unknown.

3.6. Remarks

In summary, there is now overwhelming evidence on the presence of myosin isoforms in the cell nucleus and their potentially huge impact on nuclear structure and function. One of the most remarkable aspects is that both unconventional and conventional myosin forms are genuinely localized

to the cell nucleus. Considering their intrinsically different ways of mediating motion on actin filaments, it is tempting to suggest that several systems of active and directional motion are in place in the cell nucleus and they all contribute to function. For instance, a myosin II-based sliding mechanism may be important for chromosome relocation during cell division or interphase, whereas a highly processive motion may be a requirement to organize coordinated movement of multiprotein machineries required during gene expression across the active gene.

In the next sections, we will focus on the role of NM1 in gene expression. Our goal is to provide experimentally testable hypotheses on the connectivity between NM1, actin, and the RNA polymerase, with respect to the chromatin and its importance in transcription.



4. NUCLEAR MYOSIN 1 IN GENE REGULATION

The nuclear myosin 1c isoform B that we herein refer to as NM1 is the best characterized in terms of nuclear functions (Fig. 4.4). As already mentioned, NM1 has been shown to be involved in nuclear organization by mediating chromosome movement, NM1 is directly involved in chromatin regulation and it is also coupled to the RNA polymerases I and II, being required for gene transcription (de Lanerolle and Serebryanny, 2011; Visa and Percipalle, 2010). In this chapter, we focus on the emerging key role for NM1 in gene regulation and support the idea that NM1 operates on the active gene as general transcription factor with a specific effect on the chromatin. This knowledge will be integrated with evidence that NM1 is also a molecular motor that functions in an actin-dependent manner.

4.1. NM1 in gene transcription

The association of NM1 with the transcription apparatus was initially suggested by *in situ* run-on assays where incorporation of nucleotide analogs in living cells was followed by microscopy (Fomproix and Percipalle, 2004; Pestic-Dragovich et al., 2000). The results revealed a general association of NM1 with transcription foci both in the nucleoplasm and in the nucleoli that was lost upon transcription repression with general and specific transcription inhibitors (Kysela et al., 2007; Pestic-Dragovich et al., 2000). Upon treatment with 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole that reversibly blocks RNA polymerase II but not RNA polymerase I, NM1 specifically localizes within RNA polymerase I transcription sites in nucleoli.

Further, treatment with low actinomycin D doses, which selectively inhibits RNA polymerase I transcription, led to a redistribution of NM1 to perinucleolar caps (Fomproix and Percipalle, 2004). It was therefore concluded that NM1 is likely to have an active role in transcription. A strong link between NM1 and eukaryotic gene transcription also relied on evidence that NM1 is in complex with both RNA polymerases I and II and that NM1 gene knockdown by RNAi led to quantitative drops in the amounts of mRNA and rRNA (Fomproix and Percipalle, 2004; Philimonenko et al., 2004; Sarshad et al., 2013; Ye et al., 2008). Although many intrinsic mechanistic details remain to be understood, NM1 commitment to gene transcription is now accepted. Our working hypothesis is that NM1 is a general transcription factor not only for RNA polymerase I transcription (Grummt, 2003) but also for all eukaryotic RNA polymerases.

The connection between NM1 and the RNA polymerase II apparatus was suggested by several reports. Results from an *in situ* analysis by immunoelectron microscopy showed that NM1 localizes very closely to nucleoplasmic transcription foci in a dynamic manner that is dependent on transcription (Kyselá et al., 2007). Compatible with these observations is the initial *in vitro* result that antibodies to NMI inhibited transcription by RNA polymerase II when incubated with a transcription-competent nuclear extract from HeLa cells (Pestic-Dragovich et al., 2000). Furthermore, in a minimal *in vitro* transcription system NMI was found to be involved in the initiation phase of RNA polymerase II transcription. By employing an abortive transcription initiation assay, NM1 resulted critical for the formation of the first phosphodiester bond catalyzed by the RNA polymerase II machinery (Hofmann et al., 2006a,b). These observations and evidence that the nuclear distribution of NM1 is dependent on nascent RNA (see Fomproix and Percipalle, 2004) suggest a role for NM1 as general transcription factor in RNA polymerase II-mediated transcription. A similar picture emerges in the case of RNA polymerase I transcription, where NM1 seems to be implicated in both initiation and postinitiation phases of gene transcription, including the elongation of nascent rRNA transcripts. Whether these functions result from cross talks between NM1 and some of the polymerase subunits, the Mediator complex or transcriptional enhancers, remains to be understood. In light of a potential role of NM1 as general transcription factor at multiple layers and beyond, in the next sections, we are going to discuss recent evidence that NM1 directly interacts with DNA and chromatin.

4.2. DNA binding properties of NM1

Following the groundbreaking discovery of myosin 1 by Pollard and Korn in *Acanthamoeba* (Pollard and Korn, 1973), it became clear that myosin 1 displays a modular architecture. While the C-terminus contains the actin-binding site and the catalytic ATP-hydrolyzing activity, the other end of the myosin molecule, the tail, specifies the precise target. NMI, like all other myosin IC proteins (Mermall et al., 1998), has a positively charged domain in the tail that binds to acidic phospholipids (Adams and Pollard, 1989; Doberstein and Pollard, 1992). It is therefore conceivable to predict that the NM1 C-terminus could directly bind the negatively charged DNA molecule. *In vitro*, this idea was addressed in DNA pull-down experiments by Hofmann et al. (2006b). Without taking into consideration any sequence-specific pattern on the DNA, single-stranded plasmid DNA from adenovirus promoter was conjugated to beads and the beads subsequently incubated with recombinant NM1 constructs. These constructs encompassed the full length NM1 open reading frame or NM1 subdomains and they were all expressed in baculovirus and purified to homogeneity using epitope tags. Consistently with the biochemical properties of myosin, the positively charged C-terminal tail domain was found in the bound fraction. It was therefore concluded that the NM1 C-terminal tail interacts with the DNA. On the contrary, deletion constructs lacking the C-terminal tail were almost quantitatively detected in the unbound fraction that suggests the other NM1 domains are not capable to interact with the DNA. This biochemical analysis opened up the exciting possibility that NM1 can interact with nucleic acids and that the C-terminal tail is important for this interaction.

To appreciate the significance of the nucleic acid binding properties of NM1, it is important to go back to the transcription process. During RNA synthesis, the RNA polymerase catalyzes the formation of a phosphodiester bond between two ribonucleosides in the 5' to 3' direction using single-stranded DNA as template. This operational definition of the RNA polymerase enzyme is general and valid both during transcription initiation and during transcription elongation as in both cases there is a requirement to link two adjacent ribonucleosides. NM1, as well as actin, has been suggested to play an essential function at both phases (Visa and Percipalle, 2010), supporting basal transcription by both RNA polymerase I and RNA polymerase II (Hofmann et al., 2006a,b; Percipalle et al., 2006). These results are consistent with the DNA-binding properties of NM1 but do not

provide a clear mechanistic framework. In fact, they have left many open questions. Although it is difficult to imagine, one cannot rule out a local catalytic function for NM1 in the formation of the phosphodiester bond (Hofmann et al., 2006a,b), reiterated through the entire transcription process. On the other hand, taking into consideration the requirement for single-stranded DNA template throughout RNA polymerase-mediated transcription, a more credible scenario suggests that during transcription initiation, NM1 is involved in the formation of the open complex generated on the single-stranded DNA. In other words, NM1 may contribute to produce the DNA template, thus priming a transcription-competent polymerase that would then catalyze the synthesis of the first phosphodiester bond. Transcription initiation by the RNA polymerase II machinery has been divided into three steps, namely, (i) promoter binding to form the closed complex, (ii) DNA melting and formation of single-stranded DNA to generate an open complex, and (iii) mRNA initiation with consequent moving away of the melted bubble from the start site. The formation of the open complex is a condition for the synthesis of the first phosphodiester bond by the RNA polymerase. The stabilization of the preinitiation complex and the formation of the open complex are regulated by the Mediator complex (Chadick and Asturias, 2005). By interacting with the single-stranded DNA, NM1 may therefore facilitate the connectivity between Mediator and RNA polymerase. This model is only partly speculative. In a proteomic analysis in search of NM1-associated proteins, we identified subunits of the Mediator complex that are specifically in complex with NM1. Whether the potential role for NM1 in regulating assembly of the transcription-competent RNA polymerase requires motor function and actin remains to be understood. In the next sections, we explore how that the DNA-binding properties of NM1 are likely to be important for transcription activation and elongation.

4.3. NM1 C-terminal tail mediates binding to chromatin

The *in vitro* DNA-binding properties of NM1 also bring up the interesting scenario that NM1 associates with the eukaryotic genome through direct contacts with the chromatin. ChIP experiments with anti-NM1 antibodies have indeed confirmed that the endogenous NM1 protein binds to the chromatin within the rDNA transcription unit (Percipalle et al., 2006; Philimonenko et al., 2004; Sarshad et al., 2013; Ye et al., 2008). To map how NM1 interacts with the chromatin a subset of epitope-tagged NM1

mutants was expressed in mammalian cells. These mutated NM1 constructs exhibited the RK605AA point mutations that specifically impair the actin-binding function (Sarshad et al., 2013; Ye et al., 2008). Alternatively, the mutated NM1 constructs had deletions of the neck region containing the IQ motifs (Δ IQ NM1) or the cargo-binding C-terminal tail (Δ C NM1) (Sarshad et al., 2013; Ye et al., 2008). ChIP experiments with antipeptide antibodies demonstrated that the constructs lacking neck domain and C-terminal tail did not coprecipitate with the rDNA chromatin, indicating that both domains are essential for chromatin association. Marginal drops in chromatin association were also revealed for the NM1 mutant lacking actin-binding activity (Sarshad et al., 2013), suggesting that impaired actin-binding function is not a condition for chromatin association.

The well-characterized modular architecture of myosin 1 correlates with a high degree of regulation required for motor function (Coluccio, 2008a,b). Considering the NM1 and the canonical myosin 1 are alternatively spliced isoforms (Pestic-Dragovich et al., 2000), it is conceivable that they are regulated in a similar manner. For instance, it is likely that calmodulin that is considered as the myosin 1 light chain and targets the IQ motifs within the neck region also interacts with NM1 in a conserved manner. The presence of calmodulin in the cell nucleus is compatible with this possibility. In fact, calmodulin is a major player in GTP-independent nuclear import of certain transcription factors (Sweitzer and Hanover, 1996). Furthermore, calmodulin accumulates in the cell nucleus where it appears to serve a function in pre-mRNA splicing (Agell et al., 1998). We therefore hypothesize that one or all of three IQ motifs within the NM1 neck region are targeted by calmodulin. We speculate that this strategy might be in place to stabilize NM1 on the active gene as well as a potential nuclear actomyosin complex (see in the succeeding text). On the other hand, the cytoplasmic myosin 1 interacts with the negatively charged lipid bilayers and regulates membranes remodeling and local actin assembly (Almeida et al., 2011). Consistent with its *in vitro* biochemical properties and DNA-binding abilities (Hofmann et al., 2006a,b), the NM1 C-terminal tail is likely to capture the chromatin. We propose that the chromatin itself represents the NM1 nuclear cargo.

The canonical cytoplasmic myosin 1 isoform is known to undergo post-translational modifications both within the neck domain and the C-terminal tail. Phosphorylation, arguably the best characterized modification (Tan et al., 1992), modulates myosin 1 function (Coluccio, 2008a,b). In vertebrates, negatively charged glutamate or aspartate residues define the TEDS sites (after the single letter amino acid code) (Bement and Mooseker, 1995),

located in the myosin 1 head domain, between the ATP- and actin-binding sites. The TEDS sites are phosphorylated by casein kinase II (CKII) to increase actin-dependent ATPase activity of myosin 1 without affecting actin-binding activity itself (Bement and Mooseker, 1995). Although we have no direct evidence, in the nucleus NM1 is a candidate substrate for CKII given that CKII is involved in transcription regulation (Lin et al., 2006; Panova et al., 2006; Schlosser et al., 2002; Voit et al., 1992). CKII may not however be the only kinase that phosphorylates NM1. A recent study identified three putative consensus sites for the glycogen synthase kinase (GSK)3- β located within the NM1 primary amino acid sequence (Taelman et al., 2010). These sites fall within the C-terminal tail and also in the neck region of the NM1 protein. We recently mapped a specific serine residue within the NM1 C-terminus which is indeed phosphorylated by GSK3 β and this phosphorylation event is required for NM1 association with the chromatin (Sarshad et al., 2014). The fact that GSK3- β activity is controlled by Wnt signaling adds an extra layer of complexity to how and why NM1 interacts with the chromatin. Whether NM1 is a bona fide phosphorylation substrate for GSK3- β and this mechanism regulates chromatin association becomes therefore a pressing issue to demonstrate. If this is the case, an exciting scenario opens up where NM1 function on the chromatin would be intimately connected to key signaling pathways, highly relevant for cellular growth and proliferation.

In summary, NM1 directly binds to the chromatin and both neck region and C-terminal tail represent a requirement. *In vitro*, the neck region is however dispensable for DNA binding whether the positively charged C-terminal tail is required. We therefore favor the idea that the C-terminal tail interacts with the chromatin, while the neck region plays a regulatory function that may contribute to stability of the NM1-chromatin association while regulating actin binding.

4.4. NM1 required for chromatin modifications during transcription

NM1 associates with the chromatin across the entire rDNA transcription unit, including promoter and transcribed regions (Percipalle et al., 2006; Philimonenko et al., 2004; Sarshad et al., 2013; Visa, 2005), which is compatible with roles for NM1 at transcription initiation and elongation of nascent transcripts. Although transcription-independent association has been suggested (Ye et al., 2008), NM1 binding to the chromatin and nuclear

localization seem to be heavily dependent upon transcription (Fomproix and Percipalle, 2004; Percipalle et al., 2006; Sarshad et al., 2013).

Mechanistically, NM1 may modulate transcription through a direct action on the polymerase. This is plausible but it remains to be validated. In the case of RNA polymerase I transcription, there is evidence that NM1 interacts with the phosphorylated form of TIF1a (Philimonenko et al., 2004). In view of the role of TIF1a (Drygin et al., 2010), these observation would account for a role of NM1 in facilitating recruitment of the RNA polymerase I at the gene promoter. However, growing evidence indicates that NM1 exerts its primary function in transcription precisely by means of its direct contact with the chromatin and as part of a large multiprotein assembly that contains the WICH chromatin remodeling complex with the subunits *WSTF* (Williams syndrome transcription factor) and the ATPase SNF2h (Cavellán et al., 2006; Percipalle et al., 2006). In living cells, both NM1 and *WSTF* gene silencing by siRNA led to reduced levels of 45S pre-rRNA (Percipalle et al., 2006; Sarshad et al., 2013). In abortive initiation transcription assays, antibodies against NM1 and *WSTF* did not affect the formation of the initial transcript by the RNA polymerase I machinery (Percipalle et al., 2006). When added to *in vitro* runoff transcription assays with chromatin as template, antibodies to NM1 and to the WICH subunits led however to a significant drop in the synthesis of runoff transcripts (Percipalle et al., 2006). These results endorsed a potential role for the B-WICH subunits in promoting a permissive chromatin state, compatible with transcription. Importantly, binding of NM1 is essential to promote some of the local structural alterations of the chromatin, which lead to transcription activation.

Two recent studies provided detailed mechanistic insights into how NM1 and the other B-WICH subunits cooperate to organize the chromatin at the onset of transcription activation. By silencing the *WSTF* gene, Vintermist et al. (2011) demonstrated that the B-WICH complex regulates chromatin both by local remodeling of the structure and by promoting histone modifications. Indeed, chromatin accessibility studies on cultured mammalian cells subjected to *WSTF* gene silencing resulted in a compaction of the chromatin structure over a 200 base pair region at the rRNA gene promoter. Furthermore, although *WSTF* gene knockdown did not affect distribution of the upstream binding factor (UBF), a reduction in the levels of acetylated histone H3, in particular H3K9, at the promoter and across the rRNA gene was observed in quantitative ChIP experiments. A secondary effect of *WSTF* gene knockdown at the gene promoter was a general drop

in the levels of histone acetyl transferases (HAT), including PCAF, p300, and GCN5. In a subsequent study, by combining chromatin accessibility and ChIP assays on mammalian cells subjected to NM1 gene knockdown or on cells expressing NM1 dominant mutants, different contributions to chromatin regulation (i.e., chromatin remodeling or histone modifications) were shown to be modulated by individual B-WICH subunits (Sarshad et al., 2013). In particular, NM1 loss of function by gene silencing led to a drop in rRNA synthesis as well as a significant decrease in the occupancies of PCAF and reduced H3K9 acetylation. Under the same conditions, a marginal decrease in the amount of the ATPase SNF2h bound to the gene was detected, whereas the levels of *WSTF* were essentially unchanged. NM1 gene knockdown did not however affect chromatin accessibility (Sarshad et al., 2013). An important take-home message of this study is that NM1 contributes to the B-WICH stability at the gene level, but above all, as part of the B-WICH complex, NM1 facilitates PCAF recruitment to the gene promoter for subsequent H3K9 acetylation (Sarshad et al., 2013).

WSTF therefore bookmarks the B-WICH position on the gene, whereas NM1 facilitates assembly of the multiprotein complex in a transcription-dependent manner. At the gene promoter, these coordinated mechanisms lead to local chromatin alterations required for rDNA transcription activation. Indeed, constitutive expression of those NM1 mutants that lack neck region and C-terminal tail function as dominant-negative mutants and do not support transcription of nascent rRNA (Sarshad et al., 2013). We therefore propose that NM1 binding to the chromatin is a condition for transcription activation. We do not know whether these mechanisms take place across the active gene. Ongoing genome-wide screens by chromatin immunoprecipitation and last generation deep sequencing will further address whether myosin-based mechanisms are likely to be conserved on both RNA polymerase I and RNA polymerase II genes.

4.5. Chromatin binding by NM1 and association of actin with active gene

One of the most important questions is whether the interaction between NM1 and the chromatin is compatible with the suggested synergy with actin in the context of transcription regulation. Another way of addressing this important issue is to ask whether NM1 binding to the chromatin supports actin association with the active rRNA gene. Using the epitope-tagged NM1 constructs described in the preceding text and constitutively expressed in mammalian cells, we found that binding of actin to NM1 heavily

contributes to actin being associated with the gene. In fact, constitutive expression of the RK605AA NM1 double-point mutant where the actin-binding site is disrupted produced a dominant-negative effect that led to a drop in the levels of actin occupancy at the gene promoter and across the active gene. This result underscores the importance of a functional interaction between actin and NM1 at the gene level. Although a marginal reduction in the amount of actin was observed, constitutive expression of ΔC NM1 and ΔIQ NM1 constructs did not produce the same dominant-negative effect observed by expressing the RK605AA NM1 mutant on the occupancy of actin. These results collectively indicate that a functional NM1 is a condition but it is not sufficient for actin association with the gene. We therefore hypothesize that NM1 and the RNA polymerase join forces to make sure that actin is maintained at gene promoters and across active genes, in line with the discovery that actin directly binds to RNA polymerases (Egly et al., 1984; Fomproix and Percipalle, 2004; Hofmann et al., 2004; Kukalev et al., 2005; Philimonenko et al., 2004).

The aforementioned model would argue that NM1 can simultaneously bind to chromatin and to the polymerase-associated actin. Although direct evidence is not yet available, all published results point to the fact that this topology is feasible. In fact, (i) NM1 interacts with the chromatin through its C-terminal tail, (ii) the interaction with the chromatin is regulated via the neck region, and (iii) actin binds to NM1 via the head domain located in the N-terminus of the protein. Considering the molecular size of NM1, steric hindrances are unlikely to interfere with simultaneous binding of NM1 to the chromatin and to the polymerase-associated actin. Based on these considerations, we therefore conclude that NM1 serves as structural link connecting RNA polymerase with the active gene by direct interactions with polymerase-associated actin and with the chromatin.

In an actin-based myosin motor, the myosin ATPase cycle modulates the affinity of binding between myosin and actin and ultimately generates the tension at the basis of motility. The precise mechanisms change depending on the type of myosin and form of movement. Given that NM1 and actin occupancy on active genes correlate, understanding the NM1 ATPase cycle is an important goal in order to find out how an actin-based NM1 motor functions in transcription regulation across the gene. Although we do not have quantitative biophysical measurements and, therefore, precise mechanistic details, when the general ATPase activity of myosin is inhibited with butanedione monoxime (BDM) in the living cell, results from ChIP analysis followed by quantitative real-time PCR show that occupancies of NM1,

actin, and SNF2h at the gene promoter and across the entire rDNA transcription unit are significantly altered (Sarshad et al., 2013). In living cells, BDM treatment leads to a drop in rDNA transcription (Fomproix and Percipalle, 2004; Ye et al., 2008), altogether supporting a possible involvement of myosin motor function during transcription. Across the rDNA transcription unit, this motor function is mediated by NM1 since NM1 mutants that lack specific functions, such as binding to ATP or calmodulin, were found to be incapable of associating with the RNA polymerase I and rDNA (Ye et al., 2008). An ongoing NM1 ATPase cycle is therefore likely to occur cotranscriptionally at the gene promoter and across the active gene, possibly in a reiterated manner.

In summary, our view is that the NM1 ATPase cycle and thus NM1 motor function operates across the active gene and it is a condition for actin association with the active gene. The overwhelming evidence that nuclear actin undergoes changes in its polymerization state is in line with this view. Nuclear actin polymerization is highly regulated by a number of monomeric G actin and filamentous F actin-binding proteins, and in our view, actin polymerization also takes place across the active gene, cotranscriptionally (Obrdlik and Percipalle, 2011; Percipalle, 2013; Ye et al., 2008). We therefore conclude that all ingredients that define an actin-based NM1 motor are in place across the gene and that an actin-based NM1 motor is intimately connected to the active RNA polymerase.



5. SYNERGY BETWEEN ACTIN AND MYOSIN IN RNA POLYMERASE I TRANSCRIPTION

In this section, we discuss evidence supporting the direct involvement of both NM1 and actin in RNA polymerase I transcription. This together with the considerations in the preceding text on the DNA and chromatin-binding properties of NM1 will provide the bases to discuss how an actin-based NM1 motor functions in RNA polymerase I transcription activation. We will also address emerging evidence that this mechanism is important for cell cycle progression and, therefore, the potentially has huge impact on cell growth and proliferation.

5.1. NM1 in RNA polymerase I transcription

In the nucleolus, NM1 is directly involved in RNA polymerase I transcription (Fig. 4.5). First evidence that myosin 1 is located in the nucleus and nucleolus was provided by Nowak et al. (1997) by using

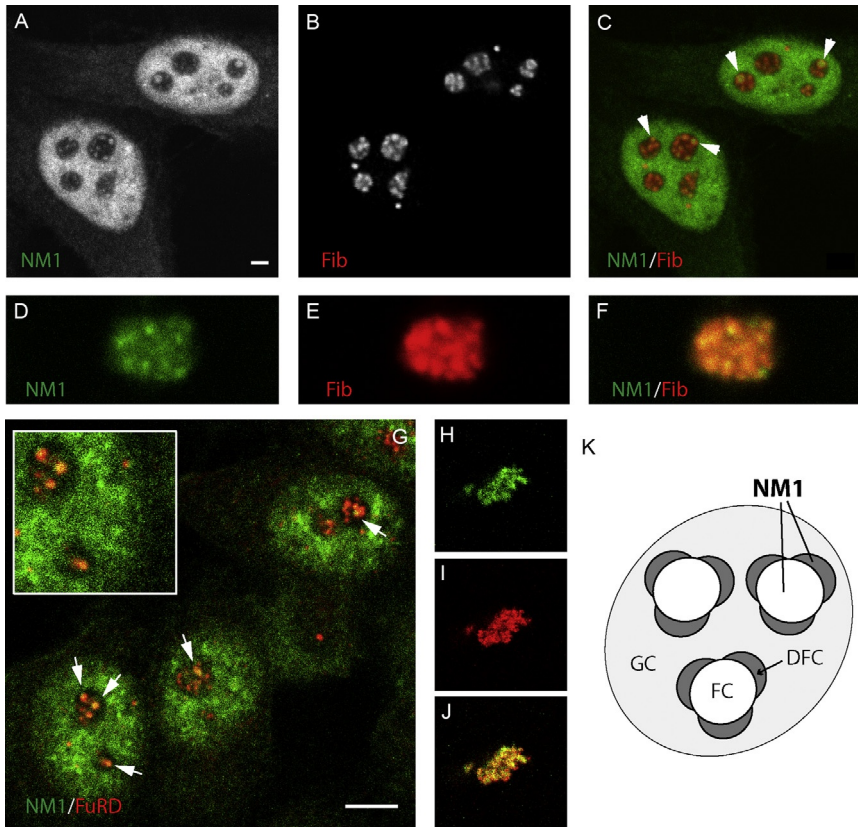


Figure 4.5 NM1 localizes to active transcription sites in the nucleolus. (A–C) NM1 is present in mammalian nucleoli. Fixed and permeabilized HeLa cells were immunostained in double-labeling experiments with antibodies against (A) NM1 and (B) fibrillarin and analyzed by confocal microscopy; (C) merged channels show colocalization of NM1 and fibrillarin in nucleolar foci (see arrowheads) (scale bar, 5 μ m). (D–F) Confocal sections of isolated nucleoli immunostained with antibodies to (D) NM1 and (E) fibrillarin; (F) merged channels show that NM1 is a genuine nucleolar protein and its distribution correlates with that of fibrillarin. (G) NM1 colocalizes with sites of active RNA polymerase I transcription. HeLa cells were incubated with BrUTP and the specific incorporation of BrUTP into nascent rRNA was visualized by immunofluorescence and confocal microscopy in double-labeling experiments with an antibody against NM1 (see arrowheads and corresponding magnification found in the inset) (scale bar, 20 μ m). (H–J) In the nucleoli, the NM1 is found in transcription sites. The nucleoli isolated from HeLa cells were subjected to BrUTP incorporation and immunostained in double-labeling experiments with antibodies against (H) NM1 and (I) BrUTP and analyzed by confocal microscopy; (J) merged channels show correlation with active transcription foci. (K) Schematic organization of the nucleolus highlighting the main compartments including fibrillar centers (FC) and dense fibrillar component (DFC) where transcription is believed to occur (Sirri et al., 2008). The newly synthesized rRNA is believed to be processed in the granular component (GC) (Sirri et al., 2008). The distribution of NM1, mostly in the FC and DFC (Percipalle et al., 2006), is compatible with its role in transcription by the RNA polymerase I.

a pan antibody against myosin 1 in immunoelectron microscopy experiments performed on cultured mammalian cells (Nowak et al., 1997). Following the discovery of NM1 (Pestic-Dragovich et al., 2000), we provided evidence for the direct coupling of NM1 with the active RNA polymerase I (Fomproix and Percipalle, 2004). In this study, using an antibody raised against the unique 16 amino acid N-terminal epitope, NM1 was localized on active RNA polymerase I transcription sites (see Fig. 4.5). This distribution was detected in both interphase and late mitotic HeLa cells when the RNA polymerase I transcription is reactivated to allow for cell cycle progression (Drygin et al., 2010; Sirri et al., 2008). Because nuclear and nucleolar localizations of NM1 are dependent on RNA and NM1 is part of the same complex with the RNA polymerase I largest subunit, a direct involvement in rRNA gene transcription was suggested. Active nucleolar transcription sites were monitored *in situ* by run-on assays. In these assays, the UTP analog used is only incorporated into elongating transcripts and cannot be used by the polymerase to initiate a new transcript. It was thus concluded that NM1 has a potential role in transcription activation and maintenance of the postinitiation phases of transcription.

A subsequent study showed that NM1 is also involved in transcription initiation (Philimonenko et al., 2004). Using a different set of NM1-specific antibodies, results from ChIP experiments convincingly showed that NM1 occupies the rRNA gene promoter. In this ChIP experiments however, NM1 was found to be excluded from all transcribed regions across the rDNA transcription unit, an observation somewhat contradictory to the type of NM1 distribution observed on active transcription sites that specifically correlates with nascent rRNAs (Fomproix and Percipalle, 2004) and, therefore, the entire rDNA transcription unit. Based on the NM1 presence at the gene promoter, Philimonenko et al. (2004) anyway proposed that NM1 interacts with the phosphorylated form of TIF1a, an RNA polymerase I-specific transcription factor required to assemble the transcription-competent polymerase at the gene promoter (Drygin et al., 2010). The phosphorylated TIF1a interacts with the promoter-bound SL1 complex (with the subunits TBP, TAFI110, TAFI63, and TAFI48) to mediate assembly of the preinitiation complex prior to transcription activation. Since actin associates with both transcriptionally active and inactive RNA polymerase I, a synergy between NM1 and actin was envisaged at an early transcriptional phase to facilitate polymerase assembly at the gene promoter (Grummt, 2003; Philimonenko et al., 2004; Visa, 2005).

The aforementioned model is very attractive but a little difficult to picture from a mechanistic point of view as it would reduce the actomyosin interaction to a strategy to simplify or coordinate protein-to-protein contacts. An actin–myosin interaction is accompanied by myosin ATPase activity, a degree of actin polymerization and cargo binding by the myosin itself. We now know that assembly of the RNA polymerase I at the gene promoter does not require the ATPase activity of myosin since RNA polymerase I occupancy at the gene promoter is not affected when the myosin ATPase activity is inhibited (Sarshad et al., 2013). Furthermore, there is growing evidence that at the gene promoter actin is likely to be monomeric (Percipalle, 2013), which would also exclude the possibility to have a canonical actin-based myosin motor. In the future, biophysical measurements may represent the way to go in order to shed light on how actin and NM1 possibly cooperate to assemble the polymerase at the gene promoter.

In contrast to RNA polymerase II transcription (Hofmann et al., 2006b), NM1 does not appear to be strictly involved transcription initiation. Abortive initiation assays performed by incubating transcription-competent lysates with a suboptimal number of nucleotides in the presence of specific anti-NM1 antibodies did not interfere with the formation of the ACU trimer, the first transcript synthesized by the mouse RNA polymerase I (Percipalle et al., 2006). In contrast, we have shown that synthesis of runoff transcripts is inhibited by the NM1 antibody (Percipalle et al., 2006). These observations, at least *in vitro*, support a primary function for NM1 in activation and postinitiation phases of transcription (Percipalle et al., 2006). This view is compatible with the distribution of NM1 across the entire rDNA transcription unit (not only within the promoter) and with lack of incorporation of the fluorine-conjugated UTP analog into nucleolar nascent transcripts in living cells subjected to NM1 gene knockdown by RNAi (Percipalle et al., 2006; Sarshad et al., 2013; Ye et al., 2008).

In summary, we favor the idea that NM1 is primarily involved in transcription activation and maintenance. Whether and how NM1 cooperates with actin is the focus of the next section.

5.2. NM1 Synergizing with actin during RNA polymerase I transcription

An actin-based NM1 motor involved in RNA polymerase I transcription activation has to fulfill a number of parameters. The motor should be based on the ATPase activity of myosin that would in turn allow for the different affinities between NM1 and actin. Secondly, but not less importantly,

myosin motors operate on polymeric actin. In other words, for a motor to support RNA polymerase-mediated transcription, we have to record myosin ATPase activity across the active gene and we have to be able to visualize dynamic actin polymerization.

We started tackling some of the aforementioned points by *in vivo* protein–protein cross-linking that allowed us to demonstrate that actin and NM1 are physically associated with each other in nucleolar lysates and that they are part of the same complex with the largest RNA polymerase I subunits (Fomproix and Percipalle, 2004). We also reported that inhibition of the general myosin ATPase activity led to a significant reduction in the synthesis of rRNA (Fomproix and Percipalle, 2004). These findings laid the bases to start discussing in terms of motors coupled to the transcription machinery. Later on, an insightful study extended these observations. Using drugs that prevent actin from polymerizing, RNA polymerase I transcription was inhibited *in vitro* as well as in living cells (Ye et al., 2008), which suggested a requirement for ongoing actin polymerization for efficient transcription (Percipalle, 2013). This hypothesis was further supported by evidence that actin mutants that stabilize the polymeric state are tightly associated with RNA polymerase I and activate transcription, whereas a polymerization-deficient actin mutant does not bind to the polymerase and does not promote rDNA transcription (Ye et al., 2008). Although actin polymerization has not been directly observed across the active gene, two recent studies have demonstrated to the nuclear actin community that actin can indeed form filamentous structures in the cell nucleus and actin polymerization is actually tightly regulated (Baarlink et al., 2013; Belin et al., 2013). As indicated in the preceding text, to date, we are still lacking quantitative measurements on the differential affinities between NM1 and actin during the myosin ATPase cycle. However, the key elements defining a molecular motor are coming into place also for NM1, that is, involvement of the ATPase activity and actin polymerization, both required for transcription.

5.3. Topology of actomyosin motor in transcription of rRNA genes

The actin-binding property of NM1 is important for RNA polymerase I transcription. As discussed in the preceding text, NM1 also facilitates transcription through its DNA-binding properties and as component of the B-WICH complex through a chromatin-based mechanism. The fact that actin is not part of the B-WICH multiprotein assembly would imply that

NM1 performs more than one functions during transcription of rRNA genes and these functions are separate from each other.

In a recent study, we showed that the functions of NM1 in complex with actin or as component of the B-WICH assembly bound to the DNA are not independent from each other, but rather, they represent different sides of the same coin. As already mentioned, by simultaneously interacting with the chromatin and the WICH complex through a direct association with the ATPase subunit SNF2h, NM1 contributes to stabilize the B-WICH complex at the gene promoter. This results in the tethering of the HAT PCAF to the chromatin for local H3K9 acetylation (Sarshad et al., 2013). This mechanism in turn allows for the establishment of permissive chromatin compatible with transcription. Interestingly, SNF2h competes with actin for NM1 binding. Indeed, the constitutively expressed RK605AA double NM1 mutant that cannot interact with actin displays increased ability to interact with SNF2h. Further, promoter occupancies of both actin and SNF2h are dependent on the myosin ATPase activity as inhibition of myosin motor function in living cells generates a domino effect. The rRNA gene promoter becomes devoid not only of NM1 but also of actin and SNF2h. Actin and SNF2h therefore compete for binding to NM1 but at the same time they are functionally connected to the NM1 motor activity.

An important question here is why actin and SNF2h compete for binding to NM1. Myosin 1 is a short-tailed, low-duty ratio motor with low affinity for actin (Coluccio, 2008a,b; Coluccio and Geeves, 1999; De La Cruz and Ostap, 2004; El Mezgueldi et al., 2002; Ostap and Pollard, 1996). Therefore, NM1 probably does not support movement of cargoes over long distances. We rather favor the idea that NM1 is involved in defining the structure and organization of the RNA polymerase I with respect to the local chromatin template. Both ATPase activity and actin binding are important for rRNA synthesis (Fomproix and Percipalle, 2004; Sarshad et al., 2013; Ye et al., 2008). It is therefore plausible that by connecting the RNA polymerase I with the chromatin, the chromatin-bound NM1 creates tension. Polymeric actin interacts with the RNA polymerase I, an interaction that is required for transcription (Ye et al., 2008). Actin polymerization is regulated along active genes by the F-actin severing protein cofilin 1 (Obrdlik and Percipalle, 2011; Ye et al., 2008). Therefore, throughout the myosin cycle (Takács et al., 2010; Yanagida and Iwane, 2000), concomitantly with the establishment of an actomyosin complex, force generation may primarily result from the direct interaction of NM1 with rDNA through its C-terminus and the simultaneous pulling of polymeric actin attached to

the polymerase. In the second step, when NM1 does not interact with actin, the rDNA associated-NM1 interacts with SNF2h in a manner that requires the bookmarking of *WSTF* on the chromatin. This provides a mechanism to stabilize the chromatin remodeling complex at active gene promoters when RNA polymerase I transcription is activated. We propose that the two-step mechanism is based on the NM1–actin interaction; it allows for polymerase motion across the active gene while providing permissive chromatin for transcription elongation by modulating B-WICH assembly and PCAF recruitment (Fig. 4.6). At the exit of mitosis, these mechanisms positively

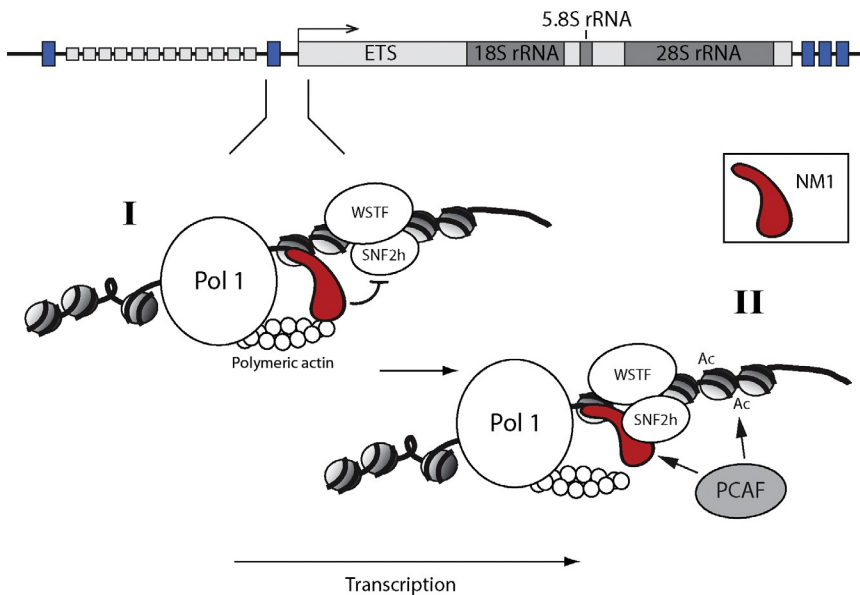


Figure 4.6 An experimental model for rDNA transcription activation by the actin-based NM1 motor. NM1 appears to be a structural link between the chromatin and the RNA polymerase I. NM1 accomplishes this function by directly contacting the chromatin through its C-terminal tail and the polymerase by an interaction with the polymerase-associated actin. Binding to actin is regulated by the ATPase cycle of myosin. When NM1 does not interact with actin, we have evidence showing that NM1 interacts with SNF2h, the ATPase subunit of the chromatin remodeling WICH. We therefore propose that NM1 functions as conformational switch that swings from one conformation to another to interact with actin or SNF2h. The consequence of this duality of action is that when bound to actin, NM1 is likely to promote local tension that facilitates RNA polymerase motion. As the other part of the coin, when bound to SNF2h, NM1 facilitates assembly of the multiprotein complex B-WICH and consequent establishment of permissive chromatin required for transcription activation. This mechanism is important for cell cycle progression at the exit of mitosis. *Adapted from Sarshad et al. (2013).*

affect cellular growth and proliferation when there is a high demand for protein synthesis and when a fraction of rDNA must be kept in an active configuration.

A molecular motor is broadly defined as a protein that uses the energy of hydrolysis of a small molecule such as nucleoside triphosphate (NTP) to complete an enzymatic cycle during the course of which the protein performs directional motion (Howard, 2001). This directional motion at the molecular level produces a substantial amount of work (i.e., motion against an opposing force) in addition to any potential biochemical product of the reaction cycle. The RNA polymerase is a typical example of molecular motor. The RNA polymerase translocates along the DNA molecule, typically reading the genetic information encoded on one strand of the double helix and rewriting a new copy of the strand. We propose that the actin-based NM1 motor function indeed helps the RNA polymerase I move across the active gene, but above all, it provides directionality to the polymerase and makes sure that the chromatin barrier is lowered in order to guarantee activation of the transcription process. Maintenance of a chromatin structure compatible with transcription is important also during the elongation of the nascent transcripts. An interesting hypothesis still speculative at this stage is that the two-step mechanism depicted in the preceding text is reiterated across the entire rDNA transcription unit in order to facilitate transcription elongation.

5.4. Actomyosin motor in rRNA transcription activation and cell cycle progression

In an insightful review, Ingrid Grummt has underscored the importance of RNA polymerase I transcription for cellular physiology (Grummt, 2003), with an impact on many regulatory pathways that adapt cellular rRNA synthesis to cell metabolism and growth, including cell cycle progression.

In mammalian cells, the levels of rRNA synthesis considerably change throughout the cell cycle. RNA polymerase I transcription peaks at S and G2 phases, is downregulated in mitosis, and slowly recovers in G1. Phosphorylation of the SL1 complex contributes to silencing the transcription machinery at mitosis (Heix et al., 1998; Kuhn et al., 1998), reducing the ability of SL1 to interact with UBF. As a consequence, promoter assembly of the RNA polymerase I is impaired and transcription cannot proceed. During mitosis, phosphorylation is also believed to target UBF. Reversible phosphorylation events are therefore used by the cellular apparatus to repress rDNA transcription during mitosis. The mechanisms that trigger

reactivation of transcription at the end of mitosis are unknown. Possibly, they require a set of mitosis-specific phosphatases that reactivate the transcription initiation factors. At the exit of mitosis, in early G1 phase, rDNA transcription remains low until UBF is further phosphorylated at two specific serine residues by G1-specific protein kinases (Voit and Grummt, 2001; Voit et al., 1992). The finding that specific cdk/cyclin complexes modulate the activity of TIF-IB/SL1 and UBF in a cell cycle-dependent manner links the control of cell cycle progression to regulation of Pol I transcription (Drygin et al., 2010).

Similar to the RNA polymerase I, UBF, and fibrillarin, NM1 was localized on active transcription foci in cells exiting mitosis (Fomproix and Percipalle, 2004) (see Fig. 4.7). This observation prompted the idea that at the end of mitosis, the synergy between NM1 and actin is a requirement to reactivate RNA polymerase I transcription and cell cycle progression.

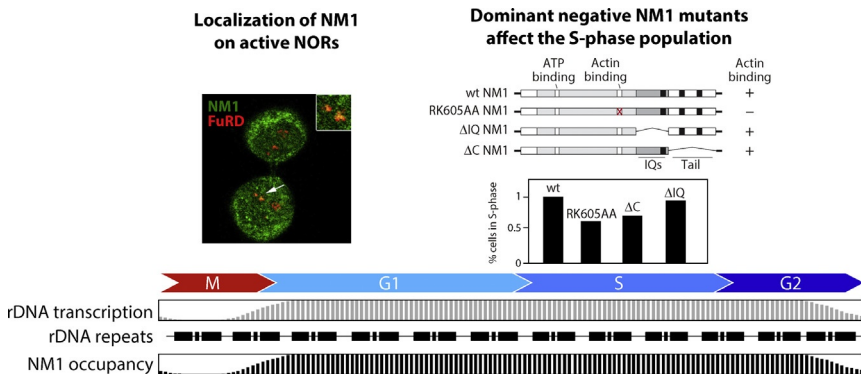


Figure 4.7 Regulation of RNA polymerase I transcription during cell cycle progression by NM1. Chromatin immunoprecipitation assays and run-on transcription experiments in living cells show that concomitantly with transcription activation at the late mitotic phases (telophase and early G1), NM1 becomes associated with the rDNA transcription units in active nucleolar organizer regions (adapted from Sarshad et al., 2013). NM1 remains associated with the rDNA throughout the interphase. This association is important to maintain rDNA transcription and promote cell cycle progression. This is believed to be achieved by NM1 through its chromatin-binding activity (performed via the C-terminal tail domain) and by its actin-binding function. In fact, flow cytometry experiments on cells constitutively expressing NM1 constructs with impaired actin-binding (RK605AA NM1) or chromatin-binding function (lacking the C-terminus as in ΔC NM1 or ΔIQ NM1) demonstrated a reduced number of cells in S phase (adapted from Sarshad et al., 2013). In mitosis when RNA polymerase I transcription is downregulated, NM1 is not associated with the rDNA transcription unit (Sarshad et al., 2013). These results suggest that NM1 is a key player for rDNA transcription activation and maintenance throughout the entire cell cycle.

Along these lines, knocking down the NM1 gene by RNAi led to a delay in cell cycle progression. Compatible with this result, we found an increase in the population of cells in the M phase when living cells released from a G1 block were treated with drugs inhibiting the ATPase activity of myosin (Sarshad et al., 2013). Another important link between cell cycle regulation and rDNA transcription is evident at G1-to-S-phase progression, with rDNA transcription peaking at S phase (Drygin et al., 2010). Remarkably, we found that NM1 gene silencing induced a drastic and significant reduction in the number of cells at S phase. This effect is amenable to impaired actin binding and chromatin binding as constitutive expression of the RK605AA NM1 and the mutant lacking the C-terminus (Δ C NM1) induced significant reduction in the number of cells at S phase (see Fig. 4.7). Although further analysis is required, these findings suggest that at the exit of mitosis, the actin-based NM1 motor is required to reactivate transcription, thus allowing the cell to normally progress through the cell cycle.

In summary, the actin-based NM1 motor by regulating transcription activation has a huge impact on cell cycle progression at the exit of mitosis and throughout the S phase when there is a high demand for protein synthesis and a considerable fraction of rDNA must be kept in an active configuration. Expression of the genes encoding the numerous constituents of ribosomes requires transcription by all three classes of nuclear RNA polymerases. Considering the involvement of actin and NM1 in transcription by all three RNA polymerases, one of the most exciting aspects for future investigations is the possibility that the interplay between transcriptional control and ribosome biogenesis is modulated and coordinated by actomyosin complexes.



6. CONCLUDING REMARKS AND PERSPECTIVES

In an earlier speculative model of a putative actin-based NM1 motor (de Lanerolle et al., 2005), a network of connectivity between NM1, actin, and the chromatin has been suggested. This model is primarily based on the hypothesis that being a myosin 1, NM1 is not likely to form filaments. Furthermore, back in 2005 when the model was proposed, nuclear actin filaments had not been detected and nuclear actin was thought to be primarily in a monomeric state. Given the DNA-binding properties of NM1, it was therefore suggested that the symbiosis between NM1 and the DNA molecule would constitute the filaments or tracks responsible

for directional movement of the RNA polymerase (de Lanerolle et al., 2005). This filamentous component, comprising both NM1 and the DNA, would then be connected to the polymerase machinery through the interaction between NM1 and the polymerase-associated actin.

The aforementioned model has many conceptual similarities to the experimental model proposed by Sarshad et al. (2013) and discussed in the chapters in the preceding text. Both models underscore the importance of NM1 as key structural element connecting the polymerase machinery with the chromatin. This is possible from a topological point of view because actin-binding and chromatin-binding functions are executed through NM1 domains whose locations are diametrically opposite from each other. The NM1 head domain, highly conserved among members of the myosin superfamily (Mermall et al., 1998), interacts with actin, whereas the positively charged C-terminal tail region that is capable of binding to acidic phospholipids (Adams and Pollard, 1989; Mermall et al., 1998) interacts with the chromatin (Sarshad et al., 2013). Considering that myosin I is believed to extend the leading edge of the cell by simultaneously binding negatively charged lipids via the tail and actin filaments via the actin-binding domain on the head (Adams and Pollard, 1989; Mermall et al., 1998), it was speculated that NMI interacts with the DNA and β -actin docked onto the polymerase to generate force that powers the sliding of polymerase complexes relative to DNA (de Lanerolle et al., 2005). This mechanism would improve the association of polymerases with the DNA and of course decrease the risk of polymerase leakage from the transcribed gene thus enhancing the entire transcriptional process.

The model is also based on the assumption that β -actin binds to and remains bound to the polymerase machinery in a monomeric configuration. Recently, however, regulated actin polymerization was visualized in the cell nucleus (Grosse and Vartiainen, 2013). Further, these polymerization events are also likely to occur across the active gene during transcription (Obdlik and Percipalle, 2011; Percipalle, 2013; Ye et al., 2008), possibly through a treadmill mechanism (Percipalle, 2013). It therefore seems unnecessary that NM1 interacts with the DNA/chromatin to generate a track system for the polymerase movement. If actin is able to polymerize (Obdlik and Percipalle, 2011; Ye et al., 2008), a dynamic filamentous support for the NM1 motor function during transcription is conceivably provided by the polymerase-associated actin polymers.

Another important aspect to be taken into consideration concerns the DNA compaction into chromatin. It is possible but not proven that binding

of NM1 to naked DNA facilitates local establishment and stabilizes the single-stranded template for transcriptional activation. However, this is hard to picture during the elongation of nascent transcripts. In the elongation phase, polymerase movement across the eukaryotic gene is however hindered by the chromatin that represents a major energetic and physical barrier for transcription. NM1 is important for local chromatin modifications that support transcription (Sarshad et al., 2013). Whether NM1 interacts with actin or with the chromatin remodeling apparatus depends on its ATPase cycle. Since myosin 1 is a nonprocessive motor that rapidly detaches and reattaches to actin, but spending most of the time detached from actin, we have proposed that the NM1 motor primarily operates through a two-step mechanism: On one end, it presumably provides local tension for movement of the polymerase across the gene, and on the other end, it leads to local chromatin modifications that generate the permissive chromatin compatible with polymerase-mediated transcription.

In the two-step model, NM1 therefore functions as classical motor protein in conjunction with actin in the sense that its ATPase cycle is central to the motor function. However, by passing from an ATP-bound state to an ADP-bound state, NM1 surely changes conformation and therefore it has potential to act as molecular switch. As discussed in the preceding text, a switch is the result of conformational changes due to enzymatic activities that generate new affinities and expand the plethora of interacting molecules. It is intriguing to speculate that as a consequence of ATP hydrolysis, NM1 swings from a configuration that has high affinity for actin to a configuration that displays higher affinity for SNF2h (see a general model in Fig. 4.2). The affinity switch would explain how NM1 guarantees both motor function and structural function that modulates assembly of the chromatin remodeling complex. Furthermore, for the affinity switch to be efficient, NMI has to stay attached to the DNA both when it pulls on the actin-polymerase complex and when it mediates recruitment of the chromatin remodeling complex.

Another important point that needs to be examined in details is whether and how NM1 binding to the DNA is regulated. We have shown that NM1 association with the rDNA is transcription-dependent. Indeed, in mitotic cells, when transcription is repressed, NM1 is released from the rDNA transcription unit. On the contrary, in early G1, when transcription is reactivated, NM1 occupies rRNA gene promoter and transcribed regions. Therefore, there must be a mechanism that regulates the DNA-binding properties of NM1. We speculate that these regulatory mechanisms consist

in posttranslational modifications that are yet to be identified. We believe that this mechanism makes sure that NMI binds DNA tightly enough to accommodate a power stroke. That is, NMI has to stay attached to the DNA when it pulls on the actin–polymerase complex. Furthermore, considering the importance of NM1 in rRNA gene transcription and the progression through the cell cycle, we speculate that the modifications that modulate the DNA-binding properties of NM1 may have a huge impact on cell growth and proliferation.

The past 15–20 years have witnessed steady increase in the number of publications focusing on the role of actin and myosin in the eukaryotic cell nucleus. All these publications from independent and prominent laboratories have finally erased the skepticism we inherited from the 1970s to 1980s culture when the concept of nuclear actin was almost taboo. A new breeze of belief was further brought in by the visualization of regulated nuclear actin polymerization.

If this is possible, we are now left with even more challenges, experimental and, above all, theoretical. Considering the importance of myosin in the cell nucleus and the connection with molecular machines such as the RNA polymerase, it will be crucial to gradually classify all other myosin species that are found in the cell nucleus. These studies should not forget that we are dealing with myosins, and in the nucleus, they have potential to produce tension or to function as transporters, forward or backward, just like in the cytoplasm. In this context, the most challenging question is whether these nuclear myosin forms whose list is bound to expand contact the DNA or function in a DNA-independent manner. NM1 is incorporated into nascent ribosomal RNPs and accompanies them throughout maturation into ribosomal subunits (Obrdlik et al., 2010). We therefore anticipate that future genome-wide screens will possibly discern whether different nuclear myosin forms attach either to the chromatin or to the transcripts. We will also need to identify the corresponding nuclear cargoes by mass spectrometry methods. In the specific case of NM1 function, many groups, including our own, have performed loss-of-function experiments in living cells subjected to RNAi-mediated NM1 gene silencing. Although difficult to achieve due to the redundancy of mechanisms that are activated when the NM1 gene is completely or partially ablated (Venit et al., 2013), in the long term, the next layer of investigations would comprise a knockout mouse model where loss-of-function and gain-of-function experiments can be performed in a physiological context.

In summary, the take-home message is that NM1 is likely to be an important transcription factor that is part of the basal machinery. Analysis of its genomic occupancy and transcriptional profiling in loss-of-function experiments will provide further mechanistic insights into the different functions across coding and possibly noncoding sequences of the genome that have been ascribed to NM1 (de Lanerolle and Serebryanny, 2011; Simon and Wilson, 2013; Visa and Percipalle, 2010). These experiments will be essential to finally award NM1 with the status of transcription factor and substantiate the idea that its activity is important for cell cycle progression.

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New Insights into the Metabolic and Molecular Mechanism of Plant Response to Anaerobiosis

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Abstract

Under anaerobic conditions, plants apply a wide spectrum of precise adaptive strategies responding to several critical challenges. The ability of efficiently sensing the oxygen presence demonstrates the existence of both direct and indirect ways of perception. The subsequent coordinate metabolic reassessment is currently under study. The complex molecular response implicates not only transcriptional and translational regulation of specific genes but also posttranscriptional and posttranslational regulatory mechanisms, each and all integrating the metabolic settings. Furthermore, the accumulation of typical metabolites during low oxygen stress condition is a key factor that suggests some critical topics in the regulation of metabolic pathways. Here, we summarize the main routes for adaptive behavior during oxygen depletion, from oxygen availability perception to recently discovered molecular mechanisms and metabolic adaptations.



1. INTRODUCTION

The Geologic Calendar is a scale in which the age of the Earth is assumed to be as long as 1 year. While *Homo sapiens* appears on the Earth in the last 4 min of this year, land plants cover the entire last month. This point of view may suggest the extent to which plants demonstrate an ability to recognize and face the wide variety of environmental conditions, even stressful and drastic, bypassing their main limit—being sessile—and colonizing the planet. Plant distinctive feature refers not only to the unavoidable necessity to face conditions without escape but also to the spread and unpredictability of seed dispersion, insomuch as second-generation plants can grow in environmental conditions that are completely different from those of their parent plants.

Both of these challenges led to the evolvement of a wide spectrum of adaptive strategies, insofar as a single plant may produce changes in metabolism in order to face an environmental state or a transient condition, inducing different metabolic responses depending on the degree of the stress conditions and involving the whole plant in a coordinated response. In particular, soil flooding—and thus anaerobiosis—is a stress condition that requires quick and precise sensing, well-coordinated signaling and an integrative response in order to bypass the stress without irreversibly impairing cell metabolism.

In responding to oxygen depletion at the molecular level, a main role has been observed for both transcriptional and translational regulations of specific genes, such as the hypoxia-related transcription factors (TFs)—in particular the family VII of the ERF (ethylene response factor) and heat shock transcription factors (HSFs), involved in oxygen sensing and stress response triggering (Licausi and Perata, 2009; Licausi et al., 2011; Pucciariello et al., 2012).

Furthermore, recent studies have highlighted the even higher complexity of this molecular response; posttranscriptional and posttranslational regulations also participate in the network of regulatory mechanisms (Mazzucotelli et al., 2008), integrating the modulation of metabolic settings. The metabolic and physiological reassessment induced by the lack of oxygen also depends on indirect sensing mechanisms involving different changes in cytosolic parameters and hormonal balances (Bailey-Serres et al., 2012). From a metabolic point of view, production of lactate and ethanol has been studied since 1974 when Davies and colleagues suggested the mechanisms regulating lactic and ethanolic fermentation.

More recently, studies of the accumulation of specific amino acids were found to belong to the adaptive response of the plant to the lack of oxygen. In particular, alanine and γ -aminobutyric acid (GABA) have been found to be the most biosynthesized amino acids during hypoxic conditions, with a role in maintaining the osmotic potential and in limiting the cytosolic acidification (Miyashita and Good, 2008). Succinate also accumulates, suggesting the last step of the noncircular TCA cycle active during oxygen depletion (Sweetlove et al., 2010), while the observed production of γ -hydroxybutyrate (GHB) during stress has been explained with the conversion of succinic semialdehyde derived from GABA-T by means of a specific reductase enzyme (Breitkreuz et al., 2003; Deleu et al., 2013; Renault et al., 2012).

Furthermore, metabolic adaptations also involve a mitochondrial role for nitrite, where it acts as an alternative electron acceptor in the electron transport chain, producing nitric oxide (Stoimenova et al., 2007) and contributing to the maintenance of mitochondrial activity and ATP synthesis during anoxic conditions (Igamberdiev et al., 2005; Stoimenova et al., 2007). Thus, plant strategies for adaptation to low oxygen conditions are defined by a complex coordination of molecular, metabolic, and physiological redefinitions, which allow the survival of plants even under strict low oxygen conditions.



2. CELLULAR OXYGEN STATUS PERCEPTION

2.1. Roots

Roots are very sophisticated organs; they ceaselessly maintain a highly complex interdependent relationship with biotic and abiotic components of soil. While root apparatus is strongly influenced by the environment, it also strongly influences the surrounding soil by means of exudates, water and oxygen uptake, and metabolic activities (Hinsinger et al., 2009). Furthermore, also among those processes operating inside roots, there is an interdependence that involves soil condition, soil condition sensing, and a subsequent redefinition of metabolic pathways, as seen, that is, for water uptake during anaerobic conditions (Tournaire-Roux et al., 2003). Actually, during flooding, plants act to redefine metabolic and molecular processes across the entire plant (Bailey-Serres and Voesenek, 2008; Christianson et al., 2010; Kreuzwieser et al., 2009). This broad rearrangement, which involves organs that are not directly suffering stress, suggests the presence of a systemic and incisive intraplant communication,

acting for the entire plant adaptation. Plant adaptation begins with the perception of stress and with the root taking on the task of perceiving the availability of oxygen. Root anatomy is composed of different zones, which are basically defined as root cap, which covers the root apices (Barlow, 2003), a meristematic zone, a transition zone, and a zone of rapid cell elongation (Baluška et al., 1990, 1996, 2001; Kieffer et al., 2009; Verbelen et al., 2006).

Focusing on the transition zone, we find that the cells of this zone have been shown to be the most intense zone for endocytic vesicle recycling and for auxin flux (Baluška et al., 2010; Sukumar et al., 2009). These cells show evidence of an electrical activity synchronized with auxin transport peaks (Masi et al., 2009) and an intense and complex hormonal network (Baluška et al., 2010). In order to sustain all of these processes, the transition zone is characterized by a very high intake of ATP and oxygen (Mancuso et al., 2000)—much higher than in the other root regions (Mancuso and Marras, 2006; Mugnai et al., 2011, 2012). Consequently, oxygen deprivation greatly affects the transition zone, which is the region of the root most sensitive to a lack of oxygen (Mugnai et al., 2012) and thus the probable point of departure of intercellular communication.

It is worth mentioning that oxygen availability sensing and intercellular signaling are a deeply intricate field of research, with many unclear points and no completely exhaustive answers. However, the key role of the transition zone in sensing and signaling oxygen deprivation may have been confirmed in 2012 when Mugnai and colleagues studied the effects of hypoxia selectively imposed on the root apex. The cells of the transition zone react to the stress by emitting an increased amount of nitric oxide, which seem to play an important role in inducing the entire root to have an acclimation response.

2.2. Multifactorial perspective of oxygen sensing and signaling

In order to favor plant survival during oxygen deprivation, the availability of oxygen can be perceived by both direct and indirect sensing, leading to a cascade of events belonging to the acclimation response (Bailey-Serres and Chang, 2005; Bailey-Serres et al., 2012). Some of the indirect mechanisms might involve the perception of the ratio of ATP and ADP, of the amounts of carbohydrates and pyruvate, of altered cytosolic pH and cytosolic calcium, and of the burst in reactive oxygen species (ROS) and nitrogen oxygen species (Bailey-Serres et al., 2012). These indicators of oxygen availability also participate in the response of other stress conditions, both

biotic and abiotic (Pucciariello and Perata, 2012). Some of them are not plant specific, but are important parameters for most living organisms.

Cytosolic-free calcium concentration is one of the most widespread signals among all living organisms. Calcium spikes are involved in the perception of many different stimuli or stresses; these spikes stimulate responses involved in gene expression, cell physiology, or metabolic pathways (Tuteja, 2009). During hypoxic and anoxic stresses, an increase in Ca^{2+} concentration has been observed in both maize and *Arabidopsis* cells (Sedbrook et al., 1996; Subbaiah et al., 2000). It has been speculated that this increase may be required for *Adh1* gene expression (Subbaiah et al., 2000).

Cytosolic pH change during anaerobiosis is a well-known effect since the lactic fermentation pathway prevails in early phases of anaerobic metabolism, resulting, first of all, in a fall of the cytoplasmic pH (Davies, 1980; Perata and Alpi, 1993). Curiously, it can be supposed that changes in pH values generated in the stressed cells of anaerobic roots might have signal roles in themselves. For example, cytosolic acidification is associated with the induction of ethanolic fermentation (Davies, 1980; Perata and Alpi, 1993), with a positive stimulus in the nitrate reductase activity, leading to an increased emission of NO (Dordas et al., 2004), and with a reduction in the hydraulic conductivity of the roots by affecting the activity of aquaporins (Bramley and Tyerman, 2010; Ehler et al., 2009; Tournaire-Roux et al., 2003).

This malfunction in the aquaporins—and the subsequent decrease of root hydraulic conductance—results in a fall in leaf hydration and thus in an induced stomata closure in order to reduce water loss (Andersen et al., 1984; Else et al., 2001; Jackson et al., 2003). This cascade of events following a change in pH values in root anoxic cells supports the suggestion that pH may have a key role in sensing oxygen deficiency and triggering responses among the whole plant. Moreover, the acidification might diffuse through root cells until reaching the xylem sap, and then move and act as a messenger in itself across the plant (Jia and Davies, 2007).

Also nitric oxide has emerged as a very significant signal molecule and is involved in many different stresses (Leterrier et al., 2012; Sun et al., 2007; Wang and Yang, 2005). NO has been shown to have various key roles during anaerobiosis. NO is an alternative product of the altered mitochondrial metabolism, since nitrite becomes an intermediate electron acceptor and is converted back to nitrate by low oxygen-induced plant hemoglobin, becoming a fundamental step in the anaerobic switch of plant mitochondria (Igamberdiev and Hill, 2009). NO is implied in the hormone-induced response by means of synergic or counteractive interactions

(Leterrier et al., 2012). NO has also been suggested as a trigger for gene expression and as an inhibitor of mitochondrial enzymes (Blokchina and Fagerstedt, 2010).

As seen before, the cells of the transition zone in the root are those with the highest intake of ATP and oxygen; thus, it is hypothesized that these cells are highly sensitive to oxygen deficiency. Interestingly, an enhanced emission of NO has also been observed in these cells—an emission which it has been speculated is involved in the metabolic switch of these cells during stress associated with a decreased demand of oxygen (Mugnai et al., 2012).

2.2.1 Reactive oxygen species

Even if it does not seem intuitive, lack of oxygen is a stress condition which induces oxidative stress. Increased levels of ROS have been observed in both tolerant and sensitive species. Pigeon pea (*Cajanus cajan*), pea (*Pisum sativum*), and soybean (*Glycine max*) have shown markedly enhanced levels of ROS, in particular H₂O₂, when subjected to a lack of oxygen (Ershova et al., 2011; Kumutha et al., 2009). H₂O₂ accumulation in response to oxygen deprivation has also been detected in *Arabidopsis thaliana* seedlings (Baxter-Burrell et al., 2002), in roots of wheat (*Triticum aestivum*), and in two different species of apple trees (*Malus hupenensis* and *Malus toringoide*) (Bai et al., 2010; Blokchina et al., 2003).

Santosa et al. (2006) evaluated the oxidative stress of submerged rice, detecting ethane production as a product of membrane peroxidation. They observed that ethane emission was enhanced during submergence, as levels of O₂ fall to 1%, highlighting the ROS production and the subsequent cell damage during the decline in the availability of oxygen. ROS production is predominantly attributed to the pathway that involves the activity of a RHO-like, small G protein (ROP) and its interaction with a NAPH oxidase (NOX) localized in the plasma membrane (Sagi and Fluhr, 2006; Sairam et al., 2008; Wong et al., 2007).

NOX generates ROS in both animal and plants, and NOX-dependent superoxide production has been suggested as a key factor for adaptation during plant hypoxia (Sairam et al., 2008). In fact, the interaction between ROP and NOX promotes a cascade of events that induces an enhanced calcium influx, an increase in the H₂O₂ levels, and a subsequent switch to anaerobic fermentation and other stress adaptive pathways (Baxter-Burrell et al., 2002; Demidchik and Maathuis, 2007; Pucciariello et al., 2012).

A mitochondrial role in ROS production has also been hypothesized. In 2012, Chang and colleagues studied ROS production in *Arabidopsis* seedlings when the electron transport chain was inhibited at the sites of

complexes III and IV (Chang et al., 2012). Experiments provided evidence about the transient activation of mitogen-activated protein kinases and the ubisemiquinone accumulation, with an associated increase in ROS production. Hydrogen peroxide, being an uncharged molecule, can easily diffuse through the mitochondrial membrane, reaching the cytosol and acting as signal of the stress condition (Han et al., 2003; Turrens, 2003). As suggested by these studies, there is evidence of a ROS role in the signaling mechanisms in plants as triggers for the stress response and as molecules involved in the cell-to-cell communication (Mittler et al., 2011). Observing the characteristics of ROS, they seem to be perfectly fitting as having a role as signal molecules. The quick production, the ability to induce specific responses, and the rapid degradation are key features for the effective signal molecule (Pauly et al., 2006).

During oxygen depletion, a set of genes related to the balance between ROS production and ROS scavenging is newly regulated, thus coordinating the ROS-dependent response (Pucciariello et al., 2012). Moreover, the lack of oxygen implies ROS-dependent TFs and heat-shock proteins (HSPs), strictly related to the presence of ROS. The involvement of all of these ROS and ROS-dependent factors during oxygen shortage also suggests a possible signaling role of ROS in themselves (Pucciariello et al., 2012). Indeed, ROS molecules have been demonstrated to have an importance in cell-to-cell signaling, following mechanisms which seem to be common between anoxic and heat stresses, since the oxidative burst belongs to both these stress conditions (Banti et al., 2010). In particular, H_2O_2 involvement has been reported for the activation of redox-sensitive TFs, such as heat shock transcription factors (HSFs) in different stress conditions involving an oxidative stress (Pucciariello et al., 2012). Hydrogen peroxide also has been shown to be involved in the low oxygen, stress-induced formation of lysigenous aerenchyma in Arabidopsis plants (Muhlenbock et al., 2007) and in the programmed cell death induced by abiotic stress (De Pinto et al., 2012). Moreover, Arabidopsis plantlets pretreated with H_2O_2 demonstrated enhanced tolerance to anoxia (Banti et al., 2010).

2.2.2 Hormonal involvement

Plant hormonal balances involve, during oxygen depletion, the rearrangement of ethylene, abscisic acid, and gibberellin (GA) levels (Yemelyanov and Shishova, 2012). Their coordinated effect is implied in the adaptive pathways of both tolerant and sensitive plants, mediating molecular and physiological responses (Bailey-Serres and Voisenek, 2008; Kende et al., 1998; Peeters et al., 2002).

Ethylene production seems to be the first hormonal regulation during low oxygen conditions, becoming the trigger for the escape strategy in tolerant species. Ethylene stimulates the growth rate of both the shoot and the leaf (Jackson, 2008), as well as the activity of specific, ethylene-dependent enzymes involved in cell loosening (Bragina et al., 2001, 2003). Ethylene plays an important role in the aerenchyma formation (Sairam et al., 2008), as demonstrated in hypoxic roots of maize, where it has been confirmed by means of exogenous application of ethylene antagonists, which were shown to exert an inhibitory effect on its development (Drew et al., 1981; Jackson et al., 1985; Konings, 1982). For most of the plants studied, ethylene production during flooding was shown to induce hyponastic leaf growth (Ridge, 1987; Voesenek and Blom, 1989). Moreover, ethylene production may have an inhibitory effect on physiological parameters during hypoxic stress, as seen in tomato plants where an increased ethylene/auxin ratio affected the formation of adventitious roots (Vidoz et al., 2010).

Ethylene also regulates the induction of hypoxia-related genes, even if only 10% of the so-called ERFs require ethylene to be regulated (Nakano et al., 2006). These ethylene-dependent ERFs seem to be fundamental for sensing oxygen, as well as for the beginning of an adaptive response (Voesenek and Sasidharan, 2013). The ethylene-inducible ERFs belonging to the family VII have been thoroughly studied in rice, since they can act as mediators for both quiescent and escape strategies. Particularly during the quiescent strategy, the group VII ERF Submergence 1A (Sub1A)-1 hinders underwater growth, inhibiting the induction of those genes codifying for proteins involved in starch breakdown and cell wall loosening (Fukao et al., 2006) but also favoring the activity of specific repressors of GA-induced growth processes (Fukao and Bailey-Serres, 2008).

In contrast, during the escape strategy, the Snorkel locus encodes for two ERF VII groups, Snorkel 1 (SK1) and Snorkel 2 (SK2), which promote fast stem elongation in order to reach the water level (Fukao and Xiong, 2013; Hattori et al., 2009).

Coordination among different hormones is clear in submerged organs of rice, where the induction of ethylene promotes a subsequent regulation of the balance between aminobutyric acid (ABA) and GAs, mediating cell elongation (Bailey-Serres et al., 2012). Reduced levels of GAs during oxygen deprivation have also been observed in *Rumex palustris* and *Rumex acetosa*. Reduced GAs, with a simultaneous increase in ABA levels, coordinate the reduced elongation (Benschop et al., 2005; Chen et al., 2010). It has been suggested that during waterlogging, both GAs and cytokinins may have

a role in root-to-shoot signaling by promoting stomatal opening, in antagonism with the ABA effect (Kumar et al., 2004; Pospíšilová, 2003). It has also been observed that NO levels rise during oxygen deprivation while GA levels decrease, suggesting a role in stomatal closure (Christianson et al., 2010).



3. MOLECULAR MECHANISMS IN ANAEROBIC RESPONSE

During hypoxic and anoxic stress, plant adaptive strategies have been divided into two broad groups: low oxygen quiescence syndrome (LOQS) and low oxygen escape syndrome (LOES) (Colmer and Voesenek, 2009). However, some responses have been shown to be conserved in all flooding-adapted plants, independently from the strategy. Since oxygen availability becomes low during submergence, plants following the LOQS strategy reduce or repress stem elongation and keep metabolic pathways in a quiescent state in order to limit energy-consuming processes and save substrate supply for the desubmergence phase. In contrast, plants responding with an LOES strategy show the ability to tolerate longer term anoxic stresses, investing in the faster elongation of submerged stems, petioles, and leaves, and thus reaching the water surface to be back in contact with air (Colmer and Voesenek, 2009; Pucciariello and Perata, 2012). Curiously, it has been proved that there is not a sharp distinction between the two strategies, since some tolerant species have the ability to “choose” the one or the other strategy depending on the seriousness of stress conditions, showing a very sophisticated stimuli response (e.g., see *Oryza sativa* spp. indica, Bailey-Serres et al., 2012). This basic differentiation in possible responses to oxygen deprivation is also shown in the numbers of genes selectively expressed during the stress, which present significant differences between flooding-sensitive plants and flooding-tolerant ones. During hypoxic stress, a flooding-tolerant plant, such as poplar, shows altered expression levels for more than 5000 genes. In contrast, the flooding-sensitive *Arabidopsis* plants show a change in the transcript abundance of about 150 genes involved in the stress response (Klok et al., 2002; Liu et al., 2005).

In particular, *Arabidopsis* and rice are the most thoroughly studied plants in their response to hypoxia and anoxia, and today, many microarray data sets provide a notable amount of information (Banti et al., 2010; Branco-Price et al., 2005; Jung et al., 2010; Lasanthi-Kudahettige et al., 2007; Lee et al., 2011; Licausi et al., 2010; Liu et al., 2005; Loreti et al., 2005; Mustroph et al., 2010; van Dongen et al., 2008). Furthermore, even in

the same plant, upregulation or downregulation of specific genes seems to depend strongly on the degree of the stress condition (i.e., from the beginning of hypoxia to a strict anoxia), involving both common and different genes for each stage of oxygen deprivation, as seen for *Arabidopsis* (Pucciariello et al., 2012). It has also been noted that there are genes whose regulation occurs throughout the entire plant, as well as specific genes differently regulated depending on the organ of the plant (Pucciariello and Perata, 2012).

In spite of these variances, a common trait for sensitive and tolerant species, as well as for different stress levels, is that a high percentage of genes involved in the stress response is composed of TFs. TFs play the part of the last regulatory step in the signal cascade, starting from the perception of the environmental change and advancing toward the metabolic response. Several hypoxia-responsive TFs have been studied, that is, the MYB (myeloblastosis), the ATAF (*Arabidopsis* transcription activation factor), the PHD (plant homeodomain), and the ERF families (Bond et al., 2009; Christianson et al., 2009; Hoeren et al., 1998; Licausi et al., 2010). But in any case, no single TF seemed to be the only one regulating the stress response. This finding suggests the existence of a more complex transcriptional network with coordinated transcriptional regulators which contribute to the stress adaptation in the molecular response to a lack of oxygen (Kreuzwieser et al., 2009; Licausi et al., 2010).

3.1. ERF VII transcription factors

3.1.1 *ERF VII as key players in anaerobic adaptation*

Among the TFs studied, particular attention has been directed to the family VII of the ethylene response factor (ERF VII) involved in stress adaptation responding to increased ethylene production (Nakano et al., 2006). Both LOES and LOQS mechanisms involve genes belonging to this family (Pucciariello and Perata, 2012), even if there are consistent differences among species. For example, ERF VII members identified in monocots such as rice and maize are double or more in respect to the numbers of those found in the dicots studied (Licausi et al., 2010; Zhang et al., 2008).

In mature rice plants using the quiescent strategy involved in the flooding response, the ERF Sub1A gene has been demonstrated to play important roles (Fukao et al., 2006; Xu et al., 2006). Rice SUB1A has a primary role related to the presence of ADH, improving the fermentative metabolism (Fukao et al., 2006). But SUB1A also induces the repression of those genes involved in sucrose degradation by means of sucrose synthase (Fukao et al.,

2006), and the inhibition of the GA-mediated underwater plant elongation, limiting cell expansion (Fukao and Bailey-Serres, 2008).

In particular, during the quiescent strategy, the submergence-induced allele Sub1A-1 activates two specific genes: the slender-rice 1 (SLR1) and the slender-rice-like 1 (SLRL1). These genes inhibit GA-induced plant elongation leading to the preservation of the energy reserve (Bailey-Serres and Voesenek, 2008; Pucciariello and Perata, 2012). In contrast, in rice plants utilizing the escape strategy, underwater plant elongation is induced by two different genes also belonging to the ERF family VII: SK1 and SK2, which have a role in promoting fast elongation, in order to reestablish gas exchange once the plant has reached the water surface (Hattori et al., 2009).

Moreover, *Arabidopsis* plants subjected to low oxygen conditions have shown an increased regulation of genes belonging to the ERF family VII, in particular five members: HRE1, HRE2, RAP2.2, RAP2.3, and RAP2.12 (Licausi et al., 2010).

The hypoxia-responsive ERF (HRE)1 and the HRE2 are both localized in the nucleus, where, as suggested by the literature, they may have the role of positively regulating gene transcription (Licausi et al., 2010; Xu et al., 2007). Both HRE1 and HRE2 are not directly induced by the increase in ethylene production, although their response to anaerobic stress quickly emerges (van Dongen et al., 2009). Experiments with transgenic plants confirmed the importance of these genes. Overexpressing HRE1 and HRE2 plants enhance their tolerance to anoxia, while the suppression of both HRE1 and HRE2 induces an increased sensitivity, lowering the expression of many hypoxic-related genes (Pucciariello and Perata, 2012). Thus, *Arabidopsis* plants lacking oxygen clearly show an increased expression of ERF VII genes and an increased expression of hypoxic genes, with a subsequent enhanced low oxygen tolerance (Hinz et al., 2010). Despite that, only plants overexpressing HRE1 show an intensified hypoxic induction of the anaerobic genes ADH, SUS1, and SUS4 and an upregulation of ADH under aerobic conditions (Licausi et al., 2010).

RAP2.2 is constitutively expressed at very high levels in roots and has a role in the induction of genes related to the sucrose metabolism and the fermentation pathways (Hinz et al., 2010). Its distribution across the plant reflects the dissemination of anaerobiosis-related genes such as ADH1, LDH1 (Dolferus et al., 2008), and AlaAT1 (Miyashita et al., 2007). Even if RAP2.2 induction under hypoxia seems not to be drastic (Hinz et al., 2010), transgenic plants overexpressing RAP2.2 showed an improved

tolerance to hypoxic stress, whereas the knockouts of the gene caused a decreased survival rate (Hinz et al., 2010).

However, overexpression of RAP2.2 did not affect the expression of ADH gene or any other anaerobic gene in air (Welsch et al., 2007). Also RAP2.12, whose role was suggested to be in the upregulation of the ADH1 expression and ADH activity (Papdi et al., 2008), did not show an increased gene expression in Arabidopsis seedlings subjected to hypoxia (Licausi et al., 2010). RAP2.3 is known to confer resistance to H₂O₂ and to positively regulate many defense genes (Ogawa et al., 2005; Papdi et al., 2008). Like RAP2.2 and RAP2.12, the expression of RAP2.3 was also unaffected by hypoxia (Licausi et al., 2010).

3.1.2 ERF VII as key players in oxygen sensing

In the past, many different hypothetical oxygen-sensing mechanisms were studied. In 2007, there emerged a possible role for prolyl hydroxylase enzymes, whose transcription seemed to increase during the oxygen deficit in *A. thaliana* and in rice (Lasanthi-Kudahettige et al., 2007; Vlad et al., 2007). In mammals, prolyl hydroxylase is an enzyme that works consuming O₂, implicated in the degradation of a TF subunit involved in oxygen-deficiency acclimation (hypoxia-inducible factor 1 α —HIF1 α). The enzyme activity is inhibited in oxygen-deficiency conditions and thus TFs related to the stress are not prevented anymore. Therefore, in mammals, prolyl hydroxylase perceives the weakening of O₂ and behaves as a direct sensor of hypoxia (Guzy and Schumacker, 2006).

In spite of these theories, no HIF1 α has been found in plants (Bailey-Serres et al., 2012). More recently, new, promising studies have shown a molecular response to hypoxia that involves the subgroup VII of the ERF and their posttranslational regulation (Licausi et al., 2011, 2013). An important role for oxygen availability sensing and hypoxia response had already been assigned to the hypoxia-related ERFs, since many different studies showed a strong relationship among oxygen conditions, induction of ERFVII genes, and subsequent reassessment of metabolic routes (Hinz et al., 2010; Licausi et al., 2010, 2011; Papdi et al., 2008).

Subsequently, a cascade of findings supported the hypothesis that ERF TFs belonging to the group VII subfamily are also hypoxia sensors in plants, suggesting that the oxygen perception involves their posttranslational regulation by the ubiquitin-dependent N-end rule pathway (NREP) for protein degradation (Licausi et al., 2010, 2011). In particular, RAP2.12, a member of the ERF VII, has been demonstrated to play a fundamental role in the

trigger of the anaerobic response (Licausi et al., 2011). In 2008, Papdi and colleagues suggested that RAP2.12 could bind the ADH1 promoter. A posttranscriptional or posttranslational regulation of RAP2.12 emerged when mutants overexpressing RAP2.12 revealed an increase in the expression of those hypoxia-responsive genes only in those plants suffering hypoxic conditions, suggesting that normoxic conditions inhibit the action of RAP2.12 (Licausi et al., 2011; Sasidharan and Mustroph, 2011). A conserved amino-terminal sequence, which includes the Met-Cys initiating motif (MCGGAI/L), is shared by the hypoxia-responsive factors HRE1, HRE2, RAP2.2, and RAP2.12 (Licausi et al., 2011, 2013). This conserved domain is a peculiar motif, a perfect target for the N-end rule degradation mechanism (Gibbs et al., 2011; Licausi et al., 2011), which is an oxygen-dependent, posttranslational pathway, able to lead the protein degradation under aerobic conditions. Thus, during hypoxic conditions, the NERP is inhibited, and RAP2.12 is not degraded and moves to the nucleus where it accumulates, inducing the expression of those genes involved into hypoxia acclimation (Fig. 5.1; Licausi, 2011; Licausi et al., 2011).

Confirming this hypothesis, it has been observed that during normoxic conditions RAP2.12 can be recruited by the membrane ACBPs (Aacyl-CoA binding proteins) and, being temporarily sequestered and protected from degradation, then can undergo degradation by means of the NERP. In contrast, during hypoxia, RAP2.12 dissociates from the membrane to move into the nucleus (Licausi et al., 2013).

3.2. Nontranscriptional regulation in low oxygen sensing and response

3.2.1 *Role of polyribosomes: Example of posttranscriptional regulation*

It is worthwhile to highlight the role of the differential mRNA translation, in particular, for those mRNAs associated with large polyribosomes (Branco-Price et al., 2005). It has been suggested an optimization of the mRNAs selective translation during stress, in order to grant an efficient translation of the hypoxia-induced mRNAs (Branco-Price et al., 2008). Interestingly, the posttranscriptional regulation also grants an increase in the translation of those mRNAs which encode enzymes that participate in the anaerobic production of ATP (Branco-Price et al., 2008), even if there is no alteration in the abundance of mRNA transcript. Moreover, during the stress, some mRNAs do not undergo translation, since they can be stabilized or degraded once sequestered into cytosolic messenger ribonucleoprotein complexes (mRNPs) (Branco-Price et al., 2008; Hoyle et al., 2007;

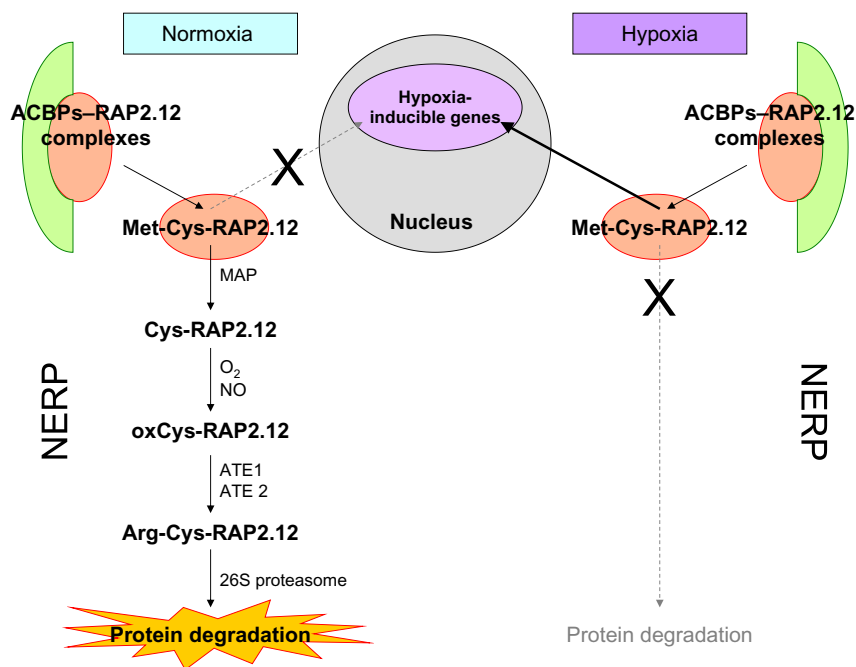


Figure 5.1 The N-end rule pathway during normoxic and hypoxic conditions. During normoxic conditions, the RAP2.12 transcription factor bound to ACBPs (acyl-CoA binding protein 1 and 2) is a target for proteasomal degradation following the N-end rule pathway (NERP). N-terminal Met cleavage catalyzed by MAP leaves the cysteine residue exposed to oxidation. Oxidized cysteine becomes then the substrate for the addition of an arginine and the subsequent degradation of the polypeptide. During oxygen deficiency, NERP is inhibited and the protein can move to the nucleus, where it triggers hypoxia-related signaling cascade enhancing plant survival. ACBPs, acyl-CoA binding proteins 1 and 2; ATE, arginyl tRNA transferase; MAP, methionine-amino peptidase; NERP, N-end rule pathway.

Parker and Sheth, 2007). This mRNP–mRNA complex allows the preservation of those mRNAs that under stress conditions are not translated, delaying their degradation (Arru and Fornaciari, 2010).

To simplify, during low oxygen conditions, a loss of correspondence between gene transcription and enzyme synthesis can be observed. Some mRNAs are sequestered by mRNPs so as not to be translated, but also not degraded. In contrast, those mRNAs useful to overcome the stress may have an unchanged expression level associated with enhanced activity of the related enzyme. Where the abundance of mRNA is unchanged, the recruitment by polyribosomes can involve a significant advantage.

The population of mRNA involved in this posttranscriptional regulation represents a considerable number of genes which are important for stress-induced metabolism (Branco-Price et al., 2005). In particular, concerning the posttranscriptional regulation of TFs, it has been reported that HRE1 and HRE2 mRNAs under hypoxia are associated with polyribosomes (Branco-Price et al., 2005, 2008), highlighting once more how transcription and translation coordinate at multiple levels. Concerning the discrepancy between gene expression variance and protein activity of the TFs RAP2.2 and RAP2.12, the issue may have a different explanation, as elucidated recently by Licausi et al. (2013).

3.2.2 N-end rule pathway: Example of posttranslational regulation

The NERP is a proteolytic mechanism which depends on the interaction of the proteasome with its target, called N-degron, which is an N-terminal residue followed by a specific sequence of amino acids (Tasaki et al., 2012). This pathway belongs to the ubiquitin–proteasome system, which determines protein stability in eukaryotes participating in many different cellular and developmental processes (Graciet and Wellmer, 2010). In particular, for plants, the NERP is involved in leaf senescence and seed germination as well as in shoot and leaf development (Graciet and Wellmer, 2010; Holman et al., 2009; Yoshida et al., 2002). In plants, the homeostatic response to hypoxia depends on the halt of the NERP related to specific ERF VII TFs. In normoxic conditions, the same proteins undergo proteolysis by means of the NERP through a mechanism which leads to a proteasome target beginning with the modification of the ERF VII tertiary destabilizing residue.

The definition of this residue depends on the classification of the N-terminal amino acids, which includes two main categories, depending on the stability conferred to the protein: stabilizing N-terminal residues and destabilizing N-terminal residues.

The stabilizing N-terminal residues assure the safety of the protein against the NERP, stunting the protein alteration and subsequent degradation. In contrast, destabilizing N-terminal residues are good targets for ubiquitin-dependent protein degradation (Varshavsky, 1997). Moreover, destabilizing residues can be further divided into primary, secondary, and tertiary destabilizing amino acids, depending on the protein modification progress status (Licausi et al., 2013; Varshavsky, 1997).

Primary, secondary, and tertiary destabilizing amino-terminal residues of amino acid sequences may be modified through different ways in order to allow protein degradation (Tasaki et al., 2012). Tertiary destabilizing

residues are those which require two steps of modification in order to become N-degrons, passing through the intermediate secondary status. The enzymatically catalyzed covalent modifications of tertiary destabilizing amino acids, such as deamidation or oxidation, alter the N-terminal residue leading to the following step: the secondary destabilizing residues. Thus, the addition of an Arg residue by means of arginyl tRNA transferases (ATEs), allows the formation of the primary destabilizing residue, which is the N-terminal proteasome target, therefore the N-degron (Fig. 5.2) (Licausi et al., 2013).

Depending on the oxygen availability status, some ERF VII TFs, for instance RAP2.12, are targets for proteasomal degradation following the NERP until oxygen is available. As shown in Fig. 5.1, the conserved amino-terminal sequence, which includes the MCGGAI/L, is the residue involved in the enzymatically catalyzed reactions. First, methionine-amino peptidase (MAP) mediates the Met cleavage (Liao et al., 2004), leaving an exposed cysteine residue, subjected to oxidation due to oxygen and nitric oxide. Consequently, oxidized cysteine becomes the substrate for the addition of an arginine and the subsequent degradation of the polypeptide (Bailey-Serres et al., 2012; Licausi et al., 2013). In contrast, during oxygen deficiency, NERP is inhibited and the protein moves to the nucleus, where it induces expressions of core hypoxia-related genes such as ADH1, PDC1, and SUS4, thus enhancing plant survival (Gibbs et al., 2011).

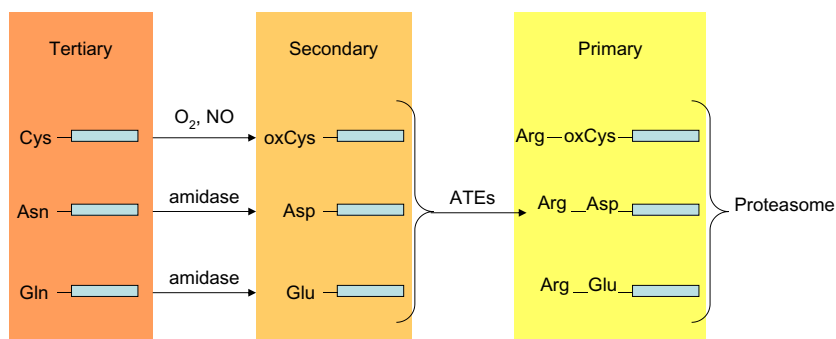


Figure 5.2 Schematic representation of oxidative and enzymatic reactions of tertiary, secondary, and primary destabilizing residues in the N-end rule pathway. Tertiary destabilizing residue can be degraded by means of deamidation or oxidation, forming an altered N-terminal residue. This secondary destabilizing residue is subjected to the addition of an Arg residue by means of arginyl tRNA transferases (ATEs). The subsequent primary destabilizing residue is then the N-terminal proteasome target.

3.3. Heat stress and oxygen-deprivation stress responses: Converging strategies

The main difference between hypoxia and anoxia response might be the greater or lesser involvement of HSFs and HSPs in the stress response. When the lack of oxygen reaches values ascribable to the anoxic condition, an overlap has been observed between heat stress-induced response and anoxia-induced response (Banti et al., 2010; Pucciariello et al., 2012). This convergence seems to be imputable to the higher amount of ROS, in particular H_2O_2 , during each of these stresses (Banti et al., 2010). The increase in HSPs transcripts during anaerobic stress is widely conserved among different plant species (Mustroph et al., 2010; Vandenbroucke et al., 2008). Members of the HSFs are believed to sense indirectly the oxygen status, acting as H_2O_2 molecular sensors (Miller and Mittler, 2006) and modulating a complex response which confers improved tolerance to anoxia (Bailey-Serres et al., 2012). Anoxic stress imposed on *Arabidopsis* seedling induces the activation of HSPs-related genes (Banti et al., 2010, 2013), whose preinduction by means of heating has been demonstrated to confer a better tolerance to a later-imposed anoxia (Banti et al., 2010).

Transgenic plants of *Arabidopsis* overexpressing the TF HsfA2 showed an increased expression of small HSPs and antioxidant-related genes and were demonstrated to better tolerate anoxia (Hinz et al., 2010; Nishizawa et al., 2006; Ogawa et al., 2007; Schramm et al., 2006). Curiously, this TF does not seem to be related to the switch to the anaerobic metabolism (Banti et al., 2010). Also at the sensing level, there is no correlation between the anoxic-induced, heat-shock, overlapping response and the hypoxic-induced, posttranslational, regulation response (Licausi et al., 2011; Sweetlove et al., 2010).

This lack of correlation may suggest that there are at least two main ways to follow to overcome oxygen-deficiency stress. A first pathway begins with a sensing of the oxygen status by means of posttranslational regulation of specific ERFs and involves the modulation of several genes among which are those related to anaerobic metabolism. A second pathway depends on a ROS-dependent sensing mechanism and involves HSFs and HSPs, increasing anoxia tolerance through other defense strategies. For example, in *Arabidopsis* plants, the expression level of ascorbate peroxidases gene depends on the activity of the HSFs (Panchuk et al., 2002), suggesting that these TFs are involved in the oxidative stress regulation by means of antioxidant activity modulation.



4. METABOLIC ADAPTATIONS

Anoxic stress induces rapid and severe metabolic and molecular adaptations in order to confront the fall in ATP production, due to the lack of oxygen as the final acceptor of electrons in oxidative phosphorylation, the fall in cytosolic pH, and the imbalance in osmotic potential. There is evidence about changes in enzyme composition in the cytosol and in mitochondria (Couee et al., 1992; Igamberdiev and Hill, 2009; Igamberdiev et al., 2004; Miyashita and Good, 2008), mainly related to the fermentation pathways, to the GABA shunt, to the switch of the TCA cycle to a non-circular TCA flux, and to the hemoglobin/nitrate cycle.

In order to elucidate these substantial metabolic changes, it would be strategic to retrace pathways, cycles, and fluxes beginning from the hypoxically induced metabolites accumulation and turnover, and then defining the events in plant cells during anaerobiosis. During root anaerobiosis, several metabolites accumulate—especially GABA and alanine (Miyashita and Good, 2008; Reggiani et al., 2000) but also GHB (Nakamura et al., 2012) and succinate (Bailey-Serres et al., 2012). Like the well-known lactate and ethanol synthesized during fermentation, their production and accumulation in plant tissues are a challenge for survival during the depletion of oxygen availability in the soil.

4.1. GABA accumulation: “GABA shunt”

GABA metabolism during anoxia mainly involves a short pathway of three enzymes moving from glutamate and generating succinate, bypassing steps of the tricarboxylic cycle, and then being called “GABA shunt” (Bouché and Fromm, 2004). The steps of this metabolic pathway begin with the cytosolic enzyme glutamate decarboxylase (GAD) which converts glutamate into GABA, then the mitochondrial enzyme GABA transaminase (GABA-T) converts GABA into succinyl semialdehyde, and finally the enzyme succinic semialdehyde dehydrogenase leads to the biosynthesis of succinate (Bouché and Fromm, 2004). Interestingly, Miyashita and Good, in 2008, demonstrated that an inhibition of the GAD and GABA-T enzymes (and thus a defect in the GABA shunt) affected also the accumulation of alanine in *Arabidopsis* roots. In Fig. 5.3, the possible ways for alanine production, both independent and dependent from the GABA-shunt pathway, are shown.

In their 2009 review, Igamberdiev and Hill noted that alanine begins to accumulate earlier than GABA. The previous year, Miyashita and Good

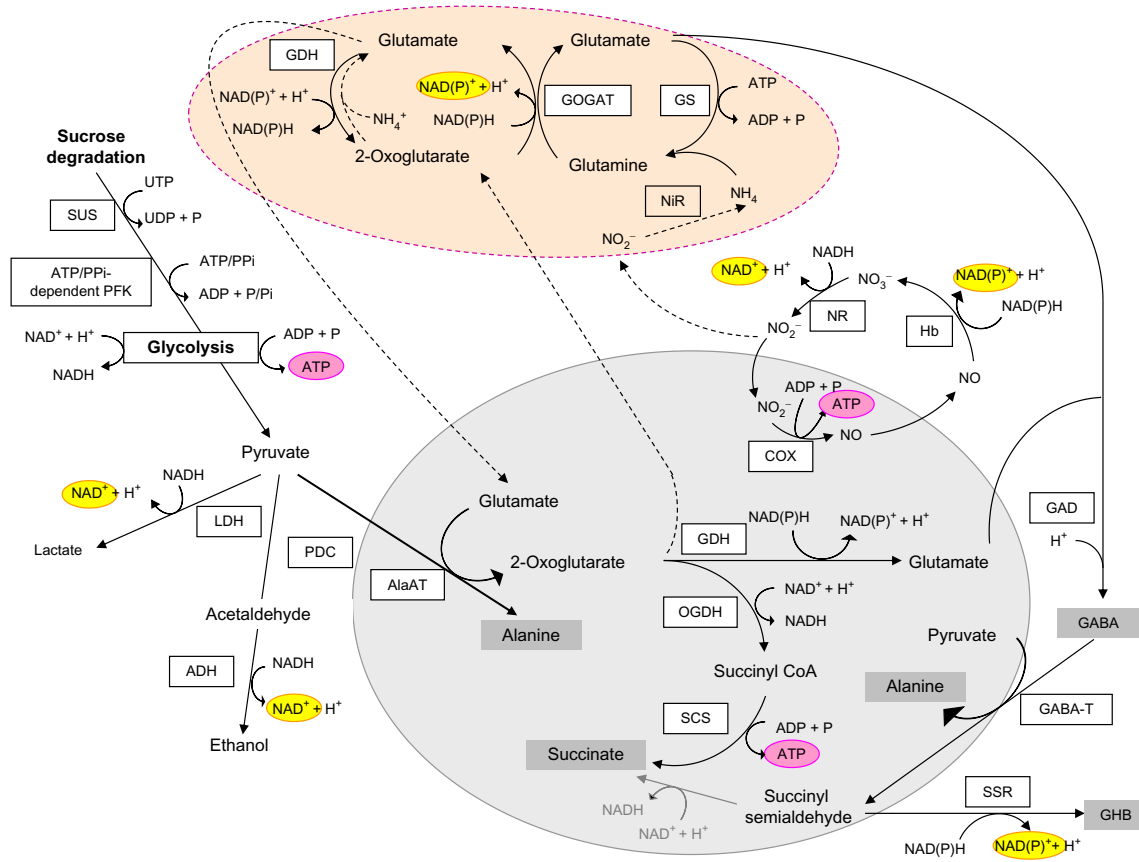


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demonstrated that in *Arabidopsis* roots, the inhibition of GAD and GABA-T was impaired (beyond the accumulation of GABA throughout the entire time of the experiment). Their study also saw the impairment of the accumulation of alanine in the second phase of the hypoxic experiment (beyond 4 h of hypoxia), while the initial rapid accumulation of alanine was not influenced by the GABA-shunt pathway. During oxygen-deficiency stress, a significant amount of succinic semialdehyde derived from the GABA-T activity is converted to GHB by means of the enzyme succinic semialdehyde reductase (SSR) (Breitkreuz et al., 2003; Deleu et al., 2013), which utilizes NADH contributing to redox regulation and providing NAD^+ , necessary for the glycolysis sustenance.

This raises the question: Which role might have GABA in the survival strategy of an anoxic cell? It is known that GABA has an important role contrasting the decrease in cytosolic pH and osmotic potential, contributing to the regulation of C:N balance, and hypothetically protecting cells from the oxidative stress (Banti et al., 2013; Bouché and Fromm, 2004; Bouché et al., 2003; Igamberdiev and Hill, 2009). In particular, it has been demonstrated (Shelp et al., 1999; Snedden et al., 1995, 1996) that GABA accumulates at low pH, GAD consumes a proton, and its activity is induced by cytosolic acidification. Moreover, accumulation of GABA is an adaptive strategy to prevent carbon loss and maintain osmotic potential, which falls as a consequence of the faster anaerobic carbohydrate consumption (Miyashita and Good, 2008; Reggiani et al., 2000).

Figure 5.3—Cont'd Metabolic reassessment during anaerobic stress. The alternative pathway responds to the energy crisis increasing ATP production and regenerating oxidizing potential. The scheme reports the main metabolic routes: enhanced sucrose degradation by means of sucrose synthase (SUS), sustained glycolysis by means of NAD^+ regeneration, fermentation, succinate accumulation, alanine and 2-oxoglutarate (2-OG) shunt and γ -aminobutyric acid (GABA) shunt, γ -hydroxybutyrate (GHB) accumulation, and enhanced GS-GOGAT cycle activity. Continuous lines indicate the followed paths during the stress; dashed lines indicate proposed active paths during the stress. Metabolites that accumulate during the stress (alanine, succinate, GABA, GHB) are written inside a dark grey squared box; newly synthesized ATP, newly regenerated NAD^+ and NAD(P)^+ are written inside elliptical-shaped boxes. 2-OG, 2-oxoglutarate; ADH, alcohol dehydrogenase; AlaAT, alanine aminotransferase; COX, cyclooxygenase; Hb, hemoglobin; GABA, γ -aminobutyric acid; GABA-T, GABA transaminase; GAD, glutamic acid decarboxylase; GDH, glutamate dehydrogenase; GHB, γ -hydroxybutyrate; GS-GOGAT, glutamate synthase cycle; LDH, lactate dehydrogenase; NiR, nitrite reductase; NR, nitrate reductase; OGDH, 2-oxoglutarate dehydrogenase; PDC, pyruvate decarboxylase; SCS, succinyl CoA ligase; SSR, succinic semialdehyde reductase; SUS, sucrose synthase.

As shown in Fig. 5.3, the GABA shunt might involve, indirectly, other metabolic pathways such as the GS/GOGAT (Bouché and Fromm, 2004; Reggiani et al., 2000).

In 2000, Reggiani and colleagues demonstrated that the use of inhibitors of the GS and GOGAT reactions significantly reduced the accumulation of alanine and GABA, proving that the GS/GOGAT cycle is necessary to assimilate nitrogen and produce those glutamate molecules that are the first step in the GABA-shunt reactions (Bouché and Fromm, 2004; Reggiani et al., 2000).

4.2. Alanine accumulation

As shown in Fig. 5.3, under anaerobic conditions, alanine production follows two possible different ways. Pyruvate can be converted to alanine by the transfer of an amino group derived from glutamate, which generates 2-oxoglutarate (2-OG) as a coproduct (Banti et al., 2013; Branco-Price et al., 2008). This reaction involves the Ala aminotransferase enzyme (AlaAT) since this enzyme is quickly induced under hypoxic conditions (Miyashita and Good, 2008; Muench and Good, 1994). However, in 2007 Miyashita et al. demonstrated that in anaerobic roots of *Arabidopsis*, AlaAT activity was not required for the alanine accumulation, suggesting the involvement of other routes which lead to alanine accumulation. In 2008, Miyashita and Good investigated the role of the GABA shunt in alanine accumulation, finding a relationship between the GABA-T activity and the alanine accumulation in hypoxic roots of *Arabidopsis*, in particular after the first 4 h from the start of the stress.

Alanine accumulation is known to be not toxic for the cells (Miyashita et al., 2007; Ricoult et al., 2005), to have a role in storing carbon and nitrogen, preventing their loss (Ricoult et al., 2005; Sato et al., 2002), and maintaining the osmotic potential (Miyashita and Good, 2008; Reggiani et al., 2000). Moreover, alanine production involves the generation of 2-OG (by means of the AlaAT activity) which can enter the partial (non-circular) TCA flux and proceed through the subsequent steps leading to ATP production by the substrate level phosphorylation. This metabolic adaptation may account for both alanine and succinate accumulation under hypoxia and anoxia.

4.3. Nitrate, nitrite, and NO: Hemoglobin/nitric oxide cycle

In anoxic root cells, nonsymbiotic plant hemoglobins (Hbs) have been demonstrated to modulate the nitric oxide (NO) produced and the amount of

cytosolic nitrate, to be involved in a metabolic process regenerating NAD^+ , and to sustain ATP synthesis due to the reduction of nitrite to NO in mitochondria (Igamberdiev and Hill, 2009; Igamberdiev et al., 2005; Stoimenova et al., 2007).

In addition to the oxygen limitation, the enhanced NO production is also responsible for the inhibition of cytochrome oxidase at the oxygen-binding site (Cooper, 2002; Dordas et al., 2003). Briefly, due to the quantities of NO and oxygen, cytochrome c oxidase (COX) is not operative in donating electrons to oxygen, and nitrite may act as an alternative electron acceptor at sites of complexes III and IV of the electron transport chain (Stoimenova et al., 2007). Both of these complexes are proton pumping, and NO formation by means of nitrite reduction seems to be responsible for the proton translocation. Since ATP production is a consequence of this proton pumping, as shown in Fig. 5.4, plant mitochondria are

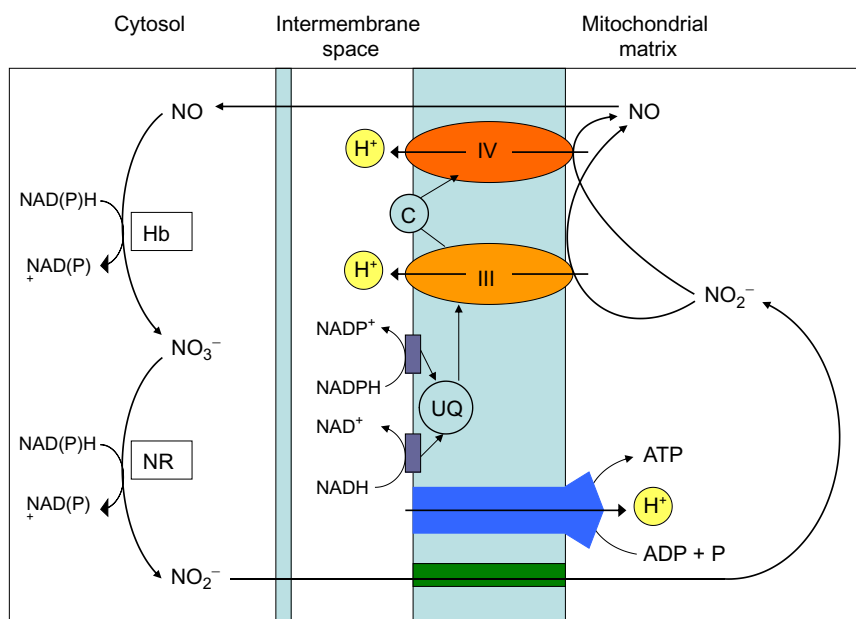


Figure 5.4 Anaerobic switch in plant mitochondria. Nitrite (NO_2^-) is the alternative electron acceptor at sites of complexes III and IV. The produced nitric oxide (NO) is converted to nitrate (NO_3^-) in the cytosol by means of hypoxically induced hemoglobin (Hb). Nitrate is then reduced to nitrite by nitrate reductase (NR) and then enters back the mitochondria. The cycle demonstrated to support the oxidation of NADH and NADPH and preserve the ATP production. C, cytochrome c ; Hb, hemoglobin; NR, nitrate reductase; Q, ubiquinone.

able to synthesize anaerobic ATP by means of NADH and NADPH as electron donors and with nitrite as a terminal electron acceptor. In this mechanism, cytosolic Hbs play a fundamental role reacting with NO formed in the mitochondria and diffused to the cytosol-generating nitrate (Igamberdiev and Hill, 2009). In the cytoplasm, nitrate can then be reduced to nitrite by nitrate reductase (NR) and nitrite can enter mitochondria, allowing the cycle to repeat.

Effectively, in hypoxic-tolerant rice subjected to oxygen-deficiency stress, NR has been shown to be upregulated at both transcriptional and enzymatic levels, while nitrite reductase (NiR) shows a very low induction (Mattana et al., 1994). Moreover, the mitochondrial COX-dependent reducing systems seem to be highly active, suggesting that most of the nitrite enters mitochondria (Planchet and Kaiser, 2006). Only a very small percentage of the nitrite generated by means of the Hb/NO cycle seems to be reduced to NH_4^+ , even though ammonia is known to be fundamental for enhanced amino acid production under anaerobic conditions (Igamberdiev and Hill, 2009).

In particular, in roots of highly tolerant species, it has been shown how the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle operates in order to reassimilate and incorporate ammonia into those metabolites typical of anaerobic conditions (Reggiani et al., 2000), and GS, Fd-GOGAT, and Fd-NADP⁺ were demonstrated to be newly synthesized during anoxic stress (Mattana et al., 1994, 1996, 1997).

4.4. Lactate and ethanol: Anaerobic fermentation

Under anaerobic conditions, since the oxidative phosphorylation pathway is inhibited, alternative pathways are necessary to regenerate NAD⁺ in order to sustain glycolysis. Fermentation is one of the first metabolic adaptations which has been studied extensively, demonstrating that two fermentative pathways, ethanolic and lactic fermentations, can regenerate NAD⁺ from NADH.

It has been suggested that cytosolic pH is the trigger which regulates the ratio between ethanolic fermentation and lactic fermentation: a decrease in cytosolic pH increases ethanolic fermentation and decreases lactic fermentation (Davies et al., 1974; Menegus et al., 1991). Also Perata and Alpi, in 1993, sustained the hypothesis that lactate biosynthesis prevails in the early phases of anaerobic metabolism, causing a cytoplasmic acidification which stimulates the activity of pyruvate decarboxylase (PDC) and alcohol

dehydrogenase (ADH) with the subsequent ethanol formation. It has also been specified that there is not—as is true for most of the adaptive metabolic changes—a common rule for all the plants; some species do not show lactic acid production before ethanol production and ethanolic fermentation are the only fermentation pathway (Andreev and Vartapetian, 1992).

More recently, in 2008, Dolferus and colleagues reported that *Arabidopsis* plants that overexpress lactate dehydrogenase (LDH) demonstrated increased root tolerance to anaerobiosis (Dolferus et al., 2008). Interestingly, these mutants showed a higher activity of PDC, confirming that in *Arabidopsis* roots, the ethanolic fermentation pathway also depends on lactate production (Dolferus et al., 2008). In *Arabidopsis* plants, four genes encoding PDC have been found, and Kürsteiner and colleagues, in 2003, demonstrated that PDC1 and PDC2 mRNA levels are induced during oxygen deprivation (Kürsteiner et al., 2003), while the quantity of PDC3 and PDC4 genes is not influenced significantly when the plant is under hypoxic stress (Mithran et al., 2014). Also in 2014, Mithran and colleagues reported that these different isoforms are organ-specific—PDC1 is root specific, while PDC2 seems to play its role in leaves.

Furthermore, it has been reported that an overexpression of PDC1 and PDC2 results in an enhanced tolerance to oxygen deficiency (Ismond et al., 2003), while the *pd1*-null mutants showed to worsen survival under the stress (Kürsteiner et al., 2003).

Concerning ADH, studies on both tolerant and intolerant species demonstrated that null or reduced ADH activity induces a higher sensitivity to the anaerobic condition. ADH knockout plants, *Arabidopsis adh1*-null mutants, rice with reduced ADH activity, and maize *adh1* mutants were in all of these cases more susceptible to anoxia (Ellis et al., 1999; Ismond et al., 2003; Jacobs et al., 1988; Johnson et al., 1994; Matsumura et al., 1995, 1998).



5. CONCLUDING REMARKS

Recent advances in plant strategies to survive anaerobic conditions gain knowledge about specific plant adaptive responses, increasing the understanding of how sensing, signaling, and thus response can quickly and successfully coordinate. The integrate regulation of molecular, metabolic, and physiological responses allows the plant survival even under strict low oxygen conditions.

Observation and deep analysis have highlighted different levels of the survival strategy, such as transcriptional and translational (i.e., TFs, such as

ERF VII and HSFs), posttranscriptional (i.e., polyribosomes), and posttranslational (i.e., NERP) regulation of specific genes involved in oxygen sensing and stress response triggering; accumulation of specific amino acids, such as alanine and γ -aminobutyric acid (GABA), succinate, and γ -hydroxybutyrate (GHB), belonging to a complex reassessment of metabolic pathways with a role in the maintenance of the osmotic potential and limiting cytosolic acidification; and a temporary role for nitrite, where it acts as an alternative mitochondrial electron acceptor in the electron transport chain for the maintenance of the mitochondrial activity and ATP synthesis during anoxic conditions.

All of these new topics become pieces of a complex puzzle, charting new courses to deeply explore and understand plant survival mechanisms under anaerobic stress.

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Beyond Expectations: Novel Insights into Epidermal Keratin Function and Regulation

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Abstract

The epidermis is a stratified epithelium that relies on its cytoskeleton and cell junctions to protect the body against mechanical injury, dehydration, and infections. Keratin intermediate filament proteins are involved in many of these functions by forming cell-specific cytoskeletal scaffolds crucial for the maintenance of cell and tissue integrity. In response to various stresses, the expression and organization of keratins are altered at transcriptional and posttranslational levels to restore tissue homeostasis. Failure to restore tissue homeostasis in the presence of keratin gene mutations results in acute and chronic skin disorders for which currently no rational therapies are available. Here,

we review the recent progress on the role of keratins in cytoarchitecture, adhesion, signaling, and inflammation. By focusing on epidermal keratins, we illustrate the contribution of keratin isotypes to differentiated epithelial functions.



1. INTRODUCTION

The cytoskeleton of most eukaryotic cell types comprises four major filamentous systems, microtubules, actin-based microfilaments, septins, and intermediate filaments (IFs) (Lazarides, 1980, 1982; Mostowy and Cossart, 2012; Steinert et al., 1984). IF proteins are encoded by a large gene family of ~70 members in mammals giving rise to α -helical proteins able to self-assemble into 10 nm wide IFs through the formation of obligatory or facultative heterodimers (Chung et al., 2013; Fuchs and Weber, 1994). Based on amino acid sequence composition, IF proteins are grouped into six different types (I–VI), or protein classes (Chung et al., 2013; Herrmann et al., 2009; Steinert et al., 1984) (Table 6.1). Type I and type II keratins (previously also called cytokeratins to name epithelial from hair-forming keratins) comprise the first two classes of IF proteins, respectively (Schweizer et al., 2006). The third group consists of vimentin, desmin, glial fibrillary acidic protein (GFAP), peripherin, and syncoilin (Clarke et al., 2010; Goldman et al., 2012; Middeldorp and Hol, 2011; Moorwood, 2008). Neurofilament triplet proteins (NF-I, NF-M, and NF-H) together with α -internexin, nestin, and synemin constitute type IV proteins, with nestin being expressed in many precursor cells (Beguín et al., 2012; Herrmann and Aebi, 2000; Lepinoux-Chambaud and Eyer, 2013). The last two groups harbor lamin proteins and two lens-specific proteins, respectively (Herrmann et al., 2009; Worman, 2012) (Table 6.1). Unlike other cytoskeletal proteins, IF proteins are differentially expressed during embryonic development and morphogenesis and upon tissue injury/regeneration, suggesting that IF proteins actively participate in these processes, a view that is confirmed by tissue-restricted pathology in human and animal disorders resulting from defects in IF genes (Bonne and Quijano-Roy, 2013; Herrmann et al., 2009; Lazarides, 1982; Simon and Wilson, 2011; Worman, 2012).

Of ~70 genes in the human genome that are coding for IF-forming proteins, 54 encode keratins. Thus, keratins evolved as the most comprehensive family of IF proteins (Schweizer et al., 2006). The total number of 54 mammalian keratins comprises 28 type I and 26 type II keratins, forming two clusters of 27 genes each on chromosomes 17q21.2 and 12q13, the gene

Table 6.1 Intermediate filament proteins are grouped into six different types

Class	Proteins	Size (kDa)	Site of expression
I + II	Type I keratins	40–60	
	K9–K28		Epithelial cells
	K31–K40		Hair cells/nail
	Type II keratins	50–70	
	K1–K8, K71–K80		Epithelial cells
	K81–K86		Hair cells
III	Vimentin	54	Cells of mesodermal origin
	Desmin	54	Muscle cells
	GFAP	50	Glial cells and astrocytes
	Peripherin	54	Peripheral and central neurons
	Syncoilin	64	Skeletal and cardiac muscle cells
IV	NF-L, NF-M, NF-H	62–112	Mature neurons
	α -Internexin	55	Neurons
	Nestin	177	Many precursor cells, neuroepithelial cells
	Synemin	173 (α) and 140 (β)	Muscle cells, mature neurons, immature and reactive astrocytes
V	Lamin A	74	Most differentiated somatic cells
	Lamin C	74	Most differentiated somatic cells
	Lamin C2	74	Germ cells
	Lamin A Δ 10	72	Unclear
	Lamin B1	66	Most or all somatic cells
	Lamin B2	68	Most or all somatic cells
	Lamin B3	53 (mouse)	Germ cells
VI	Phakinin	46	Lens epithelial cells
	Filensin	75	Lens epithelial cells

Classification is based on detailed amino acid sequence comparisons in higher vertebrates. Protein size refers to human proteins. If not indicated otherwise.

for the type I keratin K18 being located in the type II keratin gene domain (Hesse et al., 2001, 2004; Rogers et al., 2004, 2005; Schweizer et al., 2006). Like all IF proteins, keratins consist of a central α -helical rod domain of ~ 310 amino acids in length flanked by non- α -helical head and tail domains at their N- and C-termini, respectively, with the exception of the “tailless” K19 (Bader et al., 1986; Hanukoglu and Fuchs, 1983; Steinert et al., 1985). The rod domain comprises four segments with coiled coil-forming properties (1A, 1B, 2A, and 2B), separated by non- α -helical “linker” segments (L1, L12, and L2) and highly conserved motifs on both sides of the rod domain (also referred to as helix initiation and helix termination peptides, respectively), that are essential to mediate the assembly of tetrameric subunits into keratin intermediate filaments (KIFs) (Fuchs and Weber, 1994; Herrmann et al., 2003, 2009; Parry et al., 2007; Steinert et al., 1993) (Fig. 6.1).

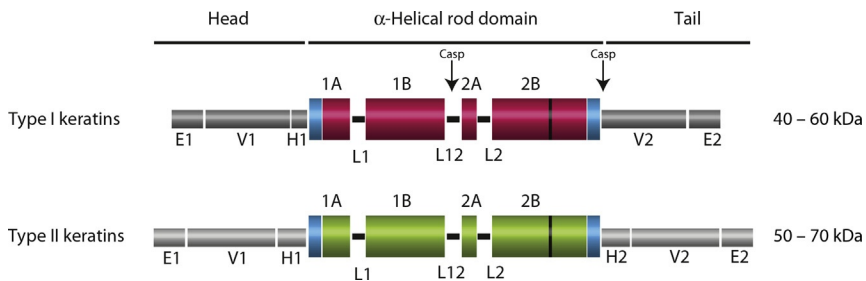


Figure 6.1 Schematic representation of type I and type II keratins. The rod domain of type I and type II keratins is highly conserved and consists of ~ 310 amino acids. It comprises four segments with coiled coil-forming properties (1A, 1B, 2A, and 2B), separated by non- α -helical “linker” segments (L1, L12, and L2). The boundaries of the rod domain (also referred to as helix initiation/helix termination motif) are highly conserved (15–20 amino acids). In both type I and type II keratins, the α -helical rod domain is flanked by nonhelical head and tail domains on the N- and C-terminus, respectively. The head and tail domains of type I and type II keratins are made up of three subdomains (H, high homology; V, special variability; E, highly charged termini) and are highly heterogeneous. In type I keratins, the H1 domain is shorter and the H2 domain is completely missing. The V1 and V2 domains are highly variable in size. The E1 and E2 subdomains have been conserved in the same keratin of different species but are highly variable between different keratins. All keratins contain within their 2B domain a so-called stutter region (black). Note that only type I keratins contain two caspase (Casp) cleavage sites. For K18, the apoptosis-specific neoepitope resulting after cleavage by caspase-9 is recognized by a specific antibody, M30 (Leers et al., 1999; Schutte et al., 2009). The antibody M65 measures soluble K18 in cell culture supernatants or blood samples, reflecting the amount of epithelial cell death. Therefore, the M30:M65 ratio indicates the proportion of apoptosis compared to total cell death (Ausch et al., 2009).

Although all keratins resemble each other in their overall organization, their head and tail domains are highly variable, for example, the V2 domain can vary between 0 and more than 100 residues from the smallest to the largest known keratins (Steinert and Roop, 1988) (www.interfil.org). Furthermore, the α -helical domains among keratins of one group share 50–99% sequence identity, whereas keratins of opposite type display only ~30% homology in these regions (Fuchs and Weber, 1994). These distinct features of keratins suggest that they might be preferentially involved in specific interactions with associated proteins (Roop et al., 1985; Steinert et al., 1985) (Fig. 6.1). While there is currently limited information about associated proteins binding exclusively to only one keratin isotype, *in vitro* data have revealed different affinities of type I and type II keratin complexes to each other (Hatzfeld and Franke, 1985; Hofmann and Franke, 1997). Thereby, keratin expression pairs endow epithelial cells with unique micro-mechanical properties.

Keratins assemble first into obligate, parallel heterodimers, next into anti-parallel tetramers, and through lateral and longitudinal interactions of tetramers into long, nonpolar IFs (Coulombe and Fuchs, 1990; Hatzfeld and Weber, 1990; Herrmann et al., 2007; Steinert, 1990) (Fig. 6.2). The head is crucial for interdimer association and filament assembly, whereas the tail may regulate filament diameter and bundling (Hatzfeld and Burba, 1994; Herrmann et al., 2009; Lee and Coulombe, 2009). In cultured cells, the assembly of KIFs into oligomeric particles starts in the periphery of the cell at focal adhesions, followed by integration into existing keratin networks and further bundling (Leube et al., 2011). However, the lack of X-ray structures of keratins and their highly insoluble nature under *in vitro* conditions have so far been major obstacles to resolve assembly stages beyond the tetramer. Given that *in vitro* keratins assemble at low nanomolar concentrations, this suggests that *in vivo*, posttranslational modifications and/or binding to regulatory proteins represents a default pathway to allow keratin assembly at distinct sites.

Keratinocytes are the predominating cells of the human skin, the largest organ of our bodies that maintains a barrier between the organism and its environment. The epidermal barrier is indispensable for the protection of the organism against mechanical injury, dehydration, infections, and other forms of stress. Together with Langerhans cells, the epidermal barrier has a major role in regulating immune responses (Simpson et al., 2011). Keratins are among the most abundant structural proteins in the cytoplasm of epithelial cells and a single keratinocyte can express between 3 and

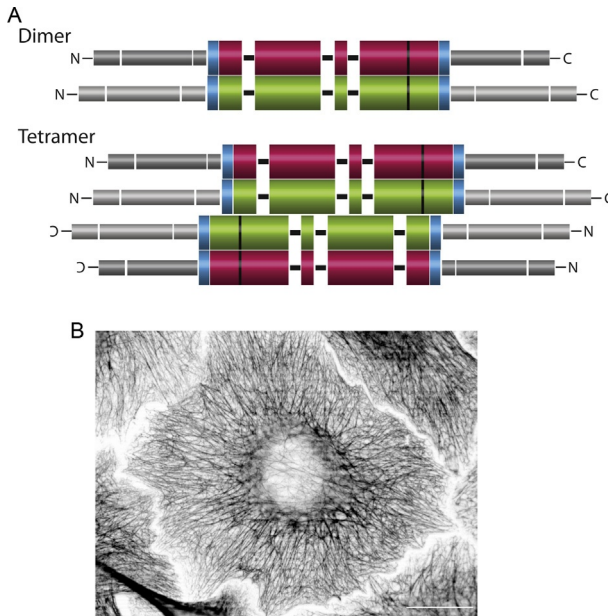


Figure 6.2 (A) Schematic representation of early keratin filament assembly stages and organization of keratin cytoskeleton. Heterodimers formed by pairing of a type I and a type II keratin are the first building blocks of a keratin filament. Heterodimers then form tetramers, which finally build long, nonpolar IF through lateral and longitudinal interactions. The exact way of how tetramers assemble remains unsolved, yet this figure shows the, to our knowledge, most plausible model. (B) Organization of keratin cytoskeleton in cultured mouse keratinocytes visualized by K14 antibody staining. Note the bundled organization of keratin filaments in the perinuclear region. Scale bar: 10 μm .

≥ 10 different “epidermal” keratins (Coulombe and Lee, 2012; Simpson et al., 2011). The expression of keratins is highly dependent on the state of development and differentiation, varies within different types of epithelia, and is changed upon injury or disease (Coulombe and Lee, 2012; Moll et al., 2008; Simpson et al., 2011). Like no other tissue, the epidermis illustrates how exquisite keratin isotype expression relates to structural and regulatory keratin functions.

In this chapter, we focus on recently discovered functions of epidermal keratins and discuss their relevance for epidermal differentiation, pathogenesis, and regeneration. When appropriate, we will discuss simple epithelial keratins to underscore general principles. For hair keratins, we refer to the recent excellent reviews (Langbein and Schweizer, 2005; Langbein et al., 2004).



2. CELL TYPE-SPECIFIC EXPRESSION OF KERATINS

Like in other epithelia, keratin expression strictly correlates with the state of differentiation. In skin epithelia, two-thirds of known keratin genes are expressed, and in a keratinocyte of stratified squamous epithelia, the keratin proteins account for up to 30–40% of the total protein (Coulombe and Lee, 2012; Nelson and Sun, 1983).

The epidermis is composed of four main layers, which undergo programmed differentiation as the skin continuously renews from stem cells (Cotsarelis, 2006; Potten and Morris, 1988; Simpson et al., 2011; Tumber et al., 2004). Attached to the extracellular matrix (ECM) through hemidesmosomes and integrin-based adhesions lies the basal, proliferative layer of the epidermis (Simpson et al., 2011; Tsuruta et al., 2011). This compartment expresses the keratin pair K5/K14, organized in loose bundles that extend from hemidesmosomes and desmosomes throughout the cytoplasm of highly prismatic basal keratinocytes. Upon terminal differentiation, keratinocytes move upward in the epithelium, and the keratin pair K5/K14 is sequentially replaced by the expression of another keratin pair, K1 and K10 in the suprabasal compartment, along with a dramatic flattening of keratinocytes and reorganization of tightly bundled keratins that run parallel to the cell surface (Fuchs and Green, 1980; Reichelt et al., 2001; Simpson et al., 2011). Depending on regional differences in the epidermis, this default pattern varies.

At sites of high mechanical strain, the keratin pair K1/K10 is supplemented by the expression of additional keratins K2e and K9 at the palms of hands and soles (Candi et al., 2005; Moll et al., 2008). Being among the first proteins expressed during cornification, the keratin pair K1/K10 is considered as an essential protein scaffold that directs the sequential deposition and cross-linking of cornified envelope proteins (Candi et al., 2005). As we will discuss later, the view that K1/K10 is a major contributor to barrier integrity and functionality is strongly supported by recent data. Epidermal injury triggers the rapid induction of K6, K16, and K17 at the wound edge, at the expense of K1/K10, to repair tissue function. This correlates with striking changes in keratin organization, morphological changes, and functional properties of activated keratinocytes (Kim et al., 2006; Reichelt and Magin, 2002; Wong and Coulombe, 2003). Notably, K6, K16, and K17 are also found to be expressed in the hair follicle and nail (De Berker et al., 2000). Epidermal stem cells are located in protected niches, such as the bulge

of the hair follicle, and are characterized by the expression of K15 (Goldstein and Horsley, 2012; Lyle et al., 1998; Watt, 1998). The vibrissae bulge harbors two types of slow-cycling stem cells, characterized by the expression of K5/K15/K17/K19 and K5/K17, respectively, that display loose keratin bundles in the former and tight bundles in the latter configuration (Larouche et al., 2008). Notably, both subpopulations lack K14 expression (Larouche et al., 2008). In view of the recent data, showing that differentiating keratinocytes are also capable of expressing K15 mediated through PKC (protein kinase C) signaling, a role of K15 as a stem cell marker has come into question (Bose et al., 2013). Neurosensory Merkel cells, which localize close to basal keratinocytes within the skin, build their IF cytoskeleton from “simple epithelial-type” keratins K8, K18, K19, and K20 (Moll et al., 1995; Sidhu et al., 2005). All these mentioned settings highlight how distinct epithelial cell populations are characterized by unique keratin expression profiles. This raises the question how this tight regulation of keratin expression can be accomplished and to what extent distinct keratins or keratin pairs contribute to context-specific functions.

2.1. Transcriptional regulation of keratin expression

The expression of keratins and other structural proteins and transcription factors according to the respective compartment and/or setting is tightly regulated, primarily at the transcriptional level (Ma et al., 1997; Sinha et al., 2000). By cotransfecting constructs expressing transcription factors with promoter constructs of K5 and K14, the activation by c-Fos and c-Jun components of activator protein 1 (AP-1), but not Fra1, was shown, and both promoters were suppressed by NF- κ B proteins, especially p65 (Ma et al., 1997). In this setting, the promoter of K17 was activated by c-Jun and the K6 promoter by all AP-1 proteins and also by p65 (Ma et al., 1997). Also, most promoters of epidermally expressed genes contain binding sites for AP-2 transcription factors, but the AP-2 element of the K5 promoter was shown to be indispensable for tissue-specific expression (Sinha et al., 2000). Furthermore, an epidermis-specific enhancer containing AP-1, AP-2, and ETS sites, being necessary and sufficient for the expression of keratinocyte-specific genes, could be identified (Sinha et al., 2000). An isoform of the transcription factor p63 (TAp63 α) is involved in inducing the expression of K14 during epidermal morphogenesis, while another isoform (Δ Np63 α) is suggested to play a role in maintaining K14 expression, supported by the fact that K14 expression is not impaired by downregulation

of p63 in mature epidermis (Candi et al., 2006; Keyes et al., 2005; Koster and Roop, 2007; Koster et al., 2006). This regulation of K14 is accomplished by direct interaction or can be mediated by AP-2 γ , a transcription factor needed to initiate the expression of K14 and being itself induced by TAp63 α (Koster et al., 2006). The expression of K1 in suprabasal cells was partially dependent on Δ Np63 α , interfering with the Notch pathway (Nguyen et al., 2006). Unlike K1 and K10, K6, K16, and K17 are quickly and reversibly regulated upon epidermal injury including oxidative stress, suggesting the presence of Nrf2 transcription factor binding sites (Kerns et al., 2007, 2010). In fact, the Nrf2 inducer sulforaphane rapidly induced the expression of the type I keratin genes *K16* and *K17* in the epidermis (Kerns et al., 2007). A follow-up study based on cultured keratinocytes indicated at least two mechanisms responsible for the upregulation of these keratins. First, the sulforaphane treatment resulted in decreased intracellular glutathione levels, coinciding with activated MAP kinases and AP-1, prompting transcription of K17. As a late effect of stimulation with sulforaphane, activated Nrf2 induced K16, additionally supported via the MAP kinase–AP-1 axis (Kerns et al., 2010; Roth et al., 2012a). These findings suggest that the highly complex regulation of keratin expression is rather accomplished by an interplay of different transcription factors. A topic not yet resolved is the pairwise regulation of type I and type II keratins. The ongoing transcription of type I keratin genes in the absence of the entire type II keratin gene cluster argues that transcription of corresponding type I keratin genes is independently regulated (Bar et al., 2014; Vijayaraj et al., 2009).



3. INTERACTION OF KERATINS WITH ASSOCIATED PROTEINS

More than three decades ago, E. Lazarides suggested that IFs “integrate mechanically the various structures of the cytoplasmic space in a way that is tailored to the differentiated state of the cell” (Lazarides, 1980). Here, we discuss how much the recent analysis of epidermal keratins supports both mechanical and regulatory keratin functions through diverse interactions and regulatory mechanisms.

3.1. Keratinocytes attach to the basal membrane via hemidesmosomes

The attachment to the basal membrane, a sheet of ECM proteins, is accomplished by type I hemidesmosomes, a complex protein junction found in

stratified epithelia like the skin, mouth, and esophagus of mammals, whereas type II hemidesmosomes predominate in simple epithelia (Margadant et al., 2008; Tsuruta et al., 2011; Zhang and Labouesse, 2010). In addition to hemidesmosomes, focal adhesions mediate ECM adhesion (Jones et al., 1998; Tsuruta et al., 2011). The 180 kDa bullous pemphigoid antigen (BP180/BPAG2) and $\alpha 6\beta 4$ integrin directly interact with laminin-332, thereby mediating the indirect linkage to the K5/K14 cytoskeleton via the 230 kDa bullous pemphigoid antigen (BP230/BPAG1e) and plectin, both members of the plakin family, in contrast to focal contacts, which are found in cultured keratinocytes and are linked to the actin cytoskeleton (Tsuruta et al., 2011). Type II hemidesmosomes display a slightly altered composition and are linked to K8/K18 filaments (Zhang and Labouesse, 2010). Hemidesmosomes of *Caenorhabditis elegans* display features similar to vertebrate hemidesmosomes and are associated with IFA-2/MUA-6, IFA-3, and IFB-1, belonging to the IF family of *C. elegans* (Zhang and Labouesse, 2010). In this model organism, it was shown that hemidesmosomes act as mechanosensors and transduce signaling processes (Zhang et al., 2011). The mechanotransduction pathway between body-wall muscles and the epidermis in this model organism involves a Rac GTPase and the proteins p21-activated kinase, G protein-coupled receptor kinase interactor, and PAK-interacting exchange factor, which are found at the hemidesmosomes (Zhang et al., 2011). Thus, hemidesmosomes are sites through which the IF cytoskeleton reacts on mechanical forces.

By transfecting mammalian cells with truncated forms of plectin, it was shown that a distinct linker region interrupting repeats in the plectin end domain plays a crucial role in binding to IFs (Karashima et al., 2012). The S4642 residue, possibly equivalent to S2849 in desmoplakin, plays an important regulatory role in binding plectin to IF. The phosphorylation of S4642 was associated with a weakened binding to IF and found to be enhanced during wound healing, a setting characterized by weaker cell matrix adhesion of keratinocytes (Bouameur et al., 2013). Sequences within the segments 1A–2A of the central rod domain of type III IFs desmin and vimentin are required for the interaction with plectin (Favre et al., 2011). This seems to be in accordance with the rod domain being the most conserved domain among IFs, in such that the binding of proteins functionally involved in the same processes also remains conserved among IF proteins. Also, this suggests that the binding of plectin to keratin might involve the central rod domain of keratins, in addition to plectin's C-terminus. Mice lacking plectin die within few days after birth, exhibiting skin blistering

due to the disruption of basal keratinocytes, along with skeletal and cardiac defects (Andra et al., 1997). Analyzing plectin-deficient basal keratinocytes suggests a role for plectin in organizing the keratin network, such that in the absence of plectin, cells display less tightly bundled keratin filaments at attachment sites to the hemidesmosome, along with a cytoprotective role against osmotic stress (Osmanagic-Myers et al., 2006). In human, a form of the skin-blistering disease epidermolysis bullosa simplex (EBS) is caused by defects in plectin expression, which underlines the role for plectin in tissue integrity (Winter and Wiche, 2013). Conversely, the absence of keratins induces relocalization of plectin away from hemidesmosomes into a more diffuse cytoplasmic distribution in keratinocytes. Thus, keratins may act upstream of plectin to regulate hemidesmosome composition and adhesion (Seltmann et al., 2013b).

In addition to plectin, distinct subdomains of the BP230 tail domain mediate interactions with K5/K14 but not with K8/K18, on the basis of yeast three-hybrid interaction data (Fontao et al., 2003). Through these interactions, keratins affect $\alpha 6\beta 4$ integrins and subsequently keratinocyte migratory behavior (Sehgal et al., 2006; Tsuruta et al., 2011).

3.2. The interdependence of keratins and desmosomes

Cell–cell contacts in the epidermis are mediated by cadherin-based junctions, like adherens junctions and desmosomes, which assemble in lateral membrane domains of adjacent keratinocytes (Simpson et al., 2011). Epidermal adherens junctions are composed of E- and P-cadherin, intracellularly interacting with p120 catenin, α - and β -catenins, and additional proteins, which provide links to the actin cytoskeleton (Simpson et al., 2011). The strong intercellular adhesion crucial for force-bearing tissues like the epidermis is provided by desmosomes that mediate adhesion with the epithelial keratin cytoskeleton.

Desmosomes, highly organized and dynamic junctions, comprise desmogleins and desmocollins (mainly Dsg1/Dsc1 and Dsg3/Dsc3), which bind to plakoglobin and plakophilins (Green and Simpson, 2007; Kitajima, 2013). Desmoplakin, like plectin (a plakin family member), links the keratin cytoskeleton to the desmosome, thereby tethering the IFs to the plasma membrane and highly contributing to mechanical integrity of the tissue (Delva et al., 2009; Green and Simpson, 2007). The domain interactions between desmoplakin and keratins are not fully resolved. As for desmoplakin, the C-terminus (DPCT) is involved in the attachment of

keratins to the desmosome (Stappenbeck and Green, 1992; Stappenbeck et al., 1993). Additionally, the phosphorylation of the aforementioned desmoplakin serine residue (S2849 of the human protein), located in the keratin-binding site of desmoplakin, decreases the interaction with keratins (Stappenbeck et al., 1994). On the side of keratins, the head domains of type II epidermal K1, K2, K5, and K6 were assumed to play a role (Kouklis et al., 1994; Meng et al., 1997). While the type II keratin K1 alone interacted with DPCT in a two-hybrid screen, K8 and K18 did not so (Meng et al., 1997). Cotransfecting of K8 and K18, typical of simple epithelia, suggested that both were required for the interaction, indicating the necessity for the heterodimer to interact with the DPCT (Meng et al., 1997). Using desmoplakin constructs lacking part of the C-terminus, the earlier-mentioned interactions with K1 or K8/K18 failed (Meng et al., 1997). Another study shows the heterodimeric association of K5/K14 to be necessary to interact with DP and claims the head and tail domains of K5/K14 to be dispensable for this interaction, indicating dependence on the tertiary structure of the keratin heterodimer and recognition sites within the rod domain (Fontao et al., 2003). This is in line with the previously discussed binding of plectin to the rod domain of type III IF proteins (Favre et al., 2011).

To address the interdependence of keratins and desmosomes *in vivo*, gene knockout studies were performed. In DP^{-/-} mice, the keratin filament appeared disorganized, assumingly due to the decreased number of desmosomes found in these mice and due to impaired attaching to the IFs in remaining desmosomes (Gallicano et al., 1998). Loss of DP in extraembryonic tissues or in the epidermis causes a collapse of the keratin cytoskeleton and weakened intercellular adhesion, suggesting a dependence of keratins on desmosomes (Gallicano et al., 1998; Vasioukhin et al., 2001). Conversely, in several keratin knockout mice, desmosomes were affected only to a limited extent (Hesse et al., 2000; Magin et al., 1998; Roth et al., 2012c; Vijayaraj et al., 2009; Wallace et al., 2012). In view of compensatory keratin expression, mice and keratinocytes lacking the entire keratin cytoskeleton are now shedding new light on the requirements of keratins for desmosome formation, maintenance, and adhesive strength. Keratinocytes lacking all keratins show destabilization of desmosomes, as a result of PKC- α -mediated phosphorylation of desmoplakin (Kroger et al., 2013). The activity of PKC- α was found to be regulated by an interaction of keratin with Rack1, a protein involved in the spatiotemporal regulation of PKC isoforms (Kroger et al., 2013). Without keratins, desmosomes assembled but were endocytosed at

accelerated rates, accompanied by weaker intercellular adhesion as revealed by the application of force to epithelial sheets (Kroger et al., 2013). *In vivo*, the lack of keratins was accompanied by significantly fewer and smaller desmosomes in all epidermal compartments. In this setting, desmosomal proteins accumulated in the cytoplasm. Remarkably, intercellular splits, likely resulting from weakened intercellular adhesion, were also noted in the skin of keratin-deficient mice (Bar et al., 2014). Although not yet verified in the presence of disease-causing keratin mutations, these data implicate more complex pathomechanisms acting in keratinopathies, with diminished intercellular adhesion playing a significant role. Analyzing a human keratinocyte cell line derived from one EBS patient (carrying a K14-R₁₂₅P mutation) revealed downregulation and mislocalization of junction proteins desmoglein 3, desmoplakin, and plakoglobin (Liovic et al., 2009). The role of desmoglein 1 and/or 3 in maintaining intercellular adhesion and tissue integrity is underscored in patients suffering from pemphigus vulgaris, a human blistering disease caused by autoantibodies against the respective proteins, manifesting with blisters and erosions on the skin (Amagai and Stanley, 2012; Koga et al., 2013). Treating primary keratinocytes with pemphigus autoantibodies leads to a p38 mitogen-activated protein kinase (MAPK)-mediated activation of EGFR (EGF receptor), another key player in regulating epidermal homeostasis, highlighting the contribution of aberrant EGFR signaling in various skin diseases and human tumors (Bektas et al., 2013; Nanba et al., 2013). In addition to EGF, insulin signaling impacts on cell adhesion and links adhesion to cell growth in a context-dependent manner. The authors reported that phosphorylation of the plakophilin 1 head domain was induced via the PI3K-AKT pathway (Hatzfeld et al., 2014; Wolf et al., 2013). This phosphorylation resulted in a cytoplasmic accumulation of plakophilin 1, going along with reduced intercellular adhesion and increased protein biosynthesis (Wolf et al., 2013). These data support the view that the deregulation of plakophilin 1, as observed in several tumors, directly contributes to hyperproliferation and carcinogenesis in a context-dependent manner. It is tempting to speculate that IGF and EGF signaling pathways, in addition to regulating desmosomal adhesion, also regulate the interaction of desmosomal components with keratins. Also, it remains to be resolved how different keratin isotypes contribute to desmosomal adhesion. This will be interesting as the coherence of desmosomal adhesion impacts on settings like wound healing or tumorigenesis.

3.3. A role of keratins in cornified envelope formation and function

The cornified envelope, a layer of $\sim 10\text{--}15$ nm thickness, is the ultimate differentiation product of epidermal keratinocytes composed of enucleated corneocytes to which a special set of lipids are covalently bound (Candi et al., 2005; Rice and Green, 1977; Steinert and Marekov, 1995). To function as an effective biological barrier, tight junctions sealing granular keratinocytes and skin-resident immune cells have to act in concert (Niessen, 2007; Simpson et al., 2011).

Initiation of terminal differentiation comprises the expression of the keratin pair K1/K10, followed by keratohyalin granules, consisting of profilaggrin, which is further processed to filaggrin monomers through specific proteolysis and dephosphorylation events (Candi et al., 2005; Resing et al., 1985, 1993). In this setting, keratin filaments together with filaggrin serve as a scaffold for the subsequent cross-linking of envoplakin, periplakin, and epiplakin, followed at later stages by involucrin, loricrin, trichohyalin, hornerin, late cornified envelope proteins, and small proline-rich proteins (Candi et al., 2005). These are believed to reinforce the cornified envelope, which is then further supported by covalent attachment of lipids (including ω -OH ceramides) that are a major contributor to epidermal barrier function (Candi et al., 2005). The generation of loricrin KO mice unexpectedly showed a rather mild phenotype, which led to the suggestion of a compensatory effect by the upregulation of other proteins of the cornified envelope (Koch et al., 2000). Knocking out involucrin in mice also had no obvious effect on the cornified envelope or epidermal morphology, but did not result in a compensatory upregulation of other components involved in the formation of the cornified envelope (Djian et al., 2000). At the same time, mice deficient in filaggrin showed dry scaly skin and altered barrier integrity, but no change in involucrin, loricrin, and K1 levels (Kawasaki et al., 2012).

In epithelial cells, six of nine members of the transglutaminase (TG) family are expressed, and three TGs are involved in cross-linking type II keratin chains (K1, K2e, and K5) at a specific lysine residue (K73 in K1) found in a conserved 22-residue window in the V1 domain of type II keratins (Candi et al., 1998, 2005; Kimonis et al., 1994; Lorand and Graham, 2003). This results in covalent cross-linking of significant amounts of K1, but not of K10 to the cornified envelope, contributing to its function (Candi et al., 2005; Roth et al., 2012c). Supporting the reliance of the cornified envelope on keratins, a missense mutation in the mentioned lysine residue affects the cornified envelope (Candi et al., 1998, 2005; Kimonis et al., 1994). TG1,

being mainly expressed in keratinocytes, becomes overexpressed during terminal differentiation and enables direct linking between the protein and lipid component of the cornified envelope (Candi et al., 2005). A disease known as lamellar ichthyosis manifesting with large dark gray or brownish thick scales covering the entire body is provoked by mutations in the gene encoding TG1 (Lorand and Graham, 2003; Terrinoni et al., 2012). The necessity for TG1 activity in the formation of the cornified envelope is further underlined by TG1 $-/-$ mice that show a defect in cell envelope assembly and significant impairment in skin barrier function, resulting in early neonatal death (Matsuki et al., 1998). TG2 is only found in the basal layer of the epidermis, plays a role for the organization of the ECM, cross-links anchoring fibrils of the dermoepidermal junction, and is believed to play a role in wound healing, but is not involved in cornification (Candi et al., 2005; Lorand and Graham, 2003). TG3 and TG5 are both expressed in differentiating keratinocytes and thus involved in cornification and cornified envelope assembly, respectively (Candi et al., 2005).

In the fully established cornified envelope, keratins have become covalently linked constituents of an insoluble network of the previously mentioned proteins and contribute to its barrier function (Candi et al., 2005). The epidermal barrier further relies on the formation of tight junctions, being build up from claudins, occludins, tricellulin, and intracellularly localized proteins of the zona occludens family (Niessen, 2007). Also, desmosomal components, desmoglein 1 and desmocollin 1 together with corneodesmosin, are cross-linked to the cornified envelope, resulting in the formation of corneodesmosomes, which link cornified cells and further support barrier function (Kitajima, 2013). In addition to covalently bound lipids and tight junctions, an immunologic barrier is provided by the Langerhans cells, which are the sole dendritic cells in the epidermis and play a key role in various inflammatory contexts (Clausen and Kel, 2010; Igyarto and Kaplan, 2013). The cross talk among Langerhans cells and keratinocytes in parts relies on the keratin-dependent release of cytokines MCP-1/CCL2, MIP-1 β /CCL19, and MIP-1 α /CCL20, all regulated by NF- κ B, from keratinocytes (Roth et al., 2009).



4. POSTTRANSLATIONAL MODIFICATIONS OF KERATINS

Like for many other proteins, posttranslational modifications are major regulators of keratin organization and functional properties. Such changes are likely to affect the assembly of KIFs, their intracellular

organization, or the interaction with associated proteins, like the components of desmosomes or the cornified envelope. The best-known keratin modifications are phosphorylation, O-glycosylation, sumoylation, and ubiquitination, whereas so far, functional analysis of phosphorylation sites has been concentrated on the head and tail domains (Omary et al., 2006, 2009; Roth et al., 2012a; Snider et al., 2011). The recent analysis, however, identified numerous phosphorylation sites also throughout the keratin rod domains, forming the basis for future analyses (www.phosphosite.org). This goes in line with various sites throughout all domains in type I and type II keratins predicted to be subjected to O-glycosylation (Roth et al., 2012a).

4.1. Phosphorylation of keratins as adaptation to distinct settings

Through posttranslational modifications, the function of single keratins can be adapted to different conditions during development or disease. In view of the well-known reorganization and dynamic behavior of keratins during wound healing and carcinogenesis/metastasis, phosphorylation by distinct kinases is likely to alter keratin organization to promote these processes. Notably, some phosphorylation sites are conserved among IF proteins, suggesting also a functional conservation, and phosphorylation is targeted by multiple signal cascades (Omary et al., 2006; Snider and Omary, 2014). Among keratins, there is less information available for posttranslational modifications of the epidermal keratin pair K5/K14 due to a higher insolubility of the proteins compared to the keratin pair K8/K18, which is the main keratin pair of simple epithelial cells like hepatocytes (Karantza, 2011).

For the simple keratin pair K8/K18, many *in vivo* phosphorylation sites, including K8-S23/S73/S431 and K18-S33/S52, have been reported (Omary et al., 1998). K8/K18 hyperphosphorylation correlates with mitosis and many settings of stress, like disease progression in patients with chronic liver disease (Ku and Omary, 2006; Roth et al., 2012a). A protective role for phosphorylation of the conserved K8-S73 in hepatocytes was supported by the finding that mice showed increased susceptibility to liver injury after expression of nonphosphorylatable K8 mutants (Ku and Omary, 2006). Therefore, a role for this conserved residue in K8 and also in other keratins as a phosphate “sponge” for stress-activated kinases was suggested, thereby preventing excess phosphorylation of other kinase targets (Ku and Omary, 2006). As participating kinases, p38 MAPK and MK2/3 could be shown to specifically phosphorylate type II and type I keratins, respectively (Menon et al., 2010). The epidermal keratins K5 and K6b were shown to be

phosphorylated *in vitro* by p38 kinase on threonine and serine residues in a conserved motif, leading to increased solubility (Toivola et al., 2002). Also, phosphorylation of keratins is observed along with different biological processes, such as specific stages of mitosis in which a phosphorylation of K5-T150 and K6-T145 is observed, in specialized compartments of the cell or upon apoptosis-associated cell stress (Omary et al., 2006; Toivola et al., 2002). Further supporting the correlation of stress and keratin phosphorylation is the disassembly of keratin IF mediated by activated PKC- δ selectively phosphorylating K8-S73 upon application of shear stress in alveolar epithelial cells (Ridge et al., 2005). In general, the hyperphosphorylation of keratins results in weakened interactions between keratins, such that phosphorylated proteins enter a nonfilamentous soluble fraction that leads to either reentering into IF or degradation (Roth et al., 2012a). Phosphorylation of K20-S13 occurs specifically in mucus-secreting goblet cells, with phosphorylation further increasing in enhanced mucus secretion upon starvation, but not in other K20-expressing enterocytes, which underlines the impact of keratin modifications for specialized cell functions (Omary et al., 2006; Zhou et al., 2006). The interaction of keratins with members of the 14-3-3 family of adapter proteins has also been shown to depend on phosphorylation on distinct residues in the keratin proteins (Omary et al., 2006; Pan et al., 2013). It was reported that K17-S44 is phosphorylated upon stimuli altering cell growth, resulting in a relocalization of 14-3-3 σ from the nucleus to the cytoplasm, which promotes activity of the mTOR pathway (Kim et al., 2006; Pan et al., 2011). Furthermore, phosphorylated K18-S33 regulates binding to 14-3-3 proteins during mitosis and hence impacts on keratin filament organization (Ku et al., 2002).

4.2. Other posttranslational modifications altering keratin function

Next to phosphorylation, other chemical modifications of keratin proteins that supposedly alter distinct functions of keratins have been identified. Glycosylation via O-linked *N*-acetylglucosamine occurs in K13, K8, and K18, the latter being modified on S30, S31, and S49 (Omary et al., 1998, 2009). These O-glycosylation sites of K18 are probably residues not phosphorylated and are involved in promoting the phosphorylation and activation of cell-survival kinases and therefore serve a protective role in epithelial injury (Ku et al., 2010). Notably, it was shown that Akt1 is able to associate with K8, but not with K18, and the interaction of K8/K18 with Akt1 did

not depend on keratin glycosylation (Ku et al., 2010). A bioinformatical approach predicted O-glycosylation sites for 13 type I and 15 type II keratins, with the majority of predicted sites being found in the head and tail domains (Roth et al., 2012a). It will be important to study the effect of keratin glycosylation on mechanical and regulatory keratin properties and link its regulation to known signaling pathways.

The acetylation of lysine residues is a highly conserved, reversible post-translational modification resulting in the neutralization of the positive charge and occurring in a broad range of proteins, thereby changing protein function and regulating various biological processes (Choudhary et al., 2009). Acetylation of K8 at the highly conserved K207 regulated filament organization and decreased keratin solubility and a site-specific phosphorylation change was found upon the inhibition of K8–K207 acetylation (Snider et al., 2013). Acetylation sites in the K8 protein have been reported to be found not only within the head and tail domains but also within the coiled-coil domains (Drake et al., 2009). In contrast to keratin acetylation, the function of which is not yet clear, K201 acetylation together with sumoylation of the nuclear IF protein lamina prevented incorporation into the nuclear envelope and increased cell death (Zhang and Sarge, 2008). Remarkably, under conditions of apoptosis and oxidative stress, sumoylated K8–K285/287, K18–K207/372, and K19–K208 were detected (Snider et al., 2011). Also, hypersumoylation of K8 variants associated with chronic liver disease was discussed to abolish the earlier-mentioned cytoprotective function (Roth et al., 2012a).

Most recently, covalent cysteine-mediated cross-linking of K14 was reported (Lee et al., 2012). Owing to structural constraints, only a *trans*-dimer homotypic disulfide bond involving C367 in the stutter region of K14 can form and is detectable in cultured keratinocytes and suprabasal epidermis *in vivo*, where it is concentrated in a keratin filament cage enveloping the nucleus. The formation of such K14–C367 cross-links impacts nuclear shape in cultured keratinocytes (Lee et al., 2012). Although many details of the regulation of K14 cross-linking remain to be determined, implications of keratin cross-linking on cytoskeletal organization and, possibly, nuclear functions could be far-ranging. Of note, the amino acid motifs surrounding the C367 residue in K14 are conserved in other epidermal keratins, whereas K8 and K18 lack cysteine residues altogether.

Taken together, the discussed posttranslational modifications are modifications that impact the dynamics of the cytoskeleton, thereby influencing processes like migration or invasion that are required upon given physiological settings like in diseased tissue. Further analysis of distinct sites subjected

to posttranslational modifications will shed light on involved signaling pathways and kinases.



5. TRANSGENIC MICE UNDERSCORE STRUCTURAL AND REGULATORY KERATIN FUNCTIONS

Knockout mice have been instrumental to reveal novel regulatory functions in addition to architectural functions of epidermal keratins. Ablating K10, the heterodimer partner of K1 that, along with K10, forms the main keratin filament of upper stratified epithelia, resulted in hyperkeratosis, impaired progression of filaggrin, but no epidermal fragility (Reichelt et al., 2001). Here, compensation by K14 was found to support tissue integrity (Reichelt et al., 2001). Considering the many different isoforms of keratins, one is tempted to wonder whether a single keratin/keratin pair provides certain unique functions. An interesting experiment, in which mice carrying a chimeric keratin protein containing the K14 rod domain and head and tail domains of K10 were generated, showed that this fusion protein did not impact on normal skin development in young mice, which suggests an unexpected compensatory role for keratin head and tail domains (Chen et al., 2006). Only when the mice got older, they developed pathological alterations including blindness and skin lesions at the age of 18 months. Following a chemical skin carcinogenesis protocol (DMBA/TPA protocol), the mice containing the hybrid protein developed benign tumor at an elevated rate (Chen et al., 2006). This finding was rather surprising, because K10 was postulated to act as a negative regulator of cell-cycle progression, mediated by an interaction of the K10 head domain with Akt (PKB) and atypical PKC- ζ ; therefore, an impact on skin development was expected (Paramio et al., 2001). Arguing against a proliferation inhibitory role of K10 is the fact that K10-deficient mice developed far less tumors upon DMBA/TPA treatment compared to their wild-type littermates (Reichelt et al., 2004). In view of the participation of several epidermal keratins in gene networks governing skin carcinogenesis and inflammation, these mice are useful to address molecular mechanisms (Quigley et al., 2009).

The ablation of both K1 and K10 leads to the apparent absence of keratins in the suprabasal layers of the epidermis. Based on a dye exclusion assay, the authors concluded that K1 and K10 were not necessary for an intact epidermal water barrier (Wallace et al., 2012). This mouse model emphasizes the contribution of keratins in the maintenance of cell adhesion and tissue integrity, as resulting animals showed severe skin fragility, accompanied by smaller desmosomes and decreased amounts of desmosomal proteins

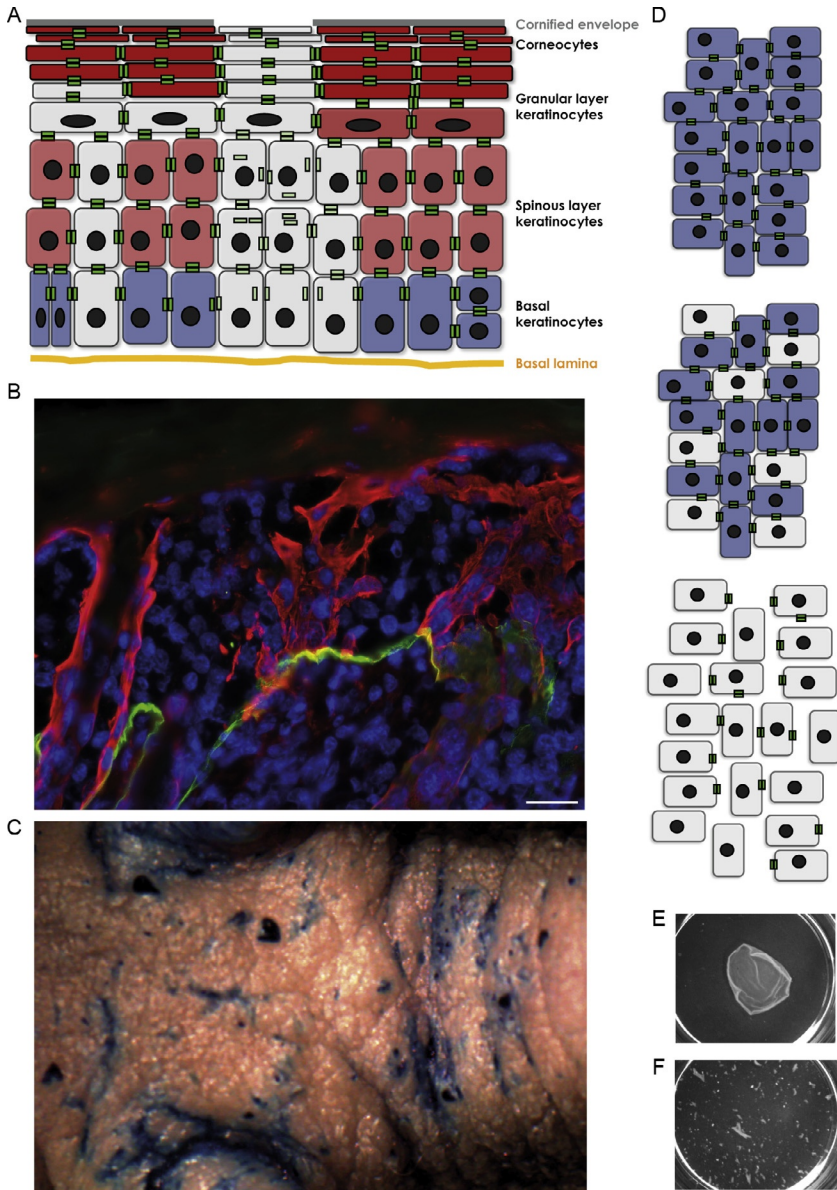


Figure 6.3 Schematic representation of the contribution of keratins to skin barrier integrity. (A) Scheme of skin displaying a keratin knockout in a mosaic fashion. Basal keratinocytes express the keratin pair K5/K14 (dark grey cells) or lack all keratins (light gray cells) and are attached to the basal membrane (basal lamina). In suprabasal layers (spinous layer and granular layer keratinocytes), wild-type keratinocytes have switched their expression profile to K1/K10 at the expense of K5/K14. Keratin-free keratinocytes in

(Wallace et al., 2012). The interdependence of desmosomes and keratins was subsequently substantiated in additional mouse and keratinocyte cell culture models. To overcome keratin redundancy, Bär et al. generated mice displaying an epidermis-specific deletion of the entire type II keratin gene locus (Bar et al., 2014). Specifically, they compared the mosaic and complete deletion of all type II keratins in mouse skin. As one might expect, the extensive epidermal damage in global keratin KO mice led to perinatal lethality, while mosaic KO mice survived about 12 days (Bar et al., 2014). In both mouse models, thickened skin, epidermal fragility, and inflammation were observed coinciding with the lack of keratins, and desmosomal proteins were found to be accumulated in the cytoplasm, which reduced the size of desmosomes and led to intercellular adhesion defects (Bar et al., 2014) (Fig. 6.3). Bringing resulting keratin-free cells into a cell culture system revealed that ~60% were needed to abide epithelial integrity in a shear stress assay (Fig. 6.3) (Bar et al., 2014).

Unlike K1 and K10, the type I keratin K9 is restricted to suprabasal keratinocytes of palmoplantar epidermis (Moll et al., 2008). Mice lacking K9 display acanthosis and hyperkeratosis accompanied by hyperpigmented calluses on their paws, a tissue fraction exposed to many stresses (Fu et al., 2014). Furthermore, terminal differentiation is impaired in the absence of K9, going along with the abnormal extended expression of K5 and K14 in the suprabasal epidermis, reduced amounts of K2, and the induction of stress-response keratins K6 and K16 (Fu et al., 2014). The deletion of K16, which is expressed in epithelial appendages and induced upon different

suprabasal layers derived from keratin-free cells in the basal layer. Wild-type keratinocytes show normal distribution of desmosomes (bars), whereas keratin-free keratinocytes show decreased desmosome size and aggregation of desmosomal proteins in the cytoplasm (also depicted as bars). For further details, see text. (B) Immunofluorescence staining of mosaic keratin KO mouse skin at postnatal day 8, corresponding to the scheme shown in (A). The basal lamina was stained with $\beta 4$ integrin. K14 staining shows patches of wild-type and keratin-free cells. Nuclei are stained with DAPI. Scale bar: 10 μm . (C) Ventral skin of mosaic keratin KO mouse at postnatal day 8 subjected to the toluidine blue dye penetration assay. Blue/dark patches on skin mark sites of defect skin barrier correlating with keratin-deficient skin patches. (D) Wild-type keratinocytes (dark grey) form an intact sheet in the shear stress assay, whereas keratin-free keratinocytes (light grey) fail to do so. In a mixed cell population, ~60% of keratin-expressing cells are sufficient to maintain sheet integrity (middle panel). Desmosomes are depicted as bars. (E) and (F) Sheet of wild-type cells and keratin-free cells, respectively, after applying rotational force in the shear stress assay. (A) Image adapted from Janina Bär; (B) and (C) image kindly provided by Janina Bär.

stresses, in a novel mouse model led to front paws showing palmoplantar keratoderma and calluses, also found in pachyonychia congenita type 1 patients, suffering from mutations in the K16 gene (Lessard and Coulombe, 2012). 60% of mutant mice died before weaning but did not display a barrier defect at young age (Lessard and Coulombe, 2012). Furthermore, the epithelium of the dorsal tongue developed lesions (Lessard and Coulombe, 2012).

Moreover, further analysis of these mice revealed a rather unexpected function for K16. In response to barrier disruption either by treatment with TPA or by tape stripping, K16-deficient mice reacted with increased expression of damage-associated molecular patterns (DAMPs) and cytokines (Lessard et al., 2013). This suggested a more complex pathomechanism for inflammatory diseases, in such that K16 might play a role in controlling signaling processes of this innate immunity (Lessard et al., 2013). Further support for the involvement of keratins in inflammatory processes was revealed by mice deficient in K1 (Roth et al., 2012c). At the same time, K17-deficient mice showed a distinctly different cytokine pattern (Lessard et al., 2013). Analysis of primary, K16-deficient keratinocytes indicated cytokine profiles similar to controls in the absence of extrinsic stimuli, suggesting complex regulation. Upon TPA treatment, K16-deficient cells showed elevated mRNAs for several DAMPs, such as S100A7A, S100A8, S100Ap, and TSLP (Lessard et al., 2013). Preliminary data suggest a cross talk between Erk1/2 and K16 underlying DAMP regulation (Lessard et al., 2013).

Further support for the involvement of keratins in inflammatory processes was revealed by mice deficient in K1 (Roth et al., 2012c). At first, these mice displayed mild skin fragility, unaltered distribution of desmosomes, and severely disrupted cornified envelopes, thus revealing a crucial function for K1 in maintaining skin barrier integrity (Roth et al., 2012c). Furthermore, the gene expression profile of K1-deficient mice resembled that of human inflammatory skin diseases atopic eczema and psoriasis (Roth et al., 2012c). Examination of the inflammation phenotype revealed the involvement of the proinflammatory cytokine IL-18, the release of which was K1-dependent (Roth et al., 2012c). Depletion of IL-18 either genetically or pharmacologically could partially rescue the phenotype resulting from K1 knockout, suggesting K1 acting upstream of IL-18 (Roth et al., 2012c). In addition to epidermal keratins, K8 has been reported to participate in modulating immune responses in part by affecting the morphology of thymic epithelial cells (Odaka et al., 2013).



6. MECHANISMS OF KERATIN-ASSOCIATED DISORDERS

Mutations in keratin genes result in a broad range of single-gene disorders affecting the skin and hair and contribute to complex diseases of the liver and the intestine (Corden and McLean, 1996; Lane and McLean, 2004; Omary et al., 2009) (Human Intermediate Filament Database; www.interfil.org). Since publication of the first K5 and K14 mutations, a more complex picture of disease mechanisms is emerging, in line with the view that keratins perform a multitude of structural and regulatory functions.

In 1991, a mutation found in the K14 gene was the first IF that could directly be linked to a disease, EBS (Bonifas et al., 1991; Coulombe et al., 1991; Lane et al., 1992; Omary, 2009). EB (Epidermolysis bullosa) is a clinically and genetically heterogeneous group of hereditary skin-blistering disorders classified into four major subtypes, depending on the epidermal level of skin cleavage and affected proteins (Fine, 2010; Intong and Murrell, 2012). The Kindler syndrome is an autosomal recessive skin disorder caused by mutations in the *FERMT1* gene (fermitin family homologue 1, also known as kindlin-1), giving rise to various skin-related symptoms like generalized blistering, keratoderma, skin atrophy, poikiloderma, and photosensitivity (Has et al., 2011; Intong and Murrell, 2012). A range of defects in collagen VII cause the dystrophic EB, being inherited either dominant or recessive, and the junctional EB is associated with mutations in the $\alpha 3$ chain of laminin-332, $\alpha 6\beta 4$ integrin, and collagen XVII (Bruckner-Tuderman and Has, 2012; Coulombe and Lee, 2012; Fine et al., 2008; Has et al., 2014; Intong and Murrell, 2012; Lane and McLean, 2004; Sawamura et al., 2010; Yuen et al., 2013). Skin cleavage occurs in these settings in the sublamina densa or the intralamina lucida, respectively (Intong and Murrell, 2012). EBS is the least severe and most prevalent form of EB and is classified into two major subtypes, depending on the site of intraepidermal rupture (Intong and Murrell, 2012). Suprabasal subtypes occur upon mutations in plakophilin-1 or desmoplakin, while basal subtypes are caused by plectin mutations (in 8% of patients) and additionally by mutations in $\alpha 6\beta 4$ integrin, dystonin, and BPAG1-e. The majority (75%) of basal EBS cases are caused by mutations in K5 or K14, the main keratin pair of basal keratinocytes (Bolling et al., 2011, 2014; Coulombe and Lee, 2012; Sawamura et al., 2010). Most subtypes follow autosomal dominant inheritance, with exceptions of some autosomal recessive inherited subtypes (Coulombe and Lee, 2012; Intong and Murrell, 2012). Patients suffer from

fluid-filled blisters on the skin, which can vary in frequency, severity, and distribution over the body (Coulombe and Lee, 2012; Lane and McLean, 2004). Also, in different subtypes, the involvement of other epithelia and the ultrastructural features of basal keratinocytes and prognosis are variable (Coulombe and Lee, 2012).

Considering the numerous members of the keratin family and their widespread expression in epithelial tissues, it is not surprising that many other keratins were identified as the underlying cause for several diseases, mainly affecting the skin and/or its appendages. As mentioned in the preceding text, upon injury, the expression of stress keratins is triggered and a mutated form of either K6a, K6b, K16, or K17 is linked to pachyonychia congenita, a disease that clinically manifests with plantar keratoderma, hypertrophic nail dystrophy with nail bed and hyponychial keratosis, and various epidermal cysts (McLean et al., 1995, 2011). Bullous congenital ichthyosiform erythroderma, also referred to as epidermolytic hyperkeratosis (EH), is caused by mutations in either K1 or K10, which are expressed in suprabasal cells of stratified, cornified epithelia and is marked by reddened and blistered skin in infancy that changes toward generalized EH in adulthood (Corden and McLean, 1996; Lane and McLean, 2004). Notably, a certain mutation in the K1 gene has been reported to be the underlying cause for diffuse nonepidermolytic palmoplantar keratoderma (Kimonis et al., 1994). For epidermolytic palmoplantar keratoderma, a mutation in the K9 gene could be identified as the underlying cause. In accordance with K9 being restricted in expression to suprabasal cells of palm and sole epidermis, patients suffer from thickened skin on respective areas (Leslie Pedrioli et al., 2012). Mutations in K2e, which is expressed late in differentiation of the interfollicular epidermis in suprabasal keratinocytes, give rise to ichthyosis bullosa of Siemens, characterized by epidermal blistering and superficial skin thickening (Akiyama et al., 2005; Lane and McLean, 2004). Mutations in the hair and nail keratins result among others in monilethrix, a disease characterized by fragile hair and nails and varying degrees of alopecia, whereas mutations in simple epithelial keratins, some of which are also expressed in Merkel cells in the skin, could not be linked to skin diseases, but have been discussed to be associated with inflammatory bowel and liver diseases (Karantza, 2011; Lane and McLean, 2004; McLean and Moore, 2011). A disease called Meesmann corneal dystrophy is caused by mutations in either K3 or K12, which are expressed in the corneal stratified epithelium (Klintworth, 2003; Lane and McLean, 2004). Remarkably, K3 is not found in the mouse genome (Hesse et al., 2004).

6.1. Mutations in K5 or K14 as the underlying cause for epidermolysis bullosa simplex

In most cases, the pathogenic effects in keratinopathies are due to missense mutations in keratin genes (Chamcheu et al., 2011). Underlying EBS, various mutations in the K5 and K14 genes have been found and the severity of the disease in any type of EBS corresponds to the position of the mutation within the keratin sequence motif. Today, for K5 and K14, various mutations in either the rod domain or head and tail domains are known (www.interfil.org). Mutations occurring in the highly conserved edges on either side of the rod domain, that is, the end domains of either 1A or 2B (helix initiation motif/helix termination motif; see Fig. 6.1), give rise to much more severe phenotypes than those found in the less conserved head or tail domains or linker segments (Lane and McLean, 2004). Probably due to deamination of methylated cytosine in the context of a CpG dinucleotide, the human K14-R125 residue located in the highly conserved part of helix 1A domain turned out to be a “hot spot” for mutations, also found in other type I keratins (Corden and McLean, 1996; Coulombe et al., 2009). Notably, there are a variety of amino acid exchanges at this and other mutation sites that also highly impact on phenotype severity (Coulombe and Lee, 2012). Recently, the case of a compound heterozygous patient with K14 and K5 mutations giving rise to EBS was reported (Wertheim-Tysarowska et al., 2014).

In vivo, the majority of dominant K5 and K14 mutations cause aggregation of the cytoskeleton, accompanied by tissue rupture when subjected to (even faint) stress, resulting in the formation of fluid-filled blisters (Coulombe et al., 2009; Lane and McLean, 2004). Using knockout mouse models for K14 and K5, it was postulated that the cell fragility in EBS emerges from a “loss-of-function” phenotype, as these mice also displayed the key features of the disease, although K14^{−/−} mice are less severely affected, possibly due to compensatory effects of type I keratins K15 and K17 (Lloyd et al., 1995; Peters et al., 2001). In the EBS Dowling-Meara subtype, the most severe form of EBS, aggregates of keratins positive for both K5 and K14 are found in the cytoplasm and along hemidesmosomes (Coulombe et al., 1991; Peters et al., 2001) (Fig. 6.4). Mice expressing K14 mutants that tend to form aggregates show earlier onset of the disease and more severe blistering compared to K14-null mice, in accordance with patients null for K14 exhibiting a less severe phenotype (Coulombe and Lee, 2012; Lloyd et al., 1995). It has been discussed that the accumulation of keratin aggregates results from a failure of the protein chaperone machinery (Loffek et al., 2010). Notably, protein aggregates are also found to be

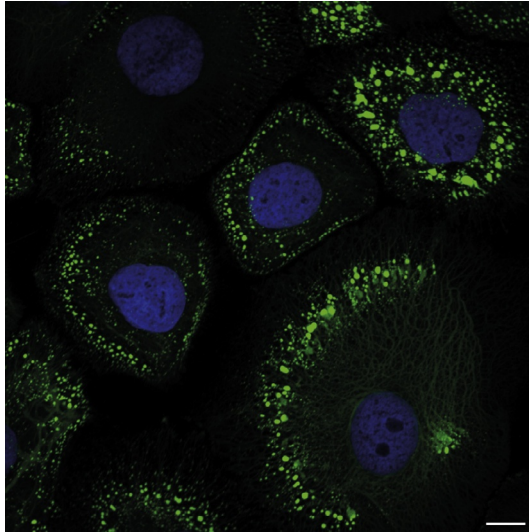


Figure 6.4 Human keratinocytes (HaCaT) showing aggregates of mutated keratin. HaCaTs were transfected with K14-R₁₂₅P fused to YFP (yellow fluorescent protein). Note that aggregates are mainly found in the periphery of the cells. Nuclei were counter-stained with DAPI. Scale bar 10 μ m. *Image kindly provided by Vinod Kumar.*

characteristic of various other diseases, for example, neurodegenerative diseases (Jucker and Walker, 2013). In support, proteasome inhibition in a cell culture model of EBS caused an increase in K14-R₁₂₅C-positive aggregates. Overexpression of the chaperone-associated ubiquitin ligase CHIP strongly reduced keratin aggregates through increased degradation of mutant K14, whereas RNA interference (RNAi)-mediated knockdown of CHIP augmented keratin aggregates. These data suggest common principles between EBS and other protein misfolding disorders, revealing that aggregation-prone keratins are targeted by components of the chaperone machinery (Loffek et al., 2010).

In order to unravel the pathomechanism of EBS, mouse and cell culture models were further analyzed. These revealed the contribution of proinflammatory cytokines interleukin-6 and interleukin-1 β in K5-deficient mice (Lu et al., 2007). An increased number in Langerhans cells, accounting for the immunologic barrier of the skin, could be found in the skin of K5 $-/-$ mice and in the skin of EBS patients carrying a K5 mutation, in line with upregulation of distinct cytokines like CCL2, CCL19, and CCL20 (Roth et al., 2009). Both of these findings could

not be found in K14^{-/-} mice and patients carrying a K14 mutation (Roth et al., 2009). Whether the cytokine production is already elevated in the skin developing with mutant keratins prior to manifestation of the disease-typical phenotype or a consequence of the skin blistered upon mechanical trauma remains to be elucidated.

6.2. Different approaches for the treatment of skin disorders

Different approaches have been undertaken to find mechanisms that positively contribute to keratinopathies. Given the inability to form a proper cytoskeleton in keratinocytes of patients suffering from EBS, the possibility of replacing this with another IF and thereby strengthen the cell came up. Due to the dominant-negative pathomechanism of most keratin mutations, which is hard to overcome with gene-therapy approaches, supplementation might be an alternative. Keratinocytes from an EBS patient with a mutation in the L1/L2 linker of K14 were immortalized and transfected with type III IF desmin, which is expressed in muscle cells and does not copolymerize with keratins (Magin et al., 2000). It was found that desmin forms typical IFs in keratinocytes, being organized independently from the endogenous keratin filament network (Magin et al., 2000). Another study showed that the ectopic expression of desmin in mouse epidermis enables the formation of a desmin filament network in basal keratinocytes and did not interfere with normal epidermal architecture (Kirfel et al., 2002). Furthermore, desmin was expressed in K5^{-/-} mice, but failed to normalize their phenotype (Kirfel et al., 2002). Another approach in supplementation therapy also used the transfection of desmin in a cell culture model of EBS and found rescue effects in responses to stress (D'Alessandro et al., 2004). By reexpression of wild-type K14 in keratinocytes from a patient with a K14-null mutation, the normal behavior profile of the keratinocytes could be rescued (D'Alessandro et al., 2011).

In an inducible mouse model, it was shown that decreasing the expression of mutant K14 restored normal morphology and functions of the skin (Cao et al., 2001). This suggests that genetic therapy approaches could play an important role in the treatment of dominantly inherited skin diseases (Cao et al., 2001). The use of the RNAi technology that enables to specifically target mutated keratins was tested in several studies. In a dominant-negative cell culture model of pachyonychia congenita, mutant K6a was targeted with small interfering RNAs (siRNAs) (Hickerson et al., 2008). Whereas in the presence of wild-type and mutated K6a normal filament formation

was disturbed, the specific knockdown of mutated K6a abolished this phenotype (Hickerson et al., 2008). A potential therapeutic route for EBS was suggested by developing siRNAs specifically targeting two different K5 mutations (Atkinson et al., 2011). These have been shown to reverse the cytoskeletal aggregation phenotype found in cells carrying the mutation (Atkinson et al., 2011). Also using RNAi technique, allele-specific knockdown of mutant K9 as therapeutic basis in treatment of epidermolytic palmoplantar keratoderma was reported (Leslie Pedrioli et al., 2012). Due to lack of a K9 mouse model, a mutant K9-luciferase reporter was coinjected with a mutant-specific siRNA into mouse footpad, which showed a higher specificity for the siRNA to the mutant than to the wild-type allele (Leslie Pedrioli et al., 2012; Roth et al., 2012b).

Another therapeutic approach toward the treatment of pachyonychia congenita is based on small molecules like members of the statin family, cholesterol-lowering drugs that have been found to downregulate K6a promoter activity (Zhao et al., 2011). The use of small molecules in the treatment of skin disorders is further supported by a study revealing the involvement of chaperone proteins in the degradation of mutant keratin aggregates (Loffek et al., 2010). It was suggested that modulating the chaperone machinery with small molecules could be a new therapeutic strategy for EBS (Loffek et al., 2010). Also, it was reported that application of the small molecule doxycycline to K5^{-/-} mice prolonged neonatal survival for a few hours (Lu et al., 2007).



7. NOVEL ARCHITECTURAL AND REGULATORY FUNCTIONS OF KERATINS

The complexity of the keratin multiprotein family has been an obstacle to unravel architectural and regulatory functions of the entire protein family and of individual members. The generation of a mouse model lacking the entire keratin multiprotein family (KtyII^{-/-} mice) was accomplished by using a targeted deletion of the entire type II keratin cluster on chromosome 15, resulting in the degradation of all type I keratins, due to the absence of dimerization partners (Vijayaraj et al., 2009). Surprisingly, KtyII^{-/-} embryos showed no defects in embryonic and extraembryonic epithelia, but resulting offspring showed lethality at embryonic day 9.5 along with severe growth retardation (Vijayaraj et al., 2009). Keratin-deficient mice revealed defects in primary hematopoiesis and vasculogenesis through reduced BMP-4 signaling, which implicates a role for keratins for the

differentiation of a nonepithelial cell lineage (Vijayaraj et al., 2010). Further examination of KtyII^{-/-} embryos revealed mislocalized GLUT1 and GLUT3, the main transporters that regulate glucose distribution in early embryos, from the apical plasma membrane in embryonic epithelia, which subsequently repressed downstream mTOR signaling via AMP kinase (Vijayaraj et al., 2009). These data proved a novel regulatory role for keratins by coordinating cell growth and protein synthesis (Vijayaraj et al., 2009).

Keratin-deficient mice then led to the establishment of keratin-free keratinocytes, enabling to address the respective contribution of keratins, actin, and microtubules to cell stiffness (Kroger et al., 2013; Seltsmann et al., 2013a,b). Using a microfluidic optical stretcher device, keratin-free cells showed about 60% higher cell deformability even for small deformations, with much smaller contributions from actin (Seltsmann et al., 2013a). Independent measurements confirmed reduced viscosity of keratin-free cytoplasm (Ramms et al., 2013). When subjected to invasion and three-dimensional colony growth assays, keratin-deficient keratinocytes showed a much greater invasion potential and outgrowth as individual cells compared to strands. In addition, keratin-deficient cells migrated two times faster compared to their wild-type counterparts (Seltsmann et al., 2013a,b). Also, without keratins, plectin is dissociated from $\beta 4$ integrin in hemidesmosomes and keratinocytes adhere much faster to ECM substrates as controls (Seltsmann et al., 2013b). As mentioned in the preceding text, the lack of keratins also resulted in diminished cell-cell adhesion and mislocalized desmoplakin (Kroger et al., 2013). The dependence on keratins in the earlier-mentioned settings could be proven by the reexpression of the single keratin pair K5/K14, which reversed the phenotypes (Kroger et al., 2011; Seltsmann et al., 2013a,b). Taken together, these findings suggest that stable keratin cytoskeletons may act as barriers against malignant transformation through maintaining stable intercellular adhesion and cell stiffness. While many studies suggest that down-regulation of keratins in addition to E-cadherin occurs during epithelial-mesenchymal transition, rendering tumor cells more motile and softer (Kalluri and Weinberg, 2009; Thiery et al., 2009), a recent study on mammary carcinoma arrived at a different conclusion. In order to identify the most invasive cancer cells in primary breast tumors, a three-dimensional organoid assay was established. This revealed that K14-positive cells led collective invasion in the major human breast cancer subtypes and knockdown of either K14 was sufficient to block collective invasion (Cheung et al., 2013).

Regarding isotype-specific functions, K17, upregulated upon stress and inflammation and in chronic disease, is particularly well characterized. It impacts on protein biosynthesis via binding to 14-3-3 σ and thereby mediates its relocalization from the nucleus to the cytoplasm (Kim et al., 2006). Subsequently, this stimulates the mTOR pathway and cell growth (Kim et al., 2006). Also, in a K17-null mouse, it was shown that K17 is essential for correct regulation of the hair cycle (Tong and Coulombe, 2006). Furthermore, keratinocytes lacking K17 displayed a higher sensitivity to TNF- α -mediated apoptosis, possibly mediated by the interaction of the TNF receptor I adapter protein TRADD (Tong and Coulombe, 2006). Further support for a role of K17 in inflammatory signaling was found by mating Gli2 overexpressing mice with K17-deficient mice (Depianto et al., 2010). The absence of K17 in this genetic background led to a reduction of Th1- and Th17-related and induction of Th2-related cytokines, with the latter mediating an anti-inflammatory response (Depianto et al., 2010; Roth et al., 2012a). As binding partner of K17 also being involved in wound repair, K6 was hypothesized to have a negative impact on signaling of Src kinase and thereby decreasing the migratory potential, whereas Src was shown to directly bind to keratin filaments (Rotty and Coulombe, 2012). Furthermore, mice deficient in either K16 or K1 further suggested an impact of keratins for the immune response (Lessard et al., 2013; Roth et al., 2012c). As mentioned in the preceding text, a role for K1 acting upstream of IL-18 was found by epistasis analysis (Roth et al., 2012c). To arrive at this conclusion, K1 was knocked down in human keratinocytes along with caspase-1, which is needed to cleave IL-18 into its active form. This prevented the release of active IL-18 from keratinocytes, while cells only deficient in caspase-1 secreted cleaved IL-18 (Roth et al., 2012c). These findings revealed more far-ranging functions of keratins next to simply structural ones, which might impact on human inflammatory skin diseases.



8. CONCLUDING REMARKS, OPEN QUESTIONS, AND FUTURE RESEARCH STRATEGIES

The analysis of numerous mouse and cell culture models for individual keratins and the entire family is finally beginning to provide a framework toward understanding architectural and regulatory keratin functions. Deletion of the entire keratin protein family showed that keratins are not essential for epithelial morphogenesis; however, without keratins, epithelia, in particular the epidermis, are much more susceptible to force, partly, because

intercellular adhesion is compromised by the absence of keratins, partly through scaffolding of PKC- α and Src kinase on keratins (Kroger et al., 2013; Rotty and Coulombe, 2012). How keratins participate in the regulation of EGF and IGF signaling toward cell adhesion and growth is a topic that promises exciting insights. The strong links of K1, K16, and K17 to inflammatory pathways, partly in connection to barrier defects, raise the following topics: What are the molecular mechanisms by which keratins regulate proinflammatory cytokines and DAMPs? What is the relevance for human disease? Given that loss-of-function mutations in the keratinocyte-resident protein filaggrin compromise the epidermal barrier and can lead to atopic dermatitis, it is conceivable that mutations or post-translational modifications of certain keratins contribute to chronic inflammatory disease and modulate the immune response. Understanding these mechanisms should also provide novel targets for the development of rational therapies for human keratin disorders. The discovery of novel posttranslational keratin modifications, including cysteine oxidation, links the major epidermal cytoskeletal proteins to the cellular redox network. It will be interesting to learn how covalent cross-linking of keratins affects cell behavior during epidermal differentiation and wound healing. Finally, while the pairwise regulation of keratins is not understood, available data support post-transcriptional mechanisms. In view of the widespread role of miRNA miR-203 in epidermal stratification, differentiation, and self-renewal, it is tempting to hypothesize that miRNAs participate in pairwise keratin regulation (Jackson et al., 2013; Yi and Fuchs, 2010; Yi et al., 2008).

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Characean Internodal Cells as a Model System for the Study of Cell Organization

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Abstract

Giant internodal cells of characean green algae have been widely used for studying cellular physiology. This review emphasizes their significance for understanding cytoarchitecture and cytoplasmic reorganization. The cytoarchitecture of internodal cells undergoes pronounced, cytoskeleton-dependent changes during development and in response to environmental cues. Under bright light, internodes develop alternating bands of acid and alkaline pH at their surface that correlate with the differential size and abundance of cortical organelles and, in the genus *Chara*, with the size and distribution of convoluted plasma membrane domains known as charasomes. Wounding induces responses ranging from chloroplast detachment to deposition of wound walls. These properties and the possibility for mechanical manipulation make the internodal cell ideal for exploring plasma membrane domains, organelle interactions, vesicle trafficking, and local cell wall deposition. The significance of this model system will further increase with the application of molecular biological methods in combination with metabolomics and proteomics.



1. INTRODUCTION

1.1. Characean green algae

The Characeae are multicellular green algae that live mainly in fresh and, occasionally, brackish water. Their thallus, sometimes referred to as a frond, consists of a regular series of long, cylindrical internodal cells and groups of small, isodiametric or flat nodal cells from which side branches and whorls of lateral cells emerge (Fig. 7.1A–E). The growth of the main axis and the side branches is indeterminate and occurs by the ordered division of a dome-shaped apical cell, which sequentially produces internodal and nodal cells. The growth of lateral cells is determinate, such that the number, shape, and spatial arrangement of internodal cells and nodes are important criteria for species identification. Characean algae were described as “*Equisetum*” in pre-Linnaean times (Bauhin, 1623) owing to their anatomy, which is indeed reminiscent of horsetails. Their German name “Armleuchteralge” relates to the candelabra-like appearance of the thallus, whereas their English common name “stonewort” refers to the lime encrustations found at the surface of many species growing in alkaline conditions (Fig. 7.1D). Characean algae can cover the bottom of lakes, slowly flowing rivers, and streams and are anchored in the sediment by delicate rhizoids. The length of their thalli ranges from several centimeters to 2 m. They prefer clear water with a low nutrient status and are therefore useful indicators of oligo- to mesotrophic habitats.

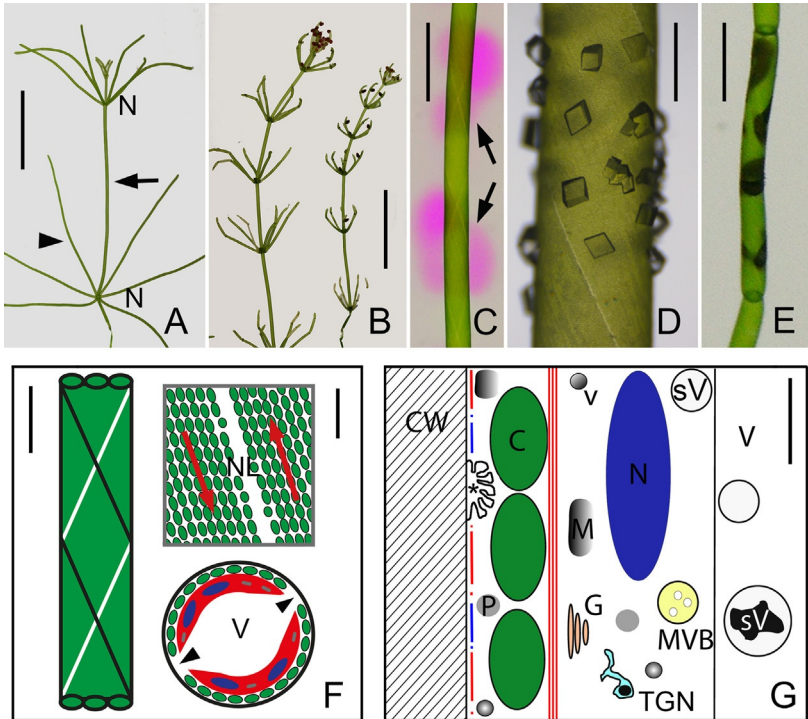


Figure 7.1 Thalli and internodal cells of *Chara australis* (A–E) and schematic representation of the cytoarchitecture of internodal cells (F and G). (A) Upper part of a young, elongating thallus. Arrow points to an internodal cell of the main axis extending between two nodes (N). Whorls extending from nodes consist of five to six branchlets (arrowhead), each with up to three internodal cells. (B) Two older, nonelongating thalli: one carrying antheridia (left thallus) the other bearing oogonia (right thallus). (C–E) pH banding pattern of branchlet internodal cells visualized by incubation in phenol red (C) where alkaline regions are marked by pink color (arrows in C), by calcite crystals (D), and by dark-brown manganese-containing cell wall incrustations (E). (F and G) Helical organization of internodal cells and schematic longitudinal section. The left image in (F) shows an internodal cell flanked by nodes (group of nodal cells) on each side. Bright and dark lines are the chloroplast-free neutral lines at the upper and lower side of the cell, respectively. The right upper image is a higher magnification of the chloroplast-containing cortex and illustrates that the neutral line (NL) separates up- and downstream endoplasm (red arrows). The right lower image is a cross section through an internodal cell showing the stationary layer of chloroplasts (green), the motile endoplasm (red) with nuclei (blue), and other organelles and the central vacuole (V). The position of the two neutral lines is indicated by arrowheads. (G) Schematic longitudinal section through a mature, nonelongating internodal cell. In the genus *Chara*, the plasma membrane adjacent to the cell wall (CW) may form convoluted domains (charasomes, asterisk). Lines and dots along the plasma membrane represent cortical microtubules (blue) and actin filaments (red). Subcortical actin filament bundles (Continued)

The Characeae are characterized by peculiar sex organs that are enclosed within a jacket of sterile cells arranged in a spiral pattern. The female “egg” becomes fertilized by spiral-shaped male gametes equipped with two long flagella of equal length (Beilby and Casanova, 2014). The developing oospore, the only diploid stage of the life cycle, has an extremely hard, calcified cell wall with characteristic spiral ridges, which allows survival in wet or dry sediment for many years or even decades. Fossilized oospores called gyrogonites date back to the Silurian period more than 400 million years ago (Villalba-Breva and Martín-Closas, 2011), indicating that characean algae are a long-established branch of the plant kingdom.

Germinating oospores develop two types of tip-growing cells: positively gravitropic rhizoids, which serve to anchor the thallus, and a delicate protonema, which grows against the gravity vector. Upon emerging from the sediment, and in response to a blue light signal, protonemata undergo an asymmetric division to initiate the transition from a tip-growing unicell to a multicellular thallus, the cells of which expand diffusely (Braun and Wasteneys, 1998). In many species, sexual reproduction does not appear to play an important role and asexual reproduction is much more common (Casanova and Brock, 1999). Vegetative propagation occurs by means of detached thallus fragments or by starch-containing nodes called “bulbils,” which may survive adverse environmental conditions in the sediment. As noted by Michael C. Grant (1990), “sexual reproduction in a monoecious species which self-fertilizes is, from the genetic perspective, precisely the same as asexual reproduction.” This is because gametes are produced mitotically, with meiosis taking place only after fertilization to give rise to the haploid thallus (Oehlkers, 1916; Shen, 1967b). Grant and Proctor (1972, 1980) suggested that the high incidence of polyploidy in charophytes is a consequence of the peculiar reproductive strategy and that this can ultimately lead to genetic variation.

Today, the characean algae form an isolated group, but molecular phylogenetic analyses suggest that they are closely related to land plants (Karol et al., 2001; Kranz et al., 1995; Leliaert et al., 2012; McCourt et al., 2004; Turmel

Figure 7.1—Cont'd (red lines) are present along the inner side of the stationary chloroplasts (C). Nuclei (N), Golgi bodies (G), *trans*-Golgi network (TGN), and multivesicular bodies (MVB) are located in the streaming endoplasm. Mitochondria (M), peroxisomes (P), and various vesicles (v) may shuttle between cortex and endoplasm. Small vacuoles (sV) with or without solid inclusions are found in the periphery of the endoplasm and within the central vacuole (V). Bars are 1 cm (A and B), 2.5 mm (C and E), 500 μ m (D and F; left bar), 20 μ m (F; right bar), and 3 μ m (G, only approximately drawn to scale).

et al., 2007) although they are no longer considered as their sister group (Wodniok et al., 2011). They share with land plants (embryophytes) a number of ultrastructural and biochemical features, for example, the fine structure and pigment composition of chloroplasts, phenylpropanoid metabolism (Markham and Porter, 1969), the structure and chemical composition of the cell wall (Peaucelle et al., 2012) and the plasmodesmata (Cook et al., 1997), the mode of cell division (Braun and Wasteneys, 1998; Cook et al., 1998; Pickett-Heaps, 1975), and the presence of plant hormones such as auxin (Boot et al., 2012; Jin et al., 2008), strigolactones (Delaux et al., 2012), and gibberellins (Kazmierczak and Stepinski, 2005; Kwiatkowska, 2003).

The Characeae family includes six genera. The most common and well-studied species belong to the genera *Chara* and *Nitella*. In nearly all *Chara* species, the internodal cell of the main axis and often also those of the lateral branches is surrounded by a layer of cortical cells that confer stability. Ecorticate *Chara* species are *Chara australis*, *Chara corallina*, and *Chara braunii*. Internodal cells in species of the genera *Nitella*, *Nitelopsis*, *Tolypella*, and *Lamprothamnium* are always ecorticate; corticate and ecorticate species have been described from the genus *Lychnothamnus* (Wood and Imahori, 1965).

1.2. The internodal cell as a model for plant cell biology

The naked (ecorticate) internodal cells have been and still are a widely used model system in plant biology. Their advantages are the large size and simple geometry, which allow extensive manipulations (see Section 4). The internodal cells are also famous for their rapid cytoplasmic streaming, which was described as early as the eighteenth century by the Italian abbot Bonaventura Corti (1774). His publication was probably one of the first papers dealing with plant cell biology. Since then, the internodal cell has served as a model for studying numerous aspects of cellular dynamics and the cytoskeleton, cell structure and morphogenesis, electrophysiology and transmembrane transport, and organelle interaction and photosynthesis (Beilby and Casanova, 2014; Braun et al., 2007; Foissner and Wasteneys, 2012; Shimmen and Yokota, 2004; Tazawa and Shimmen, 2001). Many general principles of plant cell structure and physiology were first explored and discovered in characean internodal cells before they were confirmed later in higher plant cells. We refer to some of these important findings in the following sections.

Probably, the most widely used characean species in plant cell biology is *C. australis* R. Br. This ecorticate species is often referred to as *C. corallina* Klein ex Willd., em. R.D.W. together with which it was lumped by Wood and

Imahori (1965). However, *C. australis* is dioecious and has 14 chromosomes in contrast to the monoecious *C. corallina* that has 28 chromosomes. These differences and phylogenetic analyses of several DNA regions allow the recognition of both species (M. Casanova and K. Karol, personal communication). The internodal cells of *C. australis* (and those of *C. corallina*) may reach a length of up to 15 cm and a thickness of up to 1.5 mm. More delicate species of the genera *Chara* and *Nitella* are better suited for electron microscopy having considerably smaller internodal cells. Among them are *C. braunii*, a monoecious species with 14 chromosomes and *Nitella flexilis* that can be monoecious or dioecious and, accordingly, has 12 or 6 chromosomes. The side branches of *Nitella hyalina* (monoecious with 18 chromosomes) consist of tiny internodal cells that are covered by mucilage and that are therefore suited for high-pressure freezing. The chromosome numbers of the monoecious *Nitella pseudoflabellata*, another widely used species, are not known. The salt-tolerant *Lamprothamnium papulosum* (monoecious with 50 chromosomes) is suited for the study of osmotic acclimation and turgor pressure regulation (Beilby et al., 1999; Bisson and Kirst, 1995).

A great advantage of characean algae is their easy propagation. Cultures are usually set up by planting segments of thalli into a substrate of sand, soil, and peat covering the bottom of large glass beakers, plastic containers, or glass aquaria, which are then filled with distilled water. After about 2 weeks, newly grown internodal cells of the main axis and the branchlets can be harvested for experiments. When not required, thalli are able to survive in their containers for months and even years without the need for frequent replanting. Establishing sterile cultures from (surface-sterilized) oospores, however, is an arduous and often futile process (see Section 5.2).



2. CYTOARCHITECTURE AND DEVELOPMENT OF INTERNODAL CELLS

2.1. Cortical cytoplasm and helical growth pattern

A helical organization is typical for characean algae. The male spermatozoids have a helical shape as well as those vegetative cells that surround the egg. Internodal cells are characterized by a helically organized cell wall and a helically organized cytoplasm, which is schematically illustrated in Fig. 7.1F and G.

2.1.1 Cell wall

The structure and chemistry of the cell wall of characean algae is similar to that of higher plants and consists of cellulose microfibrils embedded in an

amorphous matrix of pectic polysaccharides (Fig. 7.2A and B) (Fry et al., 2008; Homblé et al., 1989; Morrison et al., 1993; Popper and Fry, 2003; Sorensen et al., 2011). The outermost electron-dense layer is only a few nanometers thick and resembles a cuticle but has unknown chemical

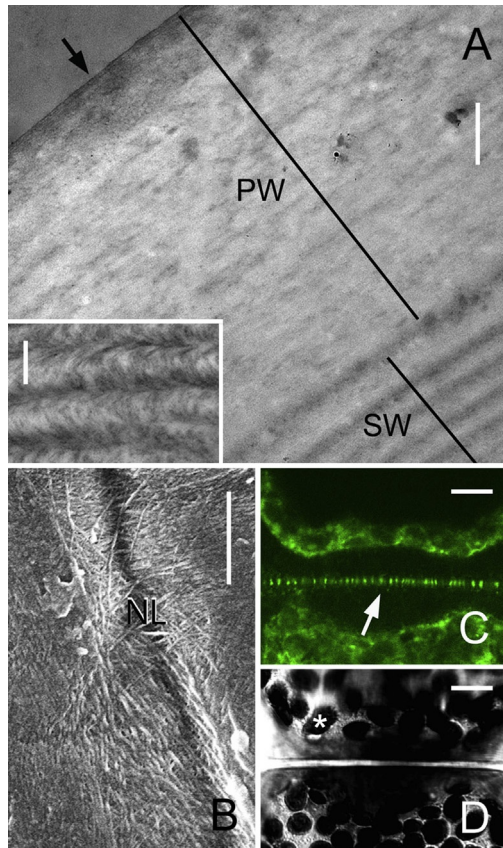


Figure 7.2 Cell walls of internodal cells of *Nitella flexilis* (A), *N. pseudoflabellata* (B), and *Chara australis* (C and D). (A) Longitudinal section of the cell wall viewed by transmission electron microscopy. The “cuticle” along the outer surface of the primary wall (PW) is indicated by an arrow. The cellulose microfibrils of the secondary cell wall (SW) are arranged in a helicoidal pattern (a higher magnification is seen at the left lower inset). (B) Cellulose microfibrils at the inner surface of the cell wall visualized by field emission scanning electron microscopy (FESEM). Note fewer microfibrils at the neutral line (NL). (C and D) Plasmodesmata (arrow) in the wall between a nodal and an internodal cell. In (C), the plasma membrane was stained with FM1-43, and (D) is the corresponding bright-field image; a chloroplast is marked by an asterisk. Bars are 10 μm (C and D), 600 nm (A and B), and 300 nm (inset in A).

composition (Fig. 7.2A) (Cook and Graham, 1998). In the primary wall of rapidly expanding internodes, the newly deposited cellulose microfibrils have a predominantly transverse orientation. The tightly packed cellulose microfibrils in the secondary wall form a distinct helicoidal pattern (Fig. 7.2A, inset). Primary and secondary cell walls together may become up to 10 μm thick. In some species, such as *N. hyalina*, mucilage is present at the surface of the apices of young thalli or covers the gametangia-bearing parts of the thallus (Wood and Imahori, 1965). A more than 10 μm thick mucilage layer has been reported from the surface of older internodes of *L. papulosum* (Beilby et al., 1999). This abundant mucilage could serve as useful model system for studying the synthesis and secretion of pectic polysaccharides, just as the seed coat model of *Arabidopsis* has been (Arsovski et al., 2010).

During their development, characean internodal cells undergo distinct phases of elongation (Wasteneys and Williamson, 1987, 1993). Initially, they grow slowly, reaching their maximum relative elemental elongation rate at about 10 days, after which they elongate at continuously decreasing relative elemental rates until reaching their final length after 30 days of growth.

The cytoplasm of internodal cells is divided into two longitudinally separated zones that accommodate the rotational flow of its contents around the large central vacuole in a direction delineated by prominent actin bundles. The separation between these zones of opposite flow is characterized by a thinner cytoplasm and lack of actin bundles and the underlying files of chloroplasts and is commonly referred to as the neutral line or indifferent zone (Figs. 7.1F, 7.2B, 7.3A, G–I, and 7.7A–C). The cell twists as it grows and the cell wall at the neutral line has distinct features, which have been described as “wall striations” (Fig. 7.2B) (Green, 1954). Measurements of cells grown under normal conditions and under various perturbations support the view that these cell wall features and also the organization of the cortical cytoplasm follow the lines of maximum cumulative growth, that is, they are strain-aligned (Green, 1959; Green and Chen, 1960). In their pioneering studies, Green (1960), Probine (1963), and Probine and Preston (1961, 1962) confirmed the multinet growth theory of Roelofsen (1959), which states that cellulose microfibrils are transversely oriented at the inner surface of the wall and become passively reoriented to the axial direction during cell elongation. Green’s early studies of the *Nitella* cell wall and the finding that the antimitotic drug colchicine caused lateral expansion (Fig. 7.4G) led to his famous prediction that the transverse deposition of cellulose microfibrils, a requirement for elongation, depends on proteins of a spindle fiber nature

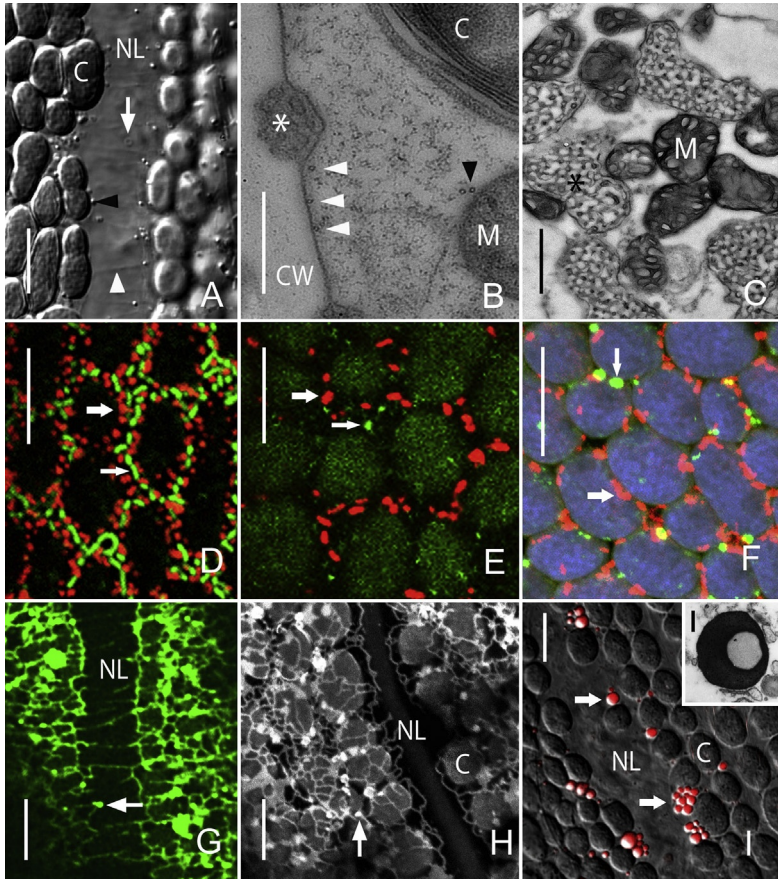


Figure 7.3 Cortex of internodal cells of *Chara australis* (A, C–F, and I), *C. braunii* (B), and *Nitella pseudoflabellata* (G and H). (A) DIC image of neutral line (NL) between chloroplast (C) files in an elongating cell. Note transverse cytoplasmic strands (white arrowhead) that probably extend along cortical microtubules. A lipid droplet is marked by a black arrowhead, and the white arrow indicates a putative Golgi body. (B) Cross section through the cortex of an elongating internodal cell that was high-pressure frozen and cryosubstituted. The transmission electron micrograph shows the cell wall (CW), a developing charasome (asterisk), cortical microtubules (white arrowheads), a chloroplast (C), and a mitochondrion (M). The black arrowhead points to a pair of microtubules far apart from the plasma membrane. (C) Tangential section through a chemically fixed, older internodal cell. The TEM image shows extended charasome (asterisk) areas intermingled with mitochondria (M). (D and E) Charasomes stained with green fluorescent plasma membrane dye FM1-43 (thin arrows) and cortical mitochondria stained with red fluorescent mitotracker (thick arrows). Note abundant charasomes and mitochondria at an acid region (D) in comparison with an alkaline zone (E) of an internodal cell. (F) Mitochondria labeled with red fluorescent MitoTracker (thick arrow), peroxisomes immunolabeled with anticatalase (green fluorescent, thin arrow), and autofluorescent
(Continued)

(Green, 1962). Newly mature internodes have randomly oriented cellulose microfibrils in the primary wall, whereas the secondary cell wall is characterized by a helicoidal arrangement of microfibrils (Fig. 7.2A and B) (Hotchkiss and Brown, 1987; Morrison et al., 1993; Neville and Levy, 1984).

Plasmodesmata are well described in characean algae. They are found at the transverse (end) cell walls and connect the cytoplasm of internodes with the cytoplasm of neighboring nodal cells (Fig. 7.2C and D), and they are also present between adjacent nodal cells. Interestingly, callose—the β -1,3-glucan that serves to plug plasmodesmata—is only found at the nodal side and never at the opening toward the internodal cell (Blackman et al., 1998). The fact that ecorticate internodal cells have extensive surface area lacking plasmodesmata has been exploited to identify plasmodesmal-associated proteins by comparing proteins extracted from nodal versus internodal cells (Blackman and Overall, 1998; Blackman et al., 1998; Faulkner et al., 2005, 2009).

2.1.2 Plasma membrane and charasomes

The internodal cells of species of the genus *Nitella* and *Nitellopsis* that have been studied so far in our and other laboratories have a smooth plasma membrane without structural peculiarities (Schmölzer et al., 2011). In species of the genus *Chara*, convoluted membrane elaborations have been described along the longitudinal cell walls. These “charasomes” consist of anastomosing membranous tubules, about 50 nm in length and 30 nm in diameter, continuous with and surrounded by the smooth plasma membrane (Fig. 7.3B and C) (Barton, 1965; Bisson et al., 1991; Chau et al., 1994; Crawley, 1965; Franceschi and Lucas, 1980; Hoepflinger et al., 2013; Lucas and Franceschi, 1981; Schmölzer et al., 2011). The continuity of the plasma membrane with the charasome membranes is confirmed by staining with fluorescent plasma membrane dyes that reveal charasomes as more or less brightly fluorescent spots depending on the amount of superimposed membranes (Fig. 7.3D–F; Schmölzer et al., 2011).

Most likely, the main function of the charasomes is to provide space for the accommodation of transporter proteins. Among these, H^+ -ATPases

Figure 7.3—Cont'd chloroplasts (false colored blue). (G) Cortical endoplasmic reticulum and lipid droplets (arrow) stained with green fluorescent phosphocholine in an elongating internodal cell. Note predominantly transverse orientation of ER meshes at the neutral line (NL). (H) Fluorescence micrograph of cortical DiOC₆-labeled endoplasmic reticulum and mitochondria (arrow) in a mature cell. Note random orientation of ER meshes. (I) Lipid droplets (stained with red fluorescent Nile red, arrows) between chloroplasts. The fluorescence image is merged with the DIC image. Inset shows an electron micrograph of a lipid droplet. Bars are 10 μ m (A, D–I), 500 nm (B and C), and 200 nm (I, inset).

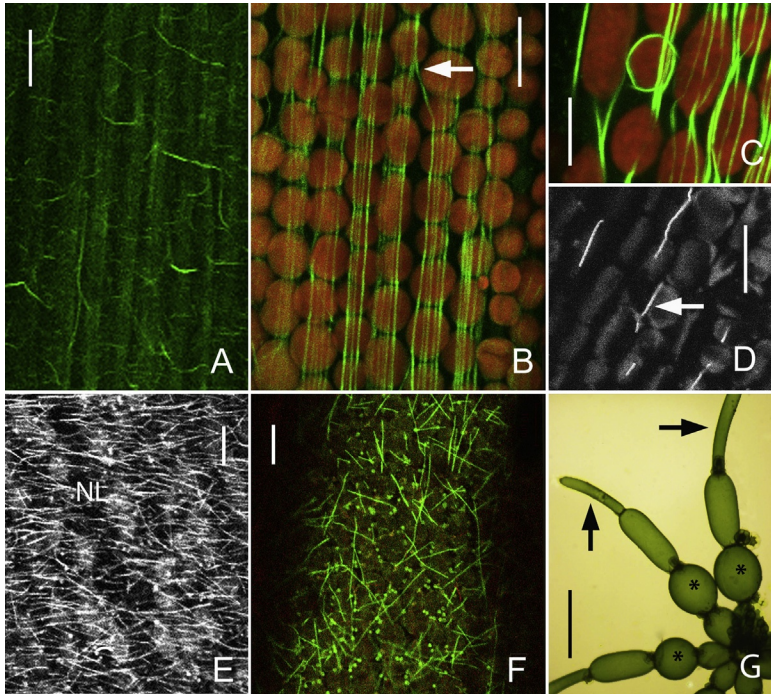


Figure 7.4 Cytoskeleton and microtubule-dependent shape of internodal cells of *Chara australis* (A, B, C, E, and F) and *Nitella pseudoflabellata* (D). (A–C) Cortical actin filaments (A) and subcortical actin bundles (B; merged with red fluorescent chloroplasts) labeled by perfusion with green fluorescent phalloidin. One chloroplast in (C) is surrounded by an actin filament ring. (D–F) Endoplasmic and cortical microtubules. (D) In young, elongating cells, endoplasmic microtubules (visualized by immunofluorescence with anti-tubulin; arrow) are occasionally present in the endoplasm and aligned parallel to the chloroplast files. (E and F) Cortical microtubules visualized by perfusion with fluorescent paclitaxel in an elongating (E) and in a mature cell (F). Note predominantly transverse orientation, even across the neutral line (NL) in the elongating cell. Punctate structures in (E) and (F) are unspecifically labeled lipid droplets. (G) Microtubule-dependent shape of internodal cells. Globular branchlet internodal cells marked by asterisks grew in the presence of the microtubule-depolymerizing oryzalin. Arrows point to elongate cells that had completed growth before treatment. Bars are 4 mm (G), 10 μ m (A, B, and D–F), and 5 μ m (C).

have been detected by cytochemical methods and immunolabeling (Schmölzer et al., 2011). The activity of these H^+ -ATPases is also thought to acidify the environment in order to facilitate uptake of HCO_3^- required for photosynthesis (Ray et al., 2003), but the absence of charasomes in cells capable of photosynthesis under highly alkaline conditions suggests otherwise (Lucas et al., 1989). It is likely that the charasomes also harbor other

transporters, for instance, protein(s) involved in chloride transport (Franceschi and Lucas, 1982). Structure and function of the charasomes are similar to the plasma membrane infoldings in transfer cells of mosses, ferns, and seed plants, which are specialized in transport of solutes and metabolites (Offler et al., 2003). The plasma membrane infoldings of transfer cells are stabilized by ingrowths of the secondary wall. In contrast, charasome membranes are stabilized by a yet unidentified proteoglycan (Pesacreta and Lucas, 1984).

The formation of charasomes requires a concerted action between exo- and endocytosis. So far, it is not clear whether the increase in plasma membrane area requires targeted exocytosis of vesicles, but it is clear that local inhibition of endocytosis is mandatory. Coated invaginations similar to clathrin-coated pits are present along the membranes of young charasomes. They do not, however, pinch off to form coated vesicles but instead appear to fuse with each other (Lucas and Franceschi, 1981). The inner, cytoplasmic membrane of mature charasomes is completely smooth. This indicates that clathrin-dependent endocytosis occurs only between charasomes where coated vesicles pinching off from coated pits are frequently observed (Lucas and Franceschi, 1981).

In young cells, charasomes are roundish with a diameter of up to 1 μm (Fig. 7.3B). In older cells, the charasomes may fuse with each other into sausage-shaped or branched structures (Fig. 7.3C) and eventually form a dense meshwork, especially in internodal cells of the branchlets. Electron micrographs show that the charasome tubules in these cells extend into the cell wall (Franceschi and Lucas, 1980). This tight association between cell wall and charasomes also becomes evident after plasmolysis, at which time the charasome-rich areas still adhere to the cell wall, while the charasome-poor zones are already detached (not shown). The formation of charasomes is light-dependent (photosynthesis-dependent) and occurs usually in close proximity to chloroplasts (Fig. 7.3D–F). Consistent with this, charasomes are absent from the chloroplast-free neutral lines and from cross walls. Charasome formation is favored by an alkaline pH of the culture medium (Lucas et al., 1986; Price et al., 1985). Interestingly, however, when charasome development is studied in branchlets isolated from their thalli and incubated in Petri dishes, charasome formation is favored by an acidic pH of the medium (Schmölzer et al., 2011). It is possible that these differences reflect the use of different carbon sources. During the culture experiments, containers are typically covered by a paraffin layer to prevent diffusion of CO_2 into the culture medium (Lucas et al., 1986) or filled with buffers to

maintain a specific $\text{CO}_2/\text{HCO}_3^-$ equilibrium (Price et al., 1985) so that cells are supplied with HCO_3^- . In contrast, cells incubated in Petri dishes are well supplied with CO_2 from the air (Schmölzer et al., 2011; see also Price et al., 1985). Further experiments, however, are necessary to prove this explanation.

Charasomes are larger and more abundant in internodal cells of the branchlets as compared with the internodal cells of the main axis. This is probably due to the fact that internodal cells of the vertical main axis are less important for photosynthesis than the internodal cells of the branchlets (Schulte et al., 1994). Charasome formation is reversible, that is, existing charasomes become degraded when photosynthesis is inhibited by darkness or inhibitors (e.g., Lucas et al., 1989; Schmölzer et al., 2011). When internodal cells are exposed to light intensities sufficient for the generation of a pH banding pattern, size, shape, and distribution of charasomes along the cell surface may become highly variable (see Section 3.1.2).

Numerous cellulose synthase complexes with a similar morphology and size to those found in other green algae and in higher plants are present at the plasma membrane in young, elongating internodal cells (McLean and Juniper, 1986) and are involved in the formation and deposition of cellulose microfibrils. Coated pits are common at smooth plasma membrane areas, especially in growing internodal cells (Lucas and Franceschi, 1981), and give rise to coated vesicles. They are, however, absent from the inner surface of mature charasomes (Lucas and Franceschi, 1981).

2.1.3 Chloroplasts

A conspicuous feature of characean internodal cells is their distinct helical organization (see earlier text and Figs. 7.1F and 7.3I). The cell wall twists as it grows and the resulting strains affect the organization of the cytoplasm. The major cytoplasmic features therefore describe a helix, which initially increases in pitch as the cell elongates before gradually decreasing over the remainder of the growth period (Green, 1959). The helical organization is most evident in the cell cortex, especially in the arrangement of chloroplasts, which are organized as helical files that can be right- or left-handed. The handedness may also differ between internodal cells of the main axis and those of the branchlets. We have noted, for instance, that in *C. australis*, the internodal cells of the main axis are right-handed, whereas those of the branchlets are left-handed.

Characean chloroplasts are similar to those of higher plants in terms of size, shape, structure, pigment composition, and genome sequence

(Fig. 7.3A) (Pickett-Heaps, 1967; Turmel et al., 2007). They are, however, firmly attached to the cell periphery and not able to reversibly change their position in response to ambient light as described for embryophytes (Königer and Bollinger, 2012; Wada, 2013). At the neutral line, which separates up- and downwardly streaming endoplasm, chloroplasts are smaller, more roundish, and less regularly arranged, and at the cross walls, chloroplasts are completely absent (McLean and Juniper, 1993). During cell growth, the number of chloroplast files remains fairly constant, but the size and the total number of chloroplasts greatly increase. The final number of chloroplast files (about 300 in *Nitella axillaris*) is already reached in very young internodal cells, which have a length of only 200 μm (Green, 1964). Chloroplasts remain relatively small during cell elongation, leaving enough space between them for vesicle trafficking toward and from the plasma membrane, which is required for deposition of cell wall components. Chloroplasts in young cells often have tapered ends and frequently appear to be connected to each other. Their division planes are always transverse to the file axis after a diffuse increase in surface area and strain-aligned elongation (Green, 1964). Chloroplast length and width nearly double over a period of 6 days until they have a length of about 7 μm and a width of about 4 μm in mature cells. In *N. axillaris*, the complete fission–fission cycle lasts about 1 day and mature internodal cells, about 80 mm long, contain about 4 million chloroplasts (Green, 1964). The chloroplasts in mature cells are also generally tightly packed, positioned obliquely to the cell periphery and partially overlapping like roof shingles. Dumbbell-shaped chloroplasts are also present in mature cells, but they do not appear to be able to complete the division furrow (Brian Gunning, personal communication). The stationary chloroplast files, together with the phenomenon of pH banding, provide an attractive model for studying various aspects of photosynthesis (e.g., Bulychev et al., 2005) (see Section 3.1).

2.2. Cytoskeleton

Characean internodal cells have been extremely useful for cytoskeletal research, and many seminal studies were conducted using *Nitella* or *Chara* internodes long before equivalent knowledge was obtained in land plant model systems. Actin was identified in *Chara* (Williamson, 1974) long before its presence was confirmed in land plants. Similarly myosin's Ca^{2+} -dependent association with actin filaments in *Chara* was described (Grolig et al., 1988) prior to myosin's identification in *Arabidopsis* (Knight and

Kendrick-Jones, 1993). In 1962, Paul Green predicted the presence of structures of spindle fiber nature would be found at the cell cortex (Green, 1962) prior to the first electron micrographs of cortical microtubules (Ledbetter and Porter, 1963). Branch-form microtubule assembly was first demonstrated in *Nitella*, and the presence of microtubule initiation factors along preexisting microtubules was predicted (Wasteneys et al., 1989) 16 years before gamma tubulin complexes were identified in angiosperms (Murata et al., 2005). The latest reviews on the cytoskeleton of internodal cells (Braun and Wasteneys, 2000; Foissner and Wasteneys, 2000a; Shimmen, 2007) reflect the abundant research carried out with these cells up until the end of the last century. Although extremely amenable for imaging and growth analysis, in recent years, the lack of a means to transform characean algae means that live-cell imaging with fluorescent cytoskeletal reporters has been conducted in other model systems such as *Arabidopsis thaliana*. Nevertheless, the contributions made with internodal cells suggest that once a genome project is complete and a transformation method established, these cells will regain their popularity for cytoskeletal research. Here, we highlight some important facts about the internodal cell actin and microtubule cytoskeleton.

2.2.1 Actin filaments and myosin motors

The early detection of microfilaments (Nagai and Rebhun, 1966) that were later confirmed to comprise actin (Palevitz and Hepler, 1975; Williamson, 1974) in characean internodal cells reflects the amenity and extensive use of these giant cells for studies on cytoplasmic streaming. Recently formed internodal cells have a meshwork of actin filaments and bundles with a random orientation and that traverse the cell through transvacuolar strands (Wasteneys et al., 1996). The transvacuolar strands disappear as internodal cells commence elongation and actin bundles become oriented in the subcortical cytoplasm approximately parallel to the newly established long axis. These subcortical actin bundles are organized in parallel groups of three to six that associate closely with the stationary chloroplast files that also run parallel to the direction of cytoplasmic streaming (Figs. 7.1F and G and 7.4B). Despite their close association with chloroplasts, several lines of evidence indicate that chloroplast files do not provide a spatial cue and are not required for the orienting the subcortical actin filament bundles. It has been shown, for example, that subcortical actin bundles maintain their parallel arrangement at the ends of cells from which chloroplasts are absent (McLean and Juniper, 1993; Wasteneys et al., 1996) and that actin bundles

remain intact after chloroplasts are dislodged from the cortex by mild detergent treatments (Williamson, 1985). Experiments in which actin bundles are induced to regenerate by wounding (Foissner and Wasteneys, 1997; Williamson and Hurley, 1986; Williamson et al., 1984) or to completely reorganize following combined treatment with cytochalasin D, which inhibits actin–myosin interaction, and oryzalin, which depolymerizes microtubules (Foissner and Wasteneys, 2000b), show that subcortical bundles establish new orientations that are often independent of the existing chloroplast files. Instead, chloroplasts readjust along the newly formed bundles or parallel to the new streaming direction (see also Section 3.3).

The prominent and highly organized subcortical actin bundles form the tracks along which ER and other organelles move in a myosin-dependent manner, driving the bulk cytoplasmic flow, which can exceed $70 \mu\text{m s}^{-1}$. Myosin's association with actin bundles was identified by immunolocalization (Grolig et al., 1988), and later studies confirmed organelle cargoes including nuclei (Wasteneys et al., 1996), mitochondria (Foissner, 2004) and endoplasmic reticulum (ER; Foissner et al., 2009). Characean myosins are acknowledged to be the fastest yet identified and are grouped with the myosin XI class, which is also found in land plants (Ito et al., 2007). The high speed of characean myosin can be attributed to the motor domain ATPase activity. In a recent study, the replacement of the *A. thaliana* myosin XI-2 motor domain by a myosin XI motor domain from *C. corallina* more than doubled the *in vitro* actin filament sliding velocity, increased the streaming velocity in *Arabidopsis* 1.7-fold, and, intriguingly, increased cell and plant size (Tominaga et al., 2013). The high velocity of characean myosins is well suited to the massive size of internodal cells since it enables efficient transport of cytoplasmic components.

Subcortical actin bundles may extend through the chloroplast files into the cortex. Most of the cortical actin filaments, however, appear to form independent of the subcortical bundles and are commonly separated from the cortical actin cytoskeleton by the chloroplast files. In the cortex of elongating internodes, a distinct array of actin strands near the plasma membrane is oriented transversely during cell growth (Fig. 7.4A), whereas the cortical actin filaments in mature cells have a random orientation (Wasteneys et al., 1996). Rings of actin filaments have also been observed around the chloroplasts bordering the neutral line (Fig. 7.4C) (Wasteneys et al., 1996).

2.2.2 Microtubules

The simple cylindrical shape, massive size, and long growth phase of internodal cells provide an ideal system for investigating the role of cortical

microtubule organization in unidirectional expansion (Wasteney, 1992). The vacuolar perfusion technique (see Section 4.3) makes these cells particularly amenable for immunofluorescence-based imaging of microtubules (Wasteney and Williamson, 1987), and they were also the first cells, along with *Tradescantia virginiana* stamen hair cells, to be successfully microinjected with fluorescently tagged brain tubulin for live-cell imaging of cortical microtubule dynamics (Wasteney et al., 1993). Despite this pedigree, characean algae remain recalcitrant to genetic transformation methods required for live-cell imaging of translational reporter fusion proteins. Consequently, there are few recent studies specifically on microtubule organization in characean internodal cells. We highlight here some of the most pertinent contributions to microtubule biology.

The vast areas of cortex and the ability to flatten out perfused internodal cells provided a unique opportunity to carry out quantification of cortical microtubule length and orientation relative to the axis of elongation and to plot this against cell age and rates of expansion (Wasteney and Williamson, 1987). This quantitative analysis demonstrated that there is a strong correlation between relative elemental elongation rates (strain) and transverse orientation of cortical microtubules (Fig. 7.4E), consistent with the earlier analysis of cellulose microfibril orientation patterns. As with microtubule arrays in land plants (Sugimoto et al., 2000), increased deviation from the transverse axis occurs as elongation rates decline and nongrowing cells have both fewer and randomly oriented microtubules (Fig. 7.4F) (Wasteney and Williamson, 1987, 1993). Microtubule orientation patterns documented by immunofluorescence generally matched the patterns for cellulose microfibrils described in earlier studies (Gertel and Green, 1977; Richmond et al., 1980). This is largely consistent with studies showing that the removal of microtubules through treatment with depolymerizing drugs leads to randomized microfibrils and isotropic growth (Fig. 7.4G) (Green, 1962; Richmond et al., 1980). Evidence, however, suggests that microtubules play no role in orienting cellulose microfibrils in mature nongrowing cells (Richmond et al., 1980) in which a helicoidal texture, likely to form through a self-assembly mechanism, has been detected (Neville and Levy, 1984).

Characean internodal cells have been useful for identifying microtubule nucleation mechanisms in plant cells, which lack centrosomes. Early studies indicated that the nuclear envelope could act as a microtubule-nucleating center in plant cells (De Mey et al., 1982; Schmit, 2002), yet this mechanism seemed problematic for populating the cortex of cells, especially when cells are rapidly expanding. In characean internodal cells, the separation of

endoplasmic nuclei from the cortex provided an ideal system to determine the site of microtubule nucleation. Vacuolar perfusion was used to first remove endogenous tubulin and then to introduce biotinylated brain tubulin, resulting in microtubule assembly both at the nuclei and at unspecified sites in the cortex (Wasteneys et al., 1989). The results of this semi-*in vitro* assay were supported by the observation that after the depolymerization of microtubules with the drug oryzalin, microtubules reappeared in the cortex (Wasteneys and Williamson, 1989a). More importantly, this latter study characterized branch-form microtubule assembly, whereby new microtubules are initiated at discrete sites along the length of preexisting microtubules. Subsequent research has confirmed that this is a common mechanism of microtubule assembly in plant as well as other eukaryotic cells and that it is dependent on protein complexes including gamma tubulin and associated proteins (Murata et al., 2005).

Branch-form microtubule assembly not only provides a means to initiate new microtubules throughout the cortex but also generates new microtubules at a wide range of angles, which could be involved in changing the predominant orientation of microtubules (Wasteneys and Ambrose, 2009). The quantification of the recovery of transverse microtubule arrays in elongating internodes showed that branch-form assembly perturbs transverse order but that once maximum polymer mass is achieved, transverse order is reestablished (Wasteneys and Williamson, 1989b). This latter finding, which strongly suggested a self-organizing mechanism called “biased turnover” (Wasteneys and Williamson, 1989b) or “selective stabilization” (Wasteneys, 2002), has been an important foundation for recent mathematical modeling on microtubule spatial organization mechanisms, in which parallel order is fostered by catastrophes induced by high-angle encounters (Allard et al., 2010; Eren et al., 2010; Wasteneys and Ambrose, 2009).

Live imaging of microtubules in *Nitella* internodal cells was achieved by microinjecting fluorescently tagged brain tubulin (Wasteneys et al., 1993). The ability to isolate individual internodes makes them ideal for microinjection work. Paradoxically, internodes of the most commonly used charophyte species were found to be too large and smaller species that were more suited to injecting and imaging needed to be collected. Brain tubulin incorporated into the *Nitella* cortical microtubules within minutes, confirming the dynamic nature of cortical microtubules. Importantly, live imaging confirmed many of the previous observations made with fixed material, including the integrated but discontinuous nature of the array and the presence of cortical assembly sites including nucleation along existing

microtubules (Wasteneys et al., 1993). It was found that microtubules have a wide range of survival times, from just a few to more than 40 min (Kropf et al., 1997; Wasteneys et al., 1993), and that disordered microtubules at wound sites are less stable than their transversely oriented counterparts (Foissner and Wasteneys, 1994). These observations, along with the observation of apparent bipolar growth of microtubules (Wasteneys et al., 1993), suggest that the structures observed by fluorescence microscopy frequently comprise two or more bundled microtubules, which is consistent with electron microscopic observations (Foissner and Wasteneys, 1999), and that bundling can confer relative stability. In retrospect, the microtubule live imaging work with characean internodal cells was limited by the computational constraints and photobleaching tendencies of first-generation point-scanning confocal microscopes.

In very young, elongating cells, microtubules are present not only in the cortex of internodal cells but also in the endoplasm where they align parallel to the actin filament bundles (Fig. 7.4D) (Wasteneys and Williamson, 1991).

2.3. Shape and dynamics of cortical organelles

The stationary chloroplast files in characean internodal cells shield the cortical cytoskeleton and organelles from the streaming endoplasm (Fig. 7.1F and G). This allows studying the dynamics of cortical organelles independently of the active and hydrodynamic forces generated by cytoplasmic streaming. Cortical actin filaments are responsible for active movement of various vesicles toward, away from, and along the plasma membrane. Cortical actin filaments and cortical microtubules cooperate in the dynamics of cortical mitochondria and cortical ER and eventually determine the shape of these organelles depending on the morphogenetic stage of the cell. Experiments with inhibitors of the actin/myosin cytoskeleton indicate that organelles use actin filaments for trafficking from the endoplasm toward the cortex or vice versa, but Brownian motion is also sufficient for crossing the interstices between the stationary chloroplasts (Klima and Foissner, 2008).

2.3.1 Cell wall-forming vesicles and endosomes

Just as in other diffusely elongating plant cells, cortical microtubules have a critical role in the deposition of cellulose microfibrils in the cell wall and thereby control cell shape (see Section 2.2.2). Actin filaments, in contrast, are responsible for the delivery of cell wall-forming vesicles toward the plasma membrane and for trafficking of endosomal organelles away from the plasma membrane.

Polysaccharide-containing vesicles with a diameter between 0.1 and 1.4 μm are abundant at the plasma membrane. In electron micrographs obtained after conventional chemical fixation and staining, these vesicles have a characteristic dense core separated from the membrane by a nonstaining space (Franceschi and Lucas, 1981b; Pickett-Heaps, 1967). Cytochemical data indicate that they contain polysaccharides for which they were called glycosomes (Franceschi and Lucas, 1981a). These vesicles appear to be released from the *trans*-Golgi network (“partially coated reticulum”; Fig. 7.6G) (Pesacreta and Lucas, 1984) and may fuse with the plasma membrane to deliver their content to the forming cell wall (Foissner, 1988a,b; Franceschi and Lucas, 1981b). The larger ones can be observed in the light microscope, and their involvement in the deposition of a wound wall has been described using video-enhanced contrast microscopy (Foissner et al., 1996, 1998). Smaller vesicles with more homogeneous, as yet unidentified content, are also found in the cortex and are probably derived from the Golgi bodies (Fig. 7.6G).

Within minutes of staining internodal cells with FM dyes such as FM1-43 or FM4-64, numerous punctate structures appear in the cortex, which then move toward the endoplasm. It is likely that these minute organelles correspond to coated vesicles and early endosomes (Ito et al, 2012; Klima and Foissner, 2008), but further studies are required to firmly reveal their identity in characean internodes and other plant cells.

2.3.2 Cortical endoplasmic reticulum

In plant cells, the ER consists of a relatively stable cortical meshwork and a highly dynamic endoplasmic tubular system (Fig. 7.3G,H and 7.6A) (Sparkes et al., 2009). In characean internodal cells, cortical (sometimes referred to as ectoplasmic) and inner (endoplasmic) ER (see Section 2.2.1) are separated from each other by stationary chloroplast files although connected by tubes winding through the chloroplast clefts. The morphology and dynamics of the cortical ER has been documented over the course of internodal cell growth (Foissner et al., 2009). In very young cells, the cortical ER covers nearly the whole inner surface of the cell with only few, small gaps. At the onset of elongation, the cortical ER forms a tight meshwork of predominantly transverse ER tubules with mesh widths rarely exceeding 500 nm and covering about 50% of the cell surface. These meshes have a predominantly transverse orientation in elongating cells and frequently change their size and their shape (Fig. 7.3G). During rapid cell growth, cortical ER dynamics and organization is dependent on the

transversely aligned cortical microtubules, as demonstrated when microtubules are depolymerized. This abolishes the transverse orientation of the ER, reduces motility, and increases mesh size. In land plant cells, no ER association with microtubules has so far been described. It is thus possible that this is a special feature of the giant internodal cells, in common with animal cells. Alternatively, microtubule–ER association could be restricted to shorter periods in the cell cycle of land plant cells and has thus escaped notice. As cells mature, the widths of the meshes increase further up to 5 μm , and the proportion of the cell surface covered by ER declines to about 20%. At this stage, the microtubule–ER association ceases and the transverse orientation is lost (Fig. 7.3H). Cortical actin filaments seem to have a stabilizing function on the ER meshes in both elongating and mature cells because of an increase in motility after actin reorganization by cytochalasins, a phenomenon also described from other plant cells (Sparkes et al., 2009).

In electron micrographs, the outer membrane of the cortical ER cisternae, facing the plasma membrane, appears smooth, whereas the inner membrane, facing the chloroplasts, is often decorated by ribosomes (own unpublished results). Continuities between the cortical ER and the outer chloroplast membrane have been described by McLean et al. (1988) and are consistent with the staining of the chloroplast envelope by DiOC₆ (3,3'-dihexyloxycarbocyanine iodide) (Fig. 7.6A) (Foissner et al., 2009). Preliminary data indicate that in *Chara* internodal cells, the cortical ER extends between charasomes and is largely absent from their inner surface (Foissner, 2009).

Live-cell imaging work shows that fluorescently stained lipid droplets appear to be closely associated with the fluorescently labeled cortical ER, suggesting that they form within or at least close to the ER tubes (Fig. 7.3G). They move along or together with the cortical ER cisternae independently of actin filaments and microtubules (Foissner, 2009). The number and size of lipid droplets increase with cell age. In internodal cells of *C. australis*, they may reach a diameter of up to 3.2 μm (Fig. 7.3I) (Foissner, 2009). On ultrathin TEM sections, the lipid droplets probably correspond to electron-dense globules with a white core (Fig. 7.3I, inset). They are, however, not surrounded by a membrane. Further studies are necessary to clarify their relationship with the ER.

2.3.3 Cortical mitochondria and peroxisomes

In characean internodal cells, shape, size, and location of mitochondria vary according to the developmental stage, environmental conditions, and the

pH banding pattern (Foissner, 2004; Schmölzer et al., 2011) (see Section 3.1.2). In cells grown under moderate or high light intensities ($\geq 10 \mu\text{Einstein m}^{-2} \text{s}^{-1}$), mitochondria are small and have the usual ellipsoid to elongate shape with a length between 1 and 2 μm and a diameter of about 500 nm (Figs. 7.3C–F and 7.5A–F). Mitochondria from cells grown under lower light intensities are often much larger and more variable in shape (Fig. 7.5G–I and Section 3.2). In very young, still slowly growing internodes harvested from the upper part of the thallus, mitochondria are nearly exclusively located in the endoplasm. Later in cellular development, an increasing number of mitochondria appear in the cortex. The maximum number of cortical mitochondria seems to be reached in rapidly elongating internodes or in internodal cells that have just ceased elongation and that are active in photosynthesis. Post maturation, the number of cortical mitochondria declines (own unpublished observations).

The migration of mitochondria between the endoplasm and the cortex mostly occurs along straight or curved tracks that are probably extensions of subcortical actin bundles. Other mitochondria appear to oscillate, indicating that Brownian movement can also drive mitochondria across the stationary

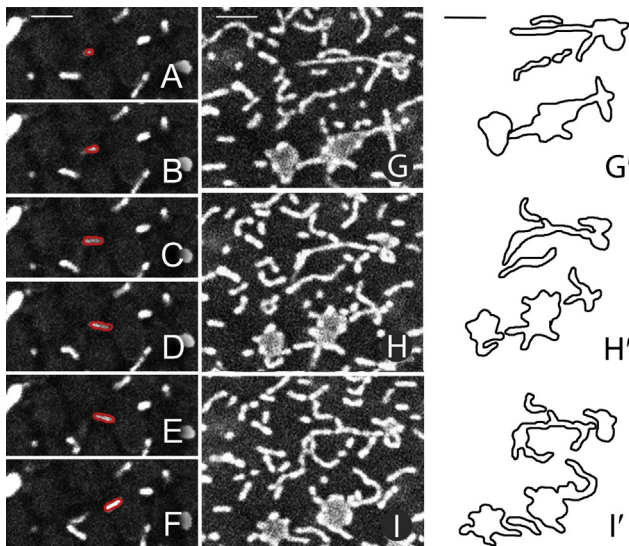


Figure 7.5 Dynamics of cortical mitochondria in an internodal cell of *Chara australis* visualized by fluorescent dyes. (A–F) Movement of a short mitochondrion (outlined white and indicated by arrows) from the endoplasm toward the cell periphery. (G–I) (fluorescence images) and (G'–I') (outlines) Shape changes, fusion, and fission of elongate and amoeboid mitochondria. Time interval between images is 10 (A–F) and 60 s (G–I). Bars are 5 μm .

chloroplast layer (Foissner, 2004). In the cortex of rapidly elongating internodes, mitochondria not only interact with actin filaments or bundles but also align at and move along the transversely oriented cortical microtubules (Fig. 7.5). Mitochondria of characean cells are therefore equipped not only with myosin motors but also with motor proteins that allow the interaction with microtubules (most likely kinesins; Li et al., 2012; Ni et al., 2005; Yang et al., 2011). This dual interaction with actin filaments and microtubules also seems to play an important role in the differential, pH banding-dependent distribution of these organelles (see section 3.1.2) and affects the dynamics and shape of the giant mitochondria formed under low light intensity or anoxic conditions (Fig. 7.5G–I and Section 3.1.2). Mitochondria of higher plant cells migrate along actin filaments and use microtubules for their positioning (anchorage; Van Gestel et al., 2002) possibly via dynamin-like proteins (Wang et al., 2012). To our knowledge, active movement of mitochondria along microtubules has so far not been described in land plants.

The fine structure of mitochondria in characean internodal cells is similar to that of land plants with irregular cristae, that is, infoldings of the inner membrane, which are slightly constricted at their bases (Fig. 7.3C).

Peroxisomes of characean internodal cells cannot be stained by vital dyes, and therefore, nothing is known about their dynamics. They are, however, easily identified on ultrathin sections because of their catalase crystals, and antibodies against catalase can be used to study their distribution by immunofluorescence at the light (fluorescence) microscopy level (Fig. 7.3F). Apart from the presence of catalase, the enzyme composition of peroxisomes in characean algae (e.g., glycolate oxidase, hydroxypyruvate reductase, acyl-CoA dehydrogenase, hydroxyacyl-CoA dehydrogenase, and enoyl-CoA hydratase) resembles that of peroxisomes from land plants (Stabenau and Winkler, 2005; Stabenau et al., 2003). It is thus reasonable to assume that they are involved in photorespiration and in fatty acid oxidation. Further studies are required to clarify their role in photosynthesis in characean internodal cells (see Section 3.1).

2.4. Organelles in the streaming endoplasm

2.4.1 Endoplasmic reticulum

The morphology and dynamics of the inner ER (Fig. 7.6A) is distinct from that of the cortical ER (Fig. 7.3G and H) (Foissner et al., 2009). Whereas cortical ER is flat and reticulate, the inner ER consists of three-dimensional branched tubes that pervade the whole endoplasm (Fig. 7.6A). The close

contact of the ER tubes with the subcortical actin bundles (Kachar and Reese, 1988) is consistent with the localization of a myosin motor anchored to the ER membrane and interacting with the actin filaments to produce rotational streaming (Kachar and Reese, 1988; Kashiyama et al., 2000; Morimatsu et al., 2000; Shimmen and Yokota, 2004; Sumiyoshi et al.,

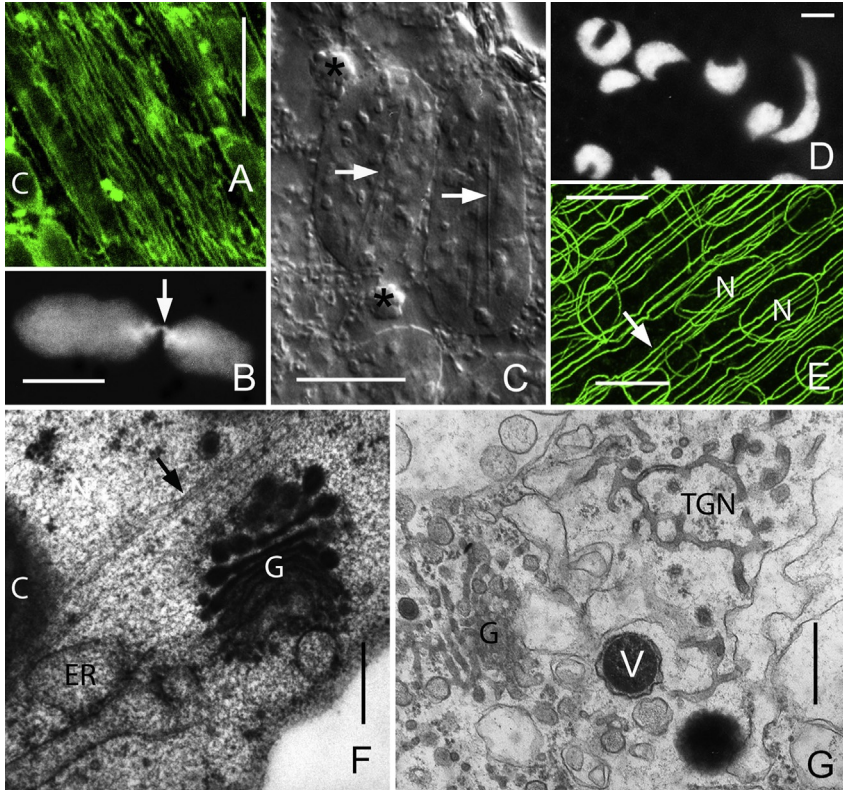


Figure 7.6 Endoplasmic organelles in internodal cells of *Nitella flexilis* (A, B, E, and G), *N. pseudoflabellata* (C), *Chara australis* (D), and *C. braunii* (F). (A) Subcortical tubes of DiOC₆-stained endoplasmic reticulum. The chloroplast (C) envelope is also stained by the fluorescent dye. (B) DAPI-labeled nucleus with a brightly fluorescent lampbrush chromosome-like structure at a nuclear fragmentation site (arrow). (C) DIC image of two nuclei with tubular inclusions (arrows) extending along and between nucleoli. Asterisks mark solid inclusions formed within ER cisternae. (D) Small and crescent-shaped nuclei in a senescent internodal cell. (E) Subcortical actin filament bundles (arrow) and nuclear (N) actin rings visualized by immunofluorescence. (F and G) Transmission electron micrographs of a high-pressure frozen and cryosubstituted (G) and a chemically fixed cell (H). In (F), a cisterna of the endoplasmic reticulum (ER) and a Golgi body (G) are seen in close contact to a subcortical actin filament bundle (arrow), which is located at the inner chloroplast (C) surface. Trans-Golgi network (TGN) and vesicles (V) are abundant in the endoplasm. Bars are 10 μm (A–E) and 400 nm (G and F).

2007). In addition, ER tubes seem to have an intrinsic motility because they perform snakelike motions in cells in which cytoplasmic streaming is arrested by cytochalasin D (Foissner et al., 2009). Continuities between the chloroplast membrane and the ER have been described from electron micrographs (McLean et al., 1988) and are obvious in the fluorescence microscope after staining with appropriate dyes (Fig. 7.6A) (Foissner, 2009).

The ER seems to be involved in the formation of solid bodies that are eventually released into the vacuole (Fig. 7.7C–G). They are up to 100 μm in diameter or length and, depending on the species or genus, have a characteristic shape. *N. flexilis* forms “echinate inclusions” (Fig. 7.7E) consisting of a polysaccharide core and a thin protein layer with traces of tannin (Overton, 1890; Silverberg and Sawa, 1974; Votava, 1914). Solid bodies with more irregular, clumpy shapes are found in the endoplasm and/or vacuole of other species (Fig. 7.7C, F, and G). The solid bodies found in *Nitella* species are released into the vacuole but remain surrounded by a membrane of ER origin as suggested by staining with DiOC₆ (Fig. 7.7C and D) (Foissner, 1988a,b; Härtel, 1951; Silverberg and Sawa, 1974). In the genus *Chara*, the solid bodies remain in the inner region of the endoplasm close to the tonoplast of the central vacuole (own unpublished results).

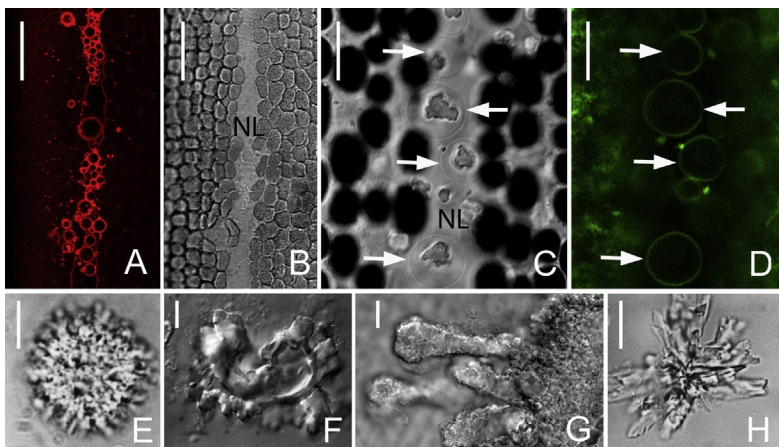


Figure 7.7 Vacuoles and vacuolar inclusions in internodal cells of *Chara australis* (A, B, and H) and in internodes of various *Nitella* species (C–G). (A and B) Small vacuoles stained by Lysotracker red along the neutral line (A, fluorescence image; B, the corresponding bright-field image). (C and D) Vacuoles with solid inclusions are surrounded by a membrane stained with DiOC₆ (arrows in the DIC image C and in the fluorescence image D). (E–G) Solid carbohydrate- and protein-containing vacuolar inclusions (E, bright field; F and G, DIC). (H) Vacuolar crystal (bright field). Bars are 20 μm (A and B), 10 μm (C and D), 5 μm (F and G), and 2 μm (E–H).

It has been speculated that the clumpy bodies or the echinate inclusions serve as reserve material, but Härtel (1951) did not observe any decrease in size or number of echinate inclusions over a period of several weeks' darkness, consistent with our own observations. It is more likely that these solid bodies are designed for wound repair because they instantaneously plug cell wall holes, thereby preventing further loss of the cytoplasm (Foissner, 1988a,b; see Section 3.3.3). Similar ER-derived protein bodies are involved in wound healing in *Bryopsis*, a multinucleate siphonous organism (Burr and Evert, 1971; Menzel, 1988). The solid bodies in internodal cells thus add another perspective concerning the amazing diversity of ER-derived compartments (Hara-Nishimura et al., 2004; Staehelin, 1997).

A special agglomeration of ER membranes, the "Spitzenkörper," is found in the apex of the tip-growing rhizoids (Limbach et al., 2008). It is involved in gravitropic signaling and in the guidance of secretory vesicles required for the immediate and precise adjustment of the growth direction in response to gravity-induced statolith sedimentation.

2.4.2 Golgi bodies, trans-Golgi network, and derived vesicles

In very young, elongating cells or in cells grown under low light intensities, Golgi bodies or *trans*-Golgi networks are occasionally seen close to the plasma membrane between the widely spaced, small chloroplasts or at the neutral line (Fig. 7.3A). In mature cells with large, densely packed chloroplasts, Golgi bodies and TGNs are exclusively located in the streaming endoplasm. The Golgi bodies of mature cells are mostly oriented parallel to the ER tubes that glide along the subcortical actin bundles (Fig. 7.6F and G). They consist of up to seven cisternae from which small vesicles with homogeneous contents detach.

The Golgi bodies are associated with the *trans*-Golgi network (Fig. 7.6G). This organelle was firstly described in characean internodal cells as "partially coated reticulum" by Pesacreta and Lucas (1984) who identified it as an autonomous organelle on the basis of structural and cytochemical data and who already suggested an involvement in membrane recycling. Only later it becomes clear that the *trans*-Golgi network is a consistent feature of plant cells and functions as an early endosome (Dettmer et al., 2006). Very likely, at least some of the FM-stained organelles in the endoplasm correspond to the *trans*-Golgi network (Ito et al., 2012; Klima and Foissner, 2008).

2.4.3 Nuclei and other organelles

Internodal cells of the main axis develop from a uninucleate segment cell (Pickett-Heaps, 1967). This nucleus and all further nuclei divide in the

absence of a spindle, a process that is referred to as amitosis (equal division) or fragmentation (unequal division) (Bucher, 1959; Kisser, 1922; Shen, 1967a). Fragmentation is nonsynchronous and appears to be mostly transverse to the longitudinal axis of nuclei, but longitudinal fragmentation has also been observed (Foissner and Wasteneys, 2000c; Shen, 1967a; Vouilloud et al., 2007). Mature internodal cells contain up to several thousand nuclei, which are exclusively localized in the streaming endoplasm. All nuclei so far investigated are flattened with a thickness that is about half to two-thirds of the width. The overall shapes of the nuclei are very characteristic for the three genera investigated up to now (Fig. 7.6B–D) (Foissner and Wasteneys, 2000c). In internodal cells of *Nitella* species, nuclei were always straight and mostly dumbbell-shaped with tapered or slightly pointed ends and tapering symmetrical constrictions (Fig. 7.6B and C). In cells that have reached their final length, the *Nitella* nuclei also have characteristic furrows along their surface that are parallel or oblique to the longitudinal axis (Foissner and Wasteneys, 2000c). Species of the genus *Chara* contain crescent-shaped, kidney-shaped, or nearly disk like nuclei (Fig. 7.6D). Nuclei of *Nitellopsis obtusa* have an ellipsoidal to allantoidal shape with characteristic lateral narrow indentations (Foissner and Wasteneys, 2000c).

Apart from these genus-specific characteristics, shape and size of nuclei vary considerably between species, between cells of the same thallus, and even within a single cell (Foissner and Wasteneys, 2000c). Species with long internodal cells tend to have larger nuclei, but in the smaller- and medium-sized species, no correlation between peak nuclear size and potential cell size can be observed. Within a given thallus, the nuclei of the smaller internodal cells of the branchlets were always smaller by 10–20% than the nuclei of internodal cells of the main axis.

Most striking are the changes in nuclear size and shape, which can be observed during cell development. Young, rapidly elongating internodal cells contain the largest nuclei with a mean length varying from 22 to 56 μm (Fig. 7.6B and C) (Foissner and Wasteneys, 2000c). These nuclei are much larger than the single nucleus of the segment cell from which the internode develops. The mean length of nuclei in senescent internodes varies between 7 and 34 μm (Fig. 7.6D), and the decrease in size often comes along with a simpler roundish or elongate shape. The increasing abundance of nuclei in mature cells is countered by a decline in the average size of nuclei. The nuclear area fraction, that is, the percentage of cytoplasmic area occupied by nuclear profiles, is highest in the youngest, rapidly elongating internodal cells, declines in slowly elongating and mature internodes, and recovers again in senescent internodes. Microspectrofluorometry shows that

the DNA content of nuclei increases up to 20-fold from the first to the fourth internode of *Chara zeylanica* and decreases again in older cells (Shen, 1967b). Nuclei are therefore polyploid only in proliferating internodes and not in senescent cells. These data suggest that the processes of nuclear fragmentation and DNA replication lag behind cell expansion and that the nucleocytoplasmic index varies in internodal cells of different age (Foissner and Wasteneys, 2000c).

The nuclei of the different species also vary in terms of the presence or absence of various inclusions and of cytoskeletal elements (actin filaments and microtubules) associated with their surface. Tubular crystals have been reported from nuclei of all internodal cells so far investigated (Fig. 7.6C) (Barton, 1967; Foissner and Wasteneys, 2000c; Pickett-Heaps, 1967; Vouilloud et al., 2007). They consist of one to several hexagonally packed tubules that have a diameter between 20 and 28 nm. This diameter is similar to that of microtubules but these tubules cannot be stained with antibodies against tubulin. The tubular crystals mostly extend along the whole nucleus, either parallel or slightly oblique to the longitudinal axis and are often associated with nucleoli. The percentage of nuclei with tubular crystals varies between species. If present, nuclei usually contain one crystal but up to 10 crystals per nucleus have also been observed. They appear to be the last structures linking the daughter nuclei during fragmentation.

In various species of *Nitella*, lampbrush chromosome-like structures with a length of up to 62 μm and a width of up to 3 μm can be observed, and their location at the constriction sites suggests involvement in the distribution of genetic material during fragmentation (Fig. 7.6B) (Foissner and Wasteneys, 2000c; Gillet and Lefebvre, 1963). Nuclei with lampbrush chromosome-like structures never contain tubular crystals, and tubular crystals exclude the presence of lampbrush chromosome-like structures. The percentage of nuclei with lampbrush chromosome-like structures in internodal cells of *N. flexilis* increases with the age of internodal cells. Spindle-shaped protein crystals up to 15 μm in length have been reported from internodal cells of the branchlets of one population of *N. flexilis* (not from others). They form during aging and are released into the cytoplasm in senescent cells (Foissner and Wasteneys, 2000c).

“True microtubules” recognized by appropriate antibodies have been described from the nuclear surface in elongating internodal cells of *C. corallina* (*australis*) and *Nitella cristata* (Wasteneys and Williamson, 1991). Actin filament bundles or more delicate actin strands are commonly seen at the nuclear surface in many species, especially in those of the genus

Nitella (Fig. 7.6E). The nuclei encircled by actin bundles are able to perform rotational movements in the streaming endoplasm or in cytoplasmic droplets (Wasteneys and Williamson, 1991). These actin bundles also support organelle movements along the nuclear surface. The function and advantage of these perinuclear cytoskeletal elements is unclear. It can, however, be excluded that they are involved in nuclear fragmentation.

Mitochondria (described in detail in Section 2.3.3) normally move as part of the rapid mass streaming. When cytoplasmic streaming is temporarily arrested, for example, after an action potential, mitochondria and other organelles (e.g., polysaccharide-containing vesicles) often move in close proximity to the subcortical actin bundles. Their velocity is then between 10 and 20 $\mu\text{m s}^{-1}$, far lower than the rate of cytoplasmic mass streaming. This close contact between mitochondria and actin filament bundles suggests direct interaction via myosin motors and can also be observed in cytoplasmic droplets squeezed out from internodal cells. The shape of long, giant mitochondria changes slowly during mass streaming, but the overall form remains the same. Active alterations in shape such as branching or amoeboid movement as described from cortical mitochondria do not occur in the endoplasm.

Chloroplasts may irreversibly detach from the cortex during wounding (see Section 3.3) and are then carried along with the streaming endoplasm. In young cells, these endoplasmic chloroplasts may increase considerably in size and become circular or cup-shaped. These observations confirm that the cortical chloroplasts elongate parallel to the direction of maximum strain (Green, 1964) and suggest that chloroplast division requires contact with components of the cortical cytoplasm and/or other chloroplasts. Chloroplasts associated with actin filaments or bundles actively rotate while being passively transported by the endoplasm and may become multiangular just as the actin filament bundles in cytoplasmic droplets, which undergo self-organization into rings and polygons (Higashi-Fujime, 1980; Jarosch, 1976; Wasteneys et al., 1996). Chloroplasts may also be released as files, still connected by subcortical actin bundles, and are then able to perform active, snakelike movements within the streaming endoplasm.

2.5. Central vacuole and vacuolar inclusions

The central vacuole of characean internodes is one of the largest eukaryotic organelles known. As with most plant cells, it occupies up to 95% of the cell volume, enabling up to 50 μl cell sap to be collected from single internodal

cells for the analysis of ions and metabolites (Oikawa et al., 2011; Sakano and Tazawa, 1984; Schulte et al., 1994). From such analysis, vacuoles have been found to accumulate large amounts of sucrose, indicative of storage rather than lytic function. Consistent with this, the tonoplast of the central vacuole is not stained by endocytic markers such as FM1-43, which are delivered to the tonoplast via membrane trafficking (Bolte et al., 2004). Distinct smaller vacuoles are found within the streaming endoplasm and occasionally at the nonstreaming neutral line where they are easier to study. Their membrane is stained by Lysotracker red and FM dyes suggesting lytic function (Fig. 7.7A and B) (Klima and Foissner, 2008). Both the central and smaller vacuoles accumulate Lysotracker yellow indicating acidic pH (Klima and Foissner, 2008).

Prior to elongation, the vacuole of young internodal cells is small and traversed by cytoplasmic strands (Wasteneys et al., 1996). Vacuole formation also occurs in cytoplasm-rich fragments, which are produced by centrifuging and ligating internodes (Beilby and Shepherd, 1989, 1991). The fact that these fragments do not undergo cell expansion suggests that vacuole formation and function extends beyond growth-related osmoregulation. A complex system of canalicular elements has been described in young cells of the salt-tolerant *L. papulosum* (Beilby et al., 1999).

The central vacuole of internodal cells not only is filled with cell sap but also contains solid inclusions (Fig. 7.7C–H). In various species of *Nitella*, solid bodies consisting of a polysaccharide core and covered by a protein layer (Silverberg and Sawa, 1974) are present within the vacuole. They are surrounded by a DiOC₆-stained membrane indicating an origin within ER cisternae (Fig. 7.7C and D) (see Section 2.4.1). The size and shape of the solid inclusions vary considerably between species and can probably be used for species identification (Fig. 7.7C–G). In *C. australis*, these polysaccharide/protein bodies remain within the ER in the perivacuolar regions of the endoplasm. In these cells, the vacuole contains spiny crystals likely of organic composition based on their solubility in ethanol or acetone (Fig. 7.7H; own unpublished observations). Whether these crystals form in the endoplasm or in the vacuole is not yet known. Both solid bodies and crystals are involved in wound healing (see Section 3.3.3).

The central vacuole is highly dynamic and its content is continuously mixed as a consequence of cytoplasmic streaming. Only the middle region where the upward and downward streaming paths meet is relatively stagnant. When streaming is arrested, the solid inclusions accumulate at the bottom of the cell. Uneven recovery of streaming may cause bulging of

endoplasm at nonstreaming or slowly streaming regions due to supply from recovered areas. These bulges may detach into the vacuole and may survive for several days as a natural cytoplasmic droplet.



3. CHANGES IN CYTOPLASMIC ORGANIZATION DUE TO ENVIRONMENTAL CONDITIONS AND IMPACTS

3.1. Photosynthetic pH banding pattern

Arens (1939) was probably the first to describe the “banding phenomenon” in internodal cells as an alternating sequence of CaCO_3 -rich and CaCO_3 -poor cell wall regions. Several decades later, Spear et al. (1969) described alternating acid and alkaline zones in the medium adjacent to the surface of internodal cells. The pH banding pattern is dependent on photosynthesis and cytoplasmic streaming (e.g., Bulychev et al., 2001b; Lucas and Dainty, 1977). The local acidification of the medium is thought to convert poorly permeable HCO_3^- into CO_2 , which diffuses into the cell where it is used for photosynthetic carbon assimilation (Chau et al., 1994). Consequently, the rate of photosynthesis is enhanced at the acid regions although chloroplasts are more or less homogeneously distributed along the lateral wall of internodal cells (Bulychev et al., 2001a; Lucas and Smith, 1973; Plieth et al., 1994).

The differences in extracellular pH of strongly banding cells can easily be demonstrated by incubating cells in media with 5–10 μM phenol red as an indicator dye (Fig. 7.1C). The detection of small differences in pH requires the use of pH electrodes. The pH banding pattern has been reported to be quite changeable in young cells (Bisson et al., 1991; Bulychev et al., 2003; Dorn and Weisenseel, 1984; Fisahn and Lucas, 1990b) and in cells that recover from dark treatment or treatment with inhibitors of photosynthesis or cytoplasmic streaming or after the generation of an action potential (Eremin et al., 2007). A stable pattern of pH bands is formed in mature and fully recovered cells. The pH of the small sharply peaked alkaline regions ranges from 8.5 to 10.5, whereas the acid bands that may extend over several dozen micrometers have a more uniform pH varying between 7.2 and 8 in *Chara* (Bulychev et al., 2003; Lucas et al., 1983). The alkaline zones are not always present as circumferential “bands” or rings extending over the whole cell surface but may also consist of smaller, punctate “patches” (Bulychev et al., 2003). At the alkaline bands, CaCO_3 encrustations are frequently found at the surface of the cell wall, and Sr and Mn ions also have been detected (Fig. 7.1D and E) (McConnaughey and Falk, 1991). CaCO_3

encrustations may account for up to 56% of the dry weight (Raven et al., 1986). Experimental studies of ion flux have shown that acidification is generated by the activity of plasma membrane bound H^+ -ATPases (Tazawa, 2003a,b; Tazawa and Shimmen, 2001). The channels responsible for the alkaline pH are not yet identified and may be responsible for either inward-directed H^+ fluxes or outward-directed OH^- fluxes (Beilby and Bisson, 1992; Lucas, 1979; Smith and Walker, 1983). Irrespective of how the pH banding pattern is generated, it has important consequences for the size and distribution of cortical organelles and even for the properties of the cell wall as outlined later.

3.1.1 pH banding-dependent variations in the morphology of chloroplasts and cell wall

Chloroplasts from acid regions are larger and have bigger starch grains than chloroplasts from alkaline zones, and the ratio of chloroplast volume in acid to alkaline zones has been reported to be about 3 (Dorn and Weisenseel, 1984; Wasteneys and Williamson, 1992). They also appear darker green, probably because of a higher concentration of chlorophyll. These differences are likely to reflect the enhanced uptake of CO_2 and consequently enhanced photosynthesis at the acid bands (Bulychev et al., 2001a). In “old” internodal cells, the chloroplasts are often not only smaller but also less abundant at alkaline zones, and the chloroplast files are less regularly arranged. This and the occasional deposition of cell wall material beneath alkaline areas suggest that the plasma membrane at alkaline zones is “leaky” and prone to an enhanced influx of external Ca^{2+} into the cytoplasm, which triggers release of chloroplasts, exocytosis, and wall deposition similar to that that happens in wounds (Foissner and Wasteneys, 2012; see Section 3.3).

At alkaline bands, the cell diameter is smaller and the cell wall is often thicker as compared with the acid regions (Bisson et al., 1991; Price et al., 1985). This is probably due to the high concentration of Ca^{2+} that cross-links pectin, inhibits wall growth, and reduces cell extension (Métraux et al., 1980; Proseus and Boyer, 2006). Differences in cell wall composition between acid and alkaline bands have been detected by Fourier transform infrared spectrophotometry (Homblé et al., 1990).

3.1.2 pH banding-dependent distribution of charasomes and cortical mitochondria

In branchlet internodal cells of *Chara*, the size and distribution of charasomes are dependent on the pH banding pattern (see Section 2.1.2). About 30 years

ago, electron microscopic studies revealed that charasomes were larger and more abundant in acid bands as compared with alkaline bands (Franceschi and Lucas, 1980; Price et al., 1985). These findings were confirmed by *in vivo* imaging of charasomes with fluorescent dyes showing that charasome size, abundance, and area fraction (% cell surface area covered by charasomes) are greater at the acid regions than at alkaline zones (Fig. 7.3D and E) (Schmölzer et al., 2011). The correlation between charasome size, density, and area fraction and the pH banding pattern is often less obvious (own unpublished data) or completely absent (Bisson et al., 1991) in internodal cells of the main axis. This and the fact that charasomes are in general smaller and less abundant in internodal cells of the main axis are consistent with the finding that internodal cells of the main axis have a less important role in photosynthesis than the branchlets (Schulte et al., 1994).

Internodal cells grown under very low light intensities, for example, at the base of the thalli or within dense canopies, have a uniform, weakly acidic pH at their surface and relatively few, evenly distributed, and relatively small charasomes. Under laboratory conditions, similar charasome patterns can be obtained within 6 days of incubating cells in pH buffers of sufficient strength to inhibit pH banding (Schmölzer et al., 2011). These treatments yield similar values for charasome size independent of whether the pH is clamped to 5.5 or to 8.5, indicating that the differential size, distribution, and area fractions in banding cells reflect active pH banding and are not (only) due to differences in external pH (Schmölzer et al., 2011). The pH buffer experiments also show that small charasomes develop even in nonbanding cells as long as photosynthesis is not inhibited, whereas 1-week treatment with the photosynthesis inhibitor DCMU or 3-week darkness results in complete loss of charasomes (Bisson et al., 1991; Chau et al., 1994; Schmölzer et al., 2011).

The present state of knowledge suggests that charasome size and distribution are responsive to pH banding but that charasomes are not required for the development of a pH banding pattern. Charasome-free internodal cells of *Nitella* and *Nitellopsis* species also develop acid and alkaline bands, and pH banding in *Chara* recovers from long-term dark treatment, DCMU, or pH buffers within a few hours or even minutes, a time period that is far too short for charasome development (Chau et al., 1994; Eremin et al., 2007; Schmölzer et al., 2011). What then is the functional significance of charasome modification in response to pH banding? It is likely that the development of these convoluted plasma membrane invaginations increases the cell surface area in order to accommodate a high number of H^+ -ATPases that serve to locally increase external acidification. Charasomes may also help

to restrict the size of the alkaline regions to minimize the loss of photosynthetic capacity. In young branchlets of *C. australis*, which have relatively few, small charasomes and in the charasome-free internodes of *N. flexilis*, the total area of alkaline regions is about half the total area of acid regions (Arens, 1939). In mature branchlet internodes of *C. australis*, the alkaline bands or spots are greatly restricted (own unpublished results). In cells of land plants, photosynthesis requires the interaction of chloroplasts with mitochondria and peroxisomes in order to detoxify metabolites. Consistent with this, the ratio of mitochondria to chloroplasts increases with the rate of photosynthesis (Padmasree et al., 2002; Wada et al., 2003). A similar relationship between the chloroplast activity and the distribution of mitochondria is seen in characean internodal cells. Cortical mitochondria are larger and more abundant at the photosynthetically more active acid regions than at the alkaline bands (Fig. 7.3D and E) (Foissner, 2004; Schmölzer et al., 2011). They are tightly associated with the chloroplasts and—if present—charasomes (Fig. 7.3C and D) (Franceschi and Lucas, 1980). Mitochondria located in acid bands are also stained more intensely by fluorescent dyes that require high membrane potential for uptake, suggesting that these mitochondria have a higher physiological activity (Schmölzer et al., 2011). In cells of land plants, chloroplasts exposed to detrimental light intensities relocate to shaded areas of the cell (e.g., Wada, 2013; Wada et al., 2003). In characean internodal cells, chloroplasts are firmly anchored in the cell periphery and are therefore unable to escape high light intensities. This drawback is likely to be compensated by the accumulation of detoxifying mitochondria. The pH band-dependent distribution of mitochondria may therefore reflect an alternative mechanism for preventing photodamage. The mechanisms by which cortical mitochondria gather at the acid regions or how they “sense” photosynthetic activity are not yet clear. Mitochondria are able to move from the endoplasm toward the cortex, but they may also migrate within the cortex to accumulate in acid band regions (see Section 2.3.3). Alternatively or in addition, mitochondrial growth and fission (Fig. 7.5) could be enhanced at the acid bands and/or inhibited at alkaline bands. The accumulation of mitochondria at the acid regions is reversible as shown by the treatment of cells with darkness or inhibitors of photosynthesis after which mitochondria are homogeneously distributed in the cell cortex (Foissner, 2004; Schmölzer et al., 2011). A reversible association between chloroplasts and mitochondria has also recently been documented in *A. thaliana* mesophyll cells (Islam et al., 2009).

Interestingly, preliminary studies indicate that peroxisomes are evenly distributed in the cortex of internodal cells although they are equipped with

enzymes involved in glycolate detoxification and thus appear to have a similar function in photorespiration as peroxisomes from higher plants (Stabenau et al., 2003).

3.1.3 pH banding and the cytoskeleton

Wasteneys and Williamson (1992) used silver-enhanced immunogold labeling in internodal cells of *C. corallina* to compare microtubule organization in relation to pH banding. Microtubule length and density were found to be higher, and microtubules had more parallel alignment in acid compared to alkaline regions. They also determined that depolymerization of microtubules did not inhibit pH band formation, in contrast to a previous study that utilized much higher concentrations of microtubule-depolymerizing drugs (Fisahn and Lucas, 1990a). Changes in microtubule organization and dynamics are therefore a consequence rather than a requirement for pH band formation. The recent finding that cortical mitochondria can interact with microtubules (see Section 2.2) suggests that the greater abundance of microtubules in acid bands could support the accumulation of mitochondria. The causal relationship between microtubule organization and charasome abundance in acid bands should also be addressed in future studies. In contrast to the findings with *Chara* internodes, microtubule patterns and dynamics in internodal cells of *N. flexilis*, visualized by microinjection of fluorescent brain tubulin, were similar along the whole cell surface and independent of the pH banding pattern (Kropf et al., 1997). These differences could reflect the absence of charasomes in *Nitella* species.

3.2. Anoxic conditions and morphology of mitochondria

The mitochondria of characean internodal cells show a remarkable variability in size and shape. Internodal cells of the main axis of *N. flexilis* grown in their natural habitat under favorable growth conditions contain roundish to elongate mitochondria that are between 1 and 2 μm long (mean: $1.4 \mu\text{m} \pm 0.4 \text{ SD}$) (Foissner, 1981). Much larger mitochondria can be observed in internodal cells of thalli collected during the cold season. Most of these giant mitochondria are elongate with a length of up to 45 μm , but mitochondria with a ring-like, disk- or cup-shaped, branched, or completely irregular morphology have also been described (Foissner, 1981, 1983; Jarosch, 1961). The formation of giant mitochondria is correlated with a reduction in total mitochondria length (volume) per unit volume of cytoplasm. An increase in mitochondrial size can be induced in cultures grown under low light conditions (Fig. 7.5) (Foissner, 1981) or

by treatment of cells with inhibitors of photosynthetic or mitochondrial electron transport (Foissner, 1983). These observations suggest that the development of giant mitochondria together with the decrease in total mitochondrial volume reflects low mitochondrial activity that is possibly induced by low oxygen pressure, as reported for cells of higher plants (e.g., Van Gestel and Verbelen, 2002).

Giant mitochondria may form either by fusion or through inhibition of fission. Both fission and fusion of mitochondria have been observed in the cortex of elongating characean internodal cells (Fig. 7.5) (Foissner, 2004). Many mitochondria, however, are rather immobile, and it is likely that their size and morphology is determined by growth in the absence of organelle division. This is consistent with the observation that the formation of giant mitochondria is prevented by inhibitors of cytosolic and mitochondrial protein synthesis (Foissner, 1983). The development of giant mitochondria in internodal cells may therefore require both fusion of individual organelles and mitochondrial growth in the absence of mitochondrial division.

Elongate and ring-, cup-, or disk-shaped giant mitochondria are present in the cortex and in the endoplasm. They slowly bend and reorient during mass streaming, but their general shape remains constant, suggesting that intrinsic factors control the shape of these organelles (Shibata *et al.*, 2009). In the cortex of elongating cells, the shape (and dynamics) of giant mitochondria is additionally controlled by motor proteins that interact with actin filaments and microtubules (see Section 2.3.3). The inhibition of actin-myosin interaction by cytochalasin treatment causes amoeba-like and disk-shaped cortical mitochondria to become elongate and to align and move transverse to the cell's long axis. Depolymerization of microtubules with oryzalin or other drugs has the opposite effect and causes vermiform mitochondria to become amoeboid or disk-shaped. These data are consistent with the presence of different motor proteins at the mitochondrial outer membrane, which interact simultaneously with transversely aligned microtubules and with a more random meshwork of cortical actin filaments (Foissner, 2004). The identity of these motors remains to be determined.

3.3. Wounding—An important tool to study reorganization of cytoarchitecture

A review about wound healing in characean internodes has been published recently (Foissner and Wasteneys, 2012), and therefore, only the most pertinent aspects are presented here. The healing of wounds is an important defense mechanism against mechanical and pathogen-induced damage

(Underwood, 2012) and therefore of general importance for the survival of plants and other organisms. In addition, the study of wound healing is an important tool for analyzing various intracellular cellular processes. Local wounding destroys existing cellular polarities and establishes new ones. At the site of injury, the actin and microtubule cytoskeleton become reorganized, which has consequences for organelle motility and distribution. Changes in cytoarchitecture may be either transient (e.g., reorganization of the cytoskeleton) or permanent (e.g., deposition of a wound wall). In the following sections, we describe different ways to induce a wound response in characean internodal cells. The available data indicate that the severity of the response can be correlated with the extent of local Ca^{2+} influx and local disturbance of Ca^{2+} homeostasis. For wounding-dependent electrophysiological responses, we refer to the review of Shimmen (2008).

3.3.1 UV light-induced chloroplast-free windows

Kamitsubo (1972) was the first to use the light of the blue or UV lamp of a fluorescence microscope for the production of chloroplast-free windows, the mildest form of a wound response resulting in a permanent change in cytoarchitecture of internodal cells. During irradiation, active cytoplasmic streaming becomes locally arrested, probably due to the inactivation of myosin motors by Ca^{2+} (Williamson and Ashley, 1982), and chloroplasts lose their anchorage in the cortex and are released into the endoplasm (Fig. 7.8A). Actin filament bundles are often dislodged together with the chloroplasts. If the time of irradiation is restricted to 7–10 min, parallel actin filament bundles regenerate by the elongation of the bundles outside the window and parallel to the direction of passive endoplasmic flow over the injured area (Fig. 7.8B) (Williamson and Hurley, 1986; Williamson et al., 1984). Notably, delicate cortical actin filaments, as present between chloroplasts and plasma membrane in control areas, are absent in regenerated windows, suggesting their recruitment to subcortical actin bundles in the absence of chloroplasts (Foissner et al., 1996). Thus, the actin organization in chloroplast-free windows resembles that of the chloroplast-free side walls adjacent to nodal cells.

Cortical microtubules disassemble immediately after the onset of irradiation and regenerate approximately at the same time as the actin bundles (Foissner and Wasteneys, 1999). The orientation of the regenerated bundles in large windows is parallel to the direction of mass streaming in contrast to their random orientation in control areas of mature cells (Fig. 7.8A) (Foissner and Wasteneys, 1999). Passive reorientation of microtubules by

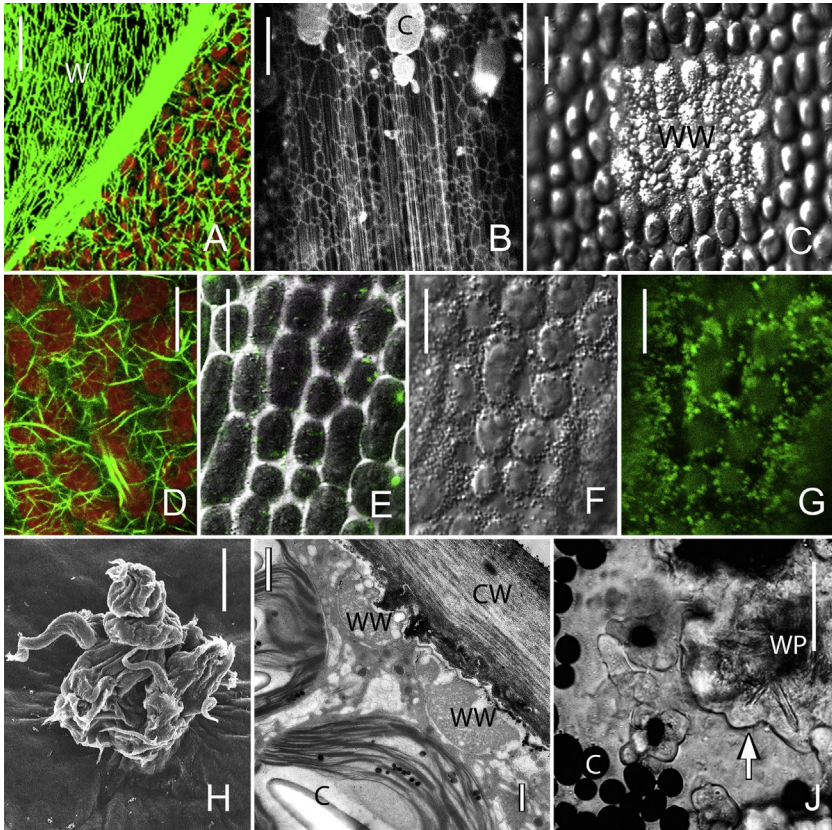


Figure 7.8 Wounding-induced changes in cytoarchitecture in internodal cells of *Nitella flexilis* (A, D, H, and I) and *Chara australis* (B, C, F, G, and J). (A) Cortical microtubules align parallel to the direction of cytoplasmic streaming in a large window (W) created by local irradiation with UV light that causes detachment of chloroplasts (red fluorescent). Note random orientation of microtubules outside the window. Microtubules visualized by immunolabeling with antitubulin and a green fluorescent second antibody. (B) In a window, the cortical ER retains its reticulate pattern in spite of close contact with the subcortical ER tubes. C, chloroplast. (C) Wound wall (WW) created by local irradiation with UV. (D–G) Early stages of wound wall deposition. A dense actin meshwork, visualized by green fluorescent phalloidin between autofluorescence chloroplasts (red) and the plasma membrane, is a prerequisite for targeted movement of organelles toward the wound site (D). Before injury, few secretory vesicles and FM1-43 fluorescent organelles are present between chloroplasts and plasma membrane in the unwounded internodal cell (E; DIC image merged with fluorescence image). After several minutes of irradiation with the UV lamp of a microscope, numerous secretory vesicles (F; DIC image) and FM1-43-stained putative recycling endosomes (G; fluorescence image) move toward the plasma membrane. (H) FESEM image of the inner surface of the cell wall onto which a wound wall was deposited after 1-day treatment of internodal cell with 50 mM CaCl_2 (cytoplasm was extracted by hypochlorite). (I) Transmission electron micrograph of wound walls

hydrodynamic forces produced by actomyosin-driven cytoplasmic streaming has later been confirmed to occur in plant cells (Sainsbury et al., 2008).

The possibility to gently remove the chloroplasts that shield the cortex at unwounded control sites not only is an excellent tool to explore cytoskeleton interactions but also can be used to find out more about the dynamics and positioning of organelles at the cortex of internodal cells, for example, the ER and mitochondria. UV irradiation for longer time periods leads to the deposition of a two-layered wound wall (Fig. 7.8C), which is similar to that obtained after mechanical injury (Klima and Foissner, 2011; see Section 3.3.3).

3.3.2 Chemically induced wound walls

An elegant method to induce the deposition of wound walls in characean internodes is the use of chemicals that cause a local influx of Ca^{2+} . This influx and the corresponding response of the cell remain local if cells produce a pH banding pattern. Ca^{2+} influx then occurs preferentially at or around the alkaline regions of the cells, whereas the acid zones are not affected (Foissner, 1989; 1990). Alternatively, local wound responses can be induced at any region of the cell by targeted application of Sephadex beads (see Section 4.2). Prior to application, the beads are soaked with solutions of appropriate chemicals and eventually adjusted to a neutral or alkaline pH (Foissner, 1991). Initially, cortical microtubules undergo disassembly and chloroplasts detach at the site of bead application (window formation; see Section 3.3.1) followed by the deposition of a wound wall onto the intact cell wall.

A requirement for wound wall deposition is the transient reorganization of the actin filament bundles into a meshwork of randomly oriented actin filaments, which guide wound wall-forming organelles toward the wound site (Fig. 7.8D, F, and G) (Foissner and Wasteneys, 1997; Foissner et al., 1996). Because of the large size of some of the wound wall-forming vesicles and the superior imaging conditions in internodal cells, the spreading of vesicular contents after exocytosis can be documented using video-enhanced interference contrast microscopy (Foissner et al., 1996). Irrespective of the type of wound wall deposited, the regeneration of the

(WW) deposited onto the normal cell wall (CW) at an alkaline region of a cell treated with 0.1 mM LaCl_3 for 1 day. (J) Puncture wound sealed by a plug of vacuolar inclusions (WP) onto which a wound wall (arrow) was deposited (bright field). C, chloroplast. Bars are 20 μm (A and J), 10 μm (B–G), 2 μm (H), and 750 nm (I).

damaged plasma membrane seems to require the fusion of FM-stained organelles (Fig. 7.8E–G) (Foissner and Wasteneys, 2012; Klima and Foissner, 2011). These organelles are probably “recycling endosomes” but further studies are required to confirm their identity.

Wound wall composition reflects the severity of the Ca^{2+} influx. “Fibrillar wound walls” consist of cellulose microfibrils embedded in a matrix of pectic polysaccharides and therefore have a structure and chemical composition similar to the normal cell wall. They can be produced when internodal cells of *N. flexilis* are treated with ≥ 50 mM CaCl_2 (Fig. 7.8H) (Foissner, 1990; Foissner and Wasteneys, 2012). During their deposition, wound wall-forming vesicles fuse with the plasma membrane and release their pectic content into the extracytoplasmic space, while the membrane delivers the cellulose synthase complexes required for microfibril formation. Excess membrane becomes recycled via coated vesicles (Foissner, 1992). The fibrillar wound walls have a characteristic shape and consist of groups of protuberances that are made up of helically ordered cellulose microfibrils. The absence of microtubules from the site of fibrillar wound wall assembly demonstrates that highly ordered cellulose arrays can form in the absence of microtubules (Foissner and Wasteneys, 2012).

In older internodal cells, similar wound walls form without chemical treatment and in all species so far investigated. They are deposited at regions where the “cuticle” and the primary wall, but not necessarily the secondary wall, become locally degraded by sessile epiphytic microorganisms. This reaction is comparable to pathogen defense, but the “elicitors” remain unknown so far. The deposition of a cell wall beneath nonpathogenic epiphytes may be in response to a mechanical disturbance. Similarly, mechanical stimuli can induce subcellular reorganization in higher plant cells (Hardham et al., 2008; Shepherd et al., 2001), possibly involving stretch-activated Ca^{2+} channels (e.g., Kaneko et al., 2005; Shepherd et al., 2001, 2008).

“Membranous wound walls” are formed when internodal cells of various species are immersed in chlortetracycline or A23187 (Foissner, 1988b, 1991, 1992). Both substances are known to act as Ca^{2+} ionophores, and, as suggested by the response of the cell, they cause a larger influx of Ca^{2+} into the cell than the application of 50 mM CaCl_2 . These chemicals induce “compound exocytosis” during which the membranes of fusing organelles (vesicles and ER) are incompletely recycled because of insufficient endocytosis (Fig. 7.8I). The deposited wound wall therefore contains not only amorphous material (pectic polysaccharides and callose) but also numerous

membrane residues, which are heavily loaded with Ca^{2+} (Foissner, 1992, 1998). For that reason, it was concluded that the formation of membranous wound walls allows the cell to detoxify incoming Ca^{2+} by transfer of Ca^{2+} -loaded organelles (mainly ER) to the extracytoplasmic space (Foissner and Wasteneys, 2012).

3.3.3 Mechanical wounding (cell wall puncture)

Coenocytic algal cells have developed effective wound-healing mechanisms to prevent the loss of the cytoplasm (Menzel, 1988). Quite common is the formation of a wound plug, which seals the cell wall hole and onto which the new cell wall is laid down. In marine algae, the plug precursor material is stored in the vacuole and coagulates upon extrusion from the cell (Menzel, 1988). Characean internodal cells have developed a similar wound-healing mechanism. They contain vacuolar crystals and large, solid inclusions that form in the endoplasm and are eventually released into the vacuole (Fig. 7.7C–H) (see Section 2.4.1). In case of injury, crystals and solid inclusions are automatically expelled due to the turgor and occlude the cell wall hole (Fig. 7.8J). The resulting wound plug prevents further loss of the cytoplasm and becomes covered by a two-layered wall. The outer layer is a membranous wound wall, whereas the inner, cytoplasm-facing layer has the properties of a fibrillar wound wall. This suggests that the outer membranous wound wall helps to recover Ca^{2+} homoeostasis, whereas the inner fibrillar wound wall confers mechanical stability.

The process of wound healing is usually completed within 2 h. Actin filament bundles and cortical microtubules regenerate along the wound wall in a similar way as described for chloroplast-free windows (Foissner and Wasteneys, 1997, 1999). Healed wounds induced by partial ligation (see Section 4.4) have been shown to become alkaline regions, possibly because of the absence of chloroplasts (Shimmen and Yamamoto, 2002).



4. EXPERIMENTAL MANIPULATIONS

The great advantages of characean internodal cells for the study of plant cell architecture and physiology include their simple geometry, the heterogeneity of the cell surface with the conspicuous pH banding pattern, and their large size, which allow various experimental manipulations and microsurgery to be conducted (Fig. 7.9).

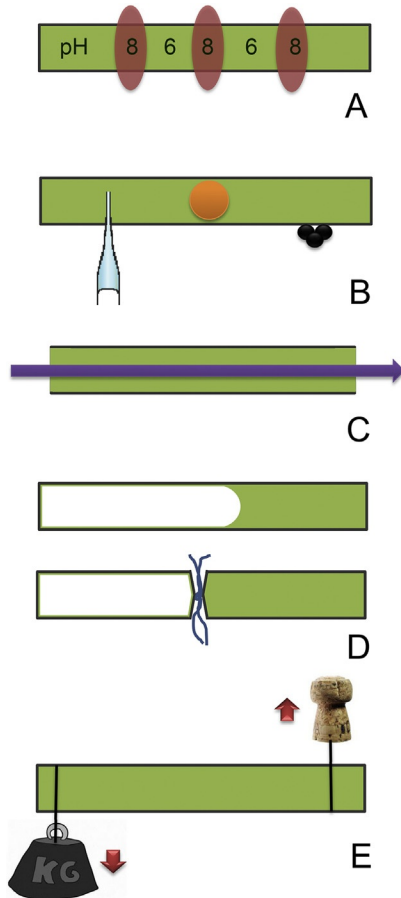


Figure 7.9 Heterogeneity of the surface of characean internodal cells and manipulations. (A) pH banding along the surface of internodal cells visualized by pH indicator phenol red. (B) Treatment with capillaries or probes for easy electrophysiological measurements, microinjection or wounding (left), local treatment with light or darkness (middle), or treatment with chemicals entrapped in sephadex beads (right). (C) Perfusion of internodal cells for exchange of vacuolar sap, for staining of cytoskeleton or organelles with plasma membrane-impermeable dyes, and for semi-*in vitro* motility assays. (D) Centrifugation and subsequent ligation for the creation of cell fragments with and without a central vacuole. (E) Application of strain by weight or buoyant force.

4.1. Easy microinjection and use of pressure probe

The large size and the simple geometry allow easy insertion of electrodes. The first intracellular measurements of membrane potentials and action potentials in plant cells were made by [Umrath \(1929\)](#) using internodal cells of *Nitella mucronata*. Since then, internodal cells have been used for

electrophysiological research and ion transport studies (Mimura, 1995; Tazawa, 2003a,b; Tazawa and Shimmen, 2001; Wayne, 1994). Indeed, *C. corallina* has been described as the plant equivalent to the squid axon when studying ion transport in plants (Trontelj et al., 2006). Internodal cells are also ideally suited for pressure probe experiments and have been valuable for exploring the effect of turgor on cell wall expansion (Boyer, 2009; Proseus et al., 2000; Wei and Lintilhac, 2007) as well as the function and properties of water channels (aquaporins; Hertel and Steudle, 1997; Schuetz and Tyerman, 1997; Ye et al., 2006).

4.2. Local illumination, local treatment with chemicals, and extracellular measurements

The large size of the internodal cells allows local illumination (Fig. 7.9B), which has been used to study the generation of pH banding patterns (Fig. 7.9A) (Bulychev et al., 2001b) and the role of endoplasmic factors affecting chloroplast activity (Dodonova and Bulychev, 2011). Chemicals can be locally applied using Sephadex beads (Fig. 7.9B) in order to study wound healing (see Section 3.3.2) (Foissner, 1989) or other local responses of cell architecture and physiology. Local application of chemicals is also possible by creating water-tight compartments with the aid of silicon grease. This method was recently used to study polar transport of ^3H -labeled auxin (Boot et al., 2012). Extracellular currents are easily measured along the cell surface by scanning pH microprobes and vibrating ion-sensitive probes (Fig. 7.9B) (Bulychev et al., 2001b; Fisahn and Lucas, 1991).

4.3. Intracellular perfusion and cytoplasmic droplets

This type of experimental manipulation was invented by Kamiya and Kuroda (1957) and further developed by other researchers (Tazawa and Shimmen, 1987 for review). Intracellular perfusion, often referred to as vacuolar perfusion, allows the control and manipulation of the internal composition of cells by the replacement of various cell compartments by artificial media. For this purpose, glass or plastic wells are fitted and sealed with vacuum grease over each cell end and then filled with isotonic medium. The middle of the cell is either exposed to the air or covered by a small drop of silicone oil. When turgor is lost, the ends of the cell are removed with small scissors (Fig. 7.9C). The open cylinder can then be perfused with different solutions, and, depending on their composition and/or the strength of perfusion, the cell sap, the tonoplast, the endoplasm, and the cortex can be

removed. Perfused cells can be ligated and turgor can be partially restored by placing the cell in hypotonic medium. This cell model has been widely used for electrophysiological measurements and for the identification and characterization of transporters (Beilby et al., 1997; Tazawa and Shimmen, 1987). Perfusion can also be used for the selective extraction of cytoskeletal and associated structures from the cell, for the introduction of foreign proteins, and for reactivation studies (Nothnagel et al., 1982; Williamson, 1975, 1985; Williamson et al., 1989). Fluorescent dyes, including dyes that are membrane-impermeable, can be used to stain the cytoskeleton and various components of the cell (see Fig. 7.4A, B, E, and F). Fixation and immunolabeling by perfusion of internodal cells are time-consuming but have many advantages as compared with the conventional methods (and as also used for multicellular plant organs or suspension culture cells) where cells are fixed as a whole before treatment with antibodies. It removes vacuolar proteases that have a detrimental effect on the protein epitopes, and it prevents the shrinkage of the cytoplasm, thereby improving the preservation of the cytoskeleton and the cell architecture (Wasteneys and Williamson, 1987).

4.4. Centrifugation and ligation

Internodal cells embedded in agar can be centrifuged, and, under appropriate g-force, endoplasmic organelles will accumulate at one end of the cell (Fig. 7.9D). If these cells are ligated prior to the recovery of normal organelle distribution, vacuole-free and vacuole-containing cell parts can be obtained. Such cytoplasm-enriched cell fragments were used for electrophysiological research and for the study of vacuole formation (Beilby and Shepherd, 1989). Mimura (1995) studied the properties of plasma membrane H^+ pumps in chloroplast-free fragments and in fragments in which the *Chara* chloroplasts were substituted by chloroplasts isolated from *Pisum* leaves.

4.5. Stretch application and constriction, isolated cell walls

In order to study various physical parameters on the distribution of cellulose microfibrils, Green (1960) used corks attached to the tips of anchored thalli or surrounded internodes with capillaries to restrict their expansion. Alternatively, weights can be used to apply stretch (Fig. 7.9E).

Empty cell wall cylinders and cell wall sheets are convenient for studying cell wall growth and properties under different experimental conditions (e.g., Berestovsky et al., 2001; Boyer, 2009; and references therein;

Proseus and Boyer, 2012; Steudle and Tyerman, 1983; Taiz et al., 1981; Toole et al., 2001, 2002; Wei and Lintilhac, 2007; Zimmermann and Steudle, 1975).

4.6. Collection of cell sap and cytoplasm

The ability to collect sizable quantities of cell sap or cytoplasm from the internodal cells of the larger species has been and will become increasingly important for single-cell metabolomics (Oikawa et al., 2011). Cytoplasmic droplets squeezed out from internodal cells are surrounded by the vacuolar membrane and have been useful for exploring the electrical properties of the tonoplast (Tazawa and Shimmen, 2001, for references). The possibility to produce droplets in which the tonoplast has different handedness will be interesting for studies addressing ligand binding and interaction of proteins within the membrane or with other regulatory factors (Berecki et al., 2001). Cytoplasmic droplets permit optical clarity of cytoplasmic organelles since refraction of light by the cell wall is not an issue (Klima and Foissner, 2008) as exemplified by early studies of actin filament-based dynamics in a semi-*in vivo* system (Higashi-Fujime, 1980; Jarosch, 1976).



5. METHODOLOGICAL LIMITATIONS OF INTERNODAL CELLS

5.1. Molecular biology in its infancy

A current deterrent for the use of the Characeae as model organisms is the lack of molecular biological data, the lack of suitable methods for genetic transformation, and the lack of mutants. Only a few annotated DNA and protein sequences from characean species have so far been published in public databases (e.g., myosin; Kashiya et al., 2000; Morimatsu et al., 2000) and the plant-specific RAB5 GTPase ARA6 (Hoepflinger et al., 2013). It is assumed that their number will rapidly increase during the next years because of the exploitation of cDNA libraries produced by 4–5–4 sequencing and related techniques, which are becoming more affordable. An important tool in the analysis of protein location and function is the expression of translational reporters, utilizing either endogenous or heterologous genes. Until now, no convincing protocol has been developed for the transformation of characean cells in spite of numerous attempts in several laboratories. The expression of heterologous ion channel proteins following microinjection of cRNA has been reported (Lühring and Witzemann, 1995), but the measured activity probably reflects that of cell's own channels (Gong and Bisson,

2002). A survey of the hitherto published data indicates that codon usage is similar to that of higher plants and cannot be a problem in expression of foreign genes.

5.2. Nonsterile cultures

Characean algae are usually vegetatively propagated by planting parts of the thallus into a substrate of sand, peat, and soil covering the bottom of large glass beakers, plastic containers, or glass aquaria. It is clear that such biotopes contain not only the algae of interest but also other organisms ranging from bacteria to multicellular animals. Organisms at the surface of internodal cells (epiphytes) may become a problem for fluorescence microscopy if they contain autofluorescent substances or avidly take up fluorescent dyes. Frequent planting and low light intensities ($\leq 10 \mu\text{Einstein m}^{-2} \text{s}^{-1}$ at the surface of the containers) reduce epiphyte development. Epiphytes are also a problem for biochemical and molecular biochemical analyses because homogenates will always contain material of non-characean origin. Sterile cultures can be obtained from surface-sterilized oospores, and a number of recipes for oospore germination have been published (e.g., Forsberg, 1965a,b,c; Kalin and Smith, 2007; Sokol and Stross, 1992). However, each species appears to need special requirements, and the reproducibility of the experiments is low.



6. CONCLUDING REMARKS

This review emphasizes the use of characean internodal cells for studying various aspects of cell architecture, which is intimately related to the organization of the cytoskeleton.

Organelles constantly exchange information and alter location and orientation and eventually even their shape to meet the changing demands of the cell. This is especially evident during photosynthesis when chloroplasts, mitochondria, and peroxisomes work together in order to exchange, detoxify, and recycle metabolites required for efficient photosynthesis. The huge characean internodal cell with its highly heterogeneous transport activities for protons at the plasma membrane (pH banding), which are sometimes coupled with complex elaborations of the plasma membrane (charasomes), together with the possibility for microsurgery, local illumination, drug application, or other treatments represents a promising model for studying signaling and interaction between organelles involved in photosynthesis and between these organelles and the cytoskeleton.

Massive changes in cytoarchitecture occur during wounding and wound healing. Because it is possible to induce different types of wound responses, internodal cells are an excellent system to explore the reorganization of the cytoskeleton and the signals eventually leading to exocytosis of vesicles involved in plasma membrane repair and deposition of wound walls, which differ not only in structure and composition but also in function.

The significance of characean internodal cells as model system for the study of cell architecture and organelle interactions will further increase with the development of molecular biological applications in combination with metabolomics and proteomics.

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