

Stem Cell Biology and Regenerative Medicine

Tiziana A.L. Brevini *Editor*

Stem Cells in Animal Species: From Pre-clinic to Biodiversity

 Humana Press

Stem Cell Biology and Regenerative Medicine

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Editor

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Part I
Stem Cells for Pre-clinical Models

Chapter 1

Stem Cells in Dystrophic Animal Models: From Preclinical to Clinical Studies

Clemetina Sitzia, Silvia Erratico, Andrea Farini, Yvan Torrente,
and Mirella Meregalli

1 Introduction

To set up a correct clinical protocol for a particular cell approach, as stem cell therapy is, the development of a complete preclinical study is essential; in fact, only if the criteria chosen for the animal model tests are evaluated in order to be correlated with the future human application, the results obtained can be prognostic.

For this reason, different aspects must be considered to select the most suitable animal model, as these aspects play an important role: (1) the genetic basis of the disease should be the same both for humans and animal models (especially it is an inherited disorder); (2) hallmarks specific of human pathology should be reiterated in animals, so that the study can evaluate eventual improvements through symptoms relief; (3) characteristics of animal itself should be correlated with the aim of the study (e.g. for a genetic screening an animal model with a large number of progeny could be handy, while for a cell therapy preclinical protocol a dog could be better compared with patients' organisms); (4) disease progression should be well characterized, so that

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reference values are available in scientific literature, in order to allow precise outcome analysis; (5) animal phenotype should be reproducible over generations, so that standard readout parameters can be used and outcomes compared (Willmann et al. 2009). The applications of animal models to clinical research are too spread; in particular, for cell therapy it has a pivotal role, especially in case of pathologies not treatable with pharmacological or conventional approaches, like neurodegenerative diseases.

Muscular dystrophies (MDs) are a heterogeneous group of disorders characterized by the degeneration/regeneration of muscle fibres, associated with progressive muscle weakness due to the development of muscle atrophy or necrosis. Genetically, these pathologies are identified by mutations in different genes of dystrophin-glycoprotein complex (DGC) that act as a bridge across the sarcolemma membrane and guarantee the connection between the extracellular matrix (ECM) and the cytoskeleton actin protein (Ibraghimov-Beskrovnaya et al. 1992).

Depending on the gene where the mutation is localized, muscular dystrophies are classified into Duchenne and Becker muscular dystrophies (DMD, BMD, dystrophin) (Hoffman et al. 1987; Koenig et al. 1987), Emery–Dreifuss (ememin, lamin A/C) (Brown et al. 2001), facioscapulohumeral muscular dystrophy (FSHD, double-homeobox protein 4 (Dux4)); oculo-pharyngeal muscular dystrophy (OPMD, poly(A)-binding protein 2 (Shanmugam et al. 2000)), Miyoshi myopathy (MM, dysferlin) (Liu et al. 1998) and limb-girdle muscular dystrophies (LGMD-2B, dysferlin (Liu et al. 1998); LGMD-2A, calpain III (Richard et al. 1995); LGMD-1C, caveolin (Minetti et al. 1998); LGMD-2C, -2D, -2E, -2F, sarcoglycan complex (Bonnemann et al. 1996)); so far, more than 30 genetically distinct types of MDs have been identified (Kaplan 2011). From a histological point of view, muscle tissue in MDs is characterized by the presence of abundant small-diameters centre-nucleated fibres, indicating regeneration, infiltration of mononucleated cells, accumulated fibrosis and fat infiltration and variation in the size of myofiber cross-sectional areas compared with healthy tissue. All these aspects are reflected in the clinical evolution of the diseases, leading to progressive muscle degeneration. Besides pharmacological approaches, focused on symptoms treatment, different therapeutic perspectives have been explored during the last decade, such as gene therapy or stem cell transplantation; although impressive improvements, nowadays no curative treatment for any MD is available. Nevertheless, to develop new therapeutic strategies, such as to confirm the existing ones or better understand the mechanisms underlining these pathologies, the use of animal models is essential; analysing a whole organism is in fact necessary to identify the effects of a preclinical strategy in a group of systemic disorders, such as MDs.

However, starting from this evidence, it is important to select the most fitting animal model depending on the aims and the characteristics of the research protocol or preclinical study. In fact, different animal models have been developed for MDs in the last years, each one with specific PROs and CONs that must be evaluated, especially in a clinical perspective.

While simple animal models, such as *Drosophila* or zebrafish, are usually used in experiments involving the genetic origin of MDs, mammalian animal models are the most employed in preclinical studies for cell and gene therapies. In particular,

compared to vertebrate models, *Drosophila* represents the most rapid and powerful model for genetic screening, thanks to its 10 days long life cycle, small size and large number of progeny; for these reasons, numerous genetic studies on DGC have been performed on *Drosophila*, especially to characterize the neuromuscular junctions (van der Plas et al. 2006; Bogdanik et al. 2008). In a similar way, also zebrafish is commonly used as a model for genetic studies, because of its easily manipulatable translucent embryos and the close resemblance of animal phenotypes to human condition (Lieschke and Currie 2007). An example is represented by the numerous models of DMD, whose application is useful both for the study of physiopathology (Berger et al. 2011; Bassett and Currie 2003; Guyon et al. 2009) and screening of antisense oligonucleotides and other small molecules (Muntoni and Wood 2011; Kawahara et al. 2011).

On the other hand, the mammalian, and in particular rodent, are the most used animal models in the preclinical protocols; even if the origin of the pathological strain is spontaneous, the possibility to genetically engineer the model in order to obtain useful characteristics is an important advantage for an experimental therapy application. In the last years a huge number of murine models for DMD have been developed, starting from the first mdx mutation identified in 1989 as a single base substitution in exon 23 (Sicinski et al. 1989); several allelic variants of the mdx-dystrophin-deficient mouse have been discovered or produced in laboratory (Araki et al. 1997), combining also other approaches to obtain more severe phenotypes, such as MyoD or utrophin lacking mdx mice (Megency et al. 1999; Deconinck et al. 1997a; Grady et al. 1997; Durbeej and Campbell 2002). Although the lifespan of these animals is shortened, their phenotype is much more benign compared with the one shown by human patients; this aspect must be taken in consideration, especially if mice are used to evaluate the treatment efficacy. Murine models are commonly used also for the dyspherlinopathies: SJL and AJ mice are characterized by mutations in dysferlin gene associated with phenotypical features of progressive muscular dystrophy (Bittner et al. 1999a; Ho et al. 2004). As regards sarcoglycanopathies, BIO 14.6 hamster is the spontaneous animal model (Straub et al. 1998), while several mouse models have been developed with null mutation in each one of the sarcoglycan genes, thanks to homologous recombination technique in embryonic stem cells (Durbeej and Campbell 2002; Duclos et al. 1998; Hack et al. 1998; Araishi et al. 1999).

Although rodents represent important models for studying molecular pathogenesis and the effects of gene manipulation and cell transplantation, other mammalian models, such as cats and dogs, may provide more clinically relevant models of different MDs that will be important for testing new therapies and evaluating benefits in terms of function. As for feline models, the reported cases are few and limited to dystrophinopathies (Carpenter et al. 2003; Gaschen et al. 1992), where a clinical presentation is reported, dogs are well known and relatively common animal model for preclinical studies of different MDs. In fact, for DMD at least three different dog breeds have been developed: the most famous and used, Golden Retriever muscular dystrophy (GRMD) (Sharp et al. 1992; Valentine et al. 1988); the beagle Canine X-linked muscular dystrophy (CXMD1), characterized by a smaller size and a

consequent easier maintenance (Shimatsu et al. 2003, 2005) and the Cavalier King Charles Spaniels with Muscular Dystrophy (CKCS-MD), recently obtained by a deletion in exon 50 (Walmsley et al. 2010). Also for sarcoglycanopathies, different canine models have been identified: a young Boston Terrier, Cocker Spaniel and Chihuahua (Walmsley et al. 2010).

In any case, before selecting a good animal model for a preclinical study, the specific PROs and CONs must be taken in consideration, as long as the consciousness that animal models are essential for the first step of in vivo trials; moreover, the results obtained from these models represent a starting point to hypothesize a correlated response in humans, but not always a precise reproduction of the evidence obtained.

In this chapter we will report the animal models for MDs nowadays available for preclinical studies, underlining the specific features of each one; furthermore, we will focus our attention on stem cell preclinical studies performed so far, showing the possible limitations of these approaches in the transition from a preclinical protocol to a clinical application.

2 Stem Cells for Therapy in Animal Models of MDs

Stem cells can be considered the most promising therapeutic approach for the treatment of MDs because of their potential to differentiate directly in the host tissue and give rise to new functional fibres. Despite gene therapy alone, whose limits are represented by the efficiency in delivering the correct gene without being recognized by immune system, the combination of this technique with an allogenic cell transplantation could represent a successful strategy; in fact, the delivery of patient's corrected cells bypasses the problem of an immune response and a rejection in a tissue already characterized by an inflammatory state. To reach this goal, different studies were performed in order to identify the stem cell type that guarantees the best performance in vivo in terms of engrafting and display of functional fibres in the host tissue. Stem cells tested in MDs animal models were derived from different tissues and can be classified into: bone marrow-derived stem cells (BMSC), adipose-derived stem cells (ASC), mesoangioblasts (MABs), satellite cells, blood- and muscle-derived CD133+ cells, side population (SP) cells, umbilical cord stem cells (UCS), Embryonic (ES) and induced pluripotent stem cells (Schuler et al. 1986).

There are advantages and disadvantages in the use of each of these cell types; some references to the specific in vivo studies are reported in Table 1.1.

BMSC, for example, are easily accessible, well characterized and systemically deliverable; on the other hand their participation in muscle regeneration is limited and below therapeutic levels. Experimental protocols were performed in different animal models of DMD (mdx mice and CXMD/GRMD dogs) and LGMD-2B (SJL mice). Also adipose tissue represents a painless and abundant source of mesenchymal stem cells; the multipotency of ASC was demonstrated by Zuk et al. (2001), proposing them as an alternative resource in cell therapy. In addition to the myogenic differentiation potential, the ASC display an immune-modulating behaviour

Table 1.1 List of specific cell-based studies performed in MD animal models

Cell type	Human pathology	Animal model	References
Bone marrow-derived stem cells (BMSC)	DMD	mdx mice	Ferrari et al. (1998), Bittner et al. (1999b), Ferrari and Mavilio (2002), Walsh et al. (2011), Leng et al. (2012)
		CXMD/GRMD dogs	Nitahara-Kasahara et al. (2012)
Adipose-derived stem cells (ASC)	LGMD-2B	SJL mice	Vieira et al. (2010)
	DMD	mdx mice	Rodriguez et al. (2005), Pinheiro et al. (2011)
		GRMD dogs	Vieira et al. (2012)
Umbilical cord stem cells (UCS)	LGMD-2B	SJL mice	Vieira et al. (2008)
	DMD	GRMD dogs	Vieira et al. (2010), Zucconi et al. (2011)
		LGMD-2B	SJL mice
Mesoangioblasts	DMD	mdx mice	Berry et al. (2007)
		GRMD dogs	Sampaolesi et al. (2006)
	LGMD-2B	SJL/BIAJ mice	Diaz-Manera et al. (2010)
	LGMD-2D	α -SG null mice	Sampaolesi et al. (2003), Guttinger et al. (2006), Galvez et al. (2006)
Satellite cells	DMD	Nude mdx mice	Montarras et al. (2005) Collins et al. (2005), Boldrin et al. (2009)
		NSG-mdx ^{4Cv}	Arpke et al. (2013)
Side population (SP)	DMD	NOD/SCID mdx mice	Gussoni et al. (1999), Motohashi et al. (2008)
		δ -sarcoglycanopathy	Sgcd ^{-/-} mice
Blood and muscle-derived CD133+ cells	DMD	SCID mdx mice	Torrente et al. (2004), Benchaouir et al. (2007), Gavina et al. (2006)
		Dysferlinopathy	SCID/AJ mice
Embryonic stem cells (ES) induced pluripotent stem cells (iPS)	DMD	mdx mice	Bhagavati and Xu (2005), Mizuno et al. (2010), Darabi et al. (2012)
		FSHD	FRG-1 mice

that could represent a further advantage in their application to cell transplantation. In this direction several studies were performed in order to evaluate the potential of ASC in treating MDs, in animal models such as mdx mice and GRMD dogs for DMD, and SJL mice for LGMD-2B.

As a third source for mesenchymal stem cells, several groups have addressed umbilical cord vein, meant as blood- or tissue-derived cells. Although in vitro potential of these cells to differentiate into myogenic lineage was demonstrated, in vivo experiments showed contradictory results. As UCS could reach host muscle

both in mice (SJL) and dog (GRMD) models, they were unable to completely differentiate into muscle cells and express the lacking protein. Only in a study it was reported that after injection of UCS in SJL mice an apparent therapeutic benefit was shown, but probably due to their immunomodulatory effect.

A particular type of stem cells is represented by blood vessel-associated progenitors called mesoangioblasts (Dellavalle et al. 2007), characterized by the expression of pericyte markers when isolated from postnatal tissues; these cells are able to cross the vessel wall and for this reason they have been used in preclinical models of systemic cell therapy for MDs. Successful results were obtained in different mice models of MDs, such as *mdx/utr^{-/-}* for DMD, α -SG null mice for LGMD-2D and SJL/BIAJ for dysferlinopathy, further than GRMD dogs. Moreover a Phase I/II clinical trial has been approved and is in progress.

As regards the cell type considered the “real” muscle stem cell, e.g. satellite cell, it was used for several *in vivo* experiments, demonstrating its ability to contribute to muscle regeneration in young and mature adult muscles. These cells, characterized by the expression of Pax7, once transplanted into an injured muscle, not only generate new muscle fibres, but also contribute to the satellite cells pool; moreover, single cell transplantation gives rise to an extensive proliferation, fibre generation and self-renewal *in vivo*. On the other hand, only a small number of cells can be derived from muscle and their regenerating efficiency is high only when transplanted fresh-isolated. Nevertheless, numerous studies were performed to evaluate the tissue regeneration and functional recovery *in vivo* after satellite cells injection, especially in DMD animal models, such as NSG-*mdx^{4Cv}* and nude *mdx* mice.

A particular case is represented by side population (SP) cells, a stem cell variant isolated by their ability to rapidly efflux the DNA-binding dye Hoechst 33342; SP cells have been so long isolated from different tissues, such as bone marrow, skin, mammary epithelium, muscle; these stem cells showed to harbour regenerative potential using varying modes of transplantation, including systemic delivery and direct organ injection (Montanaro et al. 2003; Welm et al. 2002; Hierlihy et al. 2002). Although each SP shares the ability exclude Hoechst 33342 and shows overlap of cell surface antigens such as Sca-1, it is unclear whether all tissue-derived SP cells share a common progenitor cell. The most used SP cells in animal models of MDs are the once derived from bone marrow: in fact, positive results were obtained in *mdx* and *Sgcd^{-/-}* mice, while a particular subpopulation of SP cells from muscle was transplanted in NOD/SCID *mdx* mice, all models for DMD.

The last type of somatic stem cells used in animal models of MDs is represented by the muscle and blood-derived CD133+ cells, a particular cell population characterized by the expression of surface marker CD133 and able to differentiate into muscle, hematopoietic and endothelial cell types when exposed to specific cytokines (Torrente et al. 2004). In several articles it was demonstrated that blood-derived CD133+ cells are able to participate to muscle regeneration, generating dystrophin-positive fibres, once injected both intra-arterial and intra-muscle in *scid/mdx* mice, with best results in the last case (Benchaouir et al. 2007).

A different case is constituted by embryonic stem (ES) cells and induced pluripotent stem cells (Schuler et al. 1986); these cell types, in fact, possess different

characteristics if compared with somatic stem cells: their differentiative potential is higher and wider, as their proliferation activity, as long as their teratoma possible induction once transplanted *in vivo*. This aspect represents the most important limit in the use of ES and iPS as a potential cell source in preclinical studies. Despite that, several studies were performed in order to evaluate the effects of iPS and ES in animal models of MDs: in mdx mice, models of DMD, both the cell populations displayed encouraging results, while in the FRG-1 mice, model of FSHD, the ES-based study demonstrated for the first time how the transplanted cells are capable of generating *de novo* myofibres with very little or no host contribution in such a dominant type of muscular dystrophy.

3 Animal Models in MDs

Unfortunately, despite many efforts, muscular dystrophies remain fatal. As an example, the identification and characterization of dystrophin gene led to the development of potential treatments for DMD (Bertoni 2008) but currently only the treatment with the steroid drug prednisone/prednisolone was proven to be effective on DMD patient (Backman and Henriksson 1995; Dubowitz et al. 2002). This way, myoblasts therapy obtained promising results in animal models but failed in clinical trials (Miller et al. 1997). To discover the genetic basis of unknown MDs and to develop possible treatments for such diseases, several animal models were established. In this chapter, we would like to give an overview of the applicability in preclinical studies of stem cell therapy in MDs using different animal models.

3.1 Zebrafish

In the last years, the zebrafish (*Danio rerio*) received tremendous attention owing to its advantages as a model system. Since the work of Lieschke and Currie (2007), zebrafish was used as model animal for several human diseases with the great advantage that it closely resembled the human condition. Comparative genomic studies highlighted sequence conservation of dystrophin and members of DGC in vertebrate and invertebrate species (Neuman et al. 2001; Roberts and Bobrow 1998; Gieseler et al. 2000). Moreover, Steffen and colleagues identified the orthologues of the majority of human MD disease in its genome (Steffen et al. 2007) while Catchen et al. showed the conservation among the species of the transcriptional network that activates the myogenesis (Catchen et al. 2011). In 2003, Bassett and collaborators identified a dystrophic mutant of zebrafish carrying a dystrophin null allele (Bassett and Currie 2003) while others were described later by Guyon et al. (2009) and Berger et al. (2011). It was demonstrated that as in human, zebrafish's dystrophin was firstly identified at the peripheral ends of the myofibres and after at the

non-junctional sites (Chambers et al. 2001; Clerk et al. 1992). Surprisingly, this model was very similar to DMD patients as, at the onset of the pathology, mutations in dystrophin gene caused the formation of necrotic myofibres, leading to infiltration of mononucleated cells, fibrosis and inflammation (Berger and Currie 2012). Further studies with zebrafish models demonstrated a possible role of sodium channels in preventing the development of fibre detachment. Interestingly, other works showed how dystrophin-deficient zebrafish was a suitable tool to study potential therapies for DMD. Berger et al. assessed that 20–30 % of normal dystrophin transcript levels were needed to recover a severe dystrophic pathology (Berger et al. 2011) while Kawahara and Welch isolated different compounds that ameliorated pathological phenotype of zebrafish (Kawahara et al. 2011; Welch et al. 2007).

α - (and β -) dystroglycan are components of the DGC; when glycosylated, they bind to laminin-2 and anchor the subsarcolemmal cytoskeleton to the ECM. Godfrey et al. described a mutation in the dystroglycan (DAG-1) gene, that caused an out-of-control glycosylation of α -dystroglycan, leading to a reduced binding of α -dystroglycan to laminin-2 (Godfrey et al. 2011). Recently, Hara and colleagues identified a missense mutation in DAG1 associated with limb-girdle muscular dystrophy (Hara et al. 2011). For this disease, commonly known as dystroglycanopathy, different animal models exist. Unfortunately, *Dag1* null mice were reported to be embryonic lethal due to the disruption of an extra-embryonic basement membrane (Williamson et al. 1997). However, zebrafish was able to develop normally so that this model was chosen to study the function of the orthologous *dag1* gene in muscle plasticity. Different works described the fundamental role of dystroglycan to maintain muscle integrity (Parsons et al. 2002) and isolated different mutations in *dag1* gene, associated with various muscular defects (Gupta et al. 2011; Lin et al. 2011). Importantly, it was demonstrated that in dystroglycan mutants the dystrophin was absent confirming that these proteins were necessary for the assembly and stability of the DGC (Cote et al. 1999; Fritz et al. 1995). Furthermore, in the last years, zebrafish allowed to study several genes directly involved in the glycosylation of α -dystroglycan (Avsar-Ban et al. 2010; Thornhill et al. 2008) and one of those, the ISPD gene, was found to be strictly involved in the development of the congenital MD, the Walker–Warburg syndrome (Roscioli et al. 2012).

The actin cytoskeleton of the myofibres is connected to the ECM by means of integrin, dystroglycans and laminins. Different muscle forms of laminin exist, but the laminin-2 is the most important; mutations in this isoform cause severe congenital MD and merosin deficiency (Helbling-Leclerc et al. 1995). Interesting aspects of pathology were described by using zebrafish mutants: Hall and colleagues demonstrated that myofiber detachment was not due to defects in cell signalling but due to mechanical load of the fibres, that caused apoptosis of muscle fibres, and not necrosis as in DMD patients (Hall et al. 2007). Moreover, Maselli and Jacoby suggested that missense mutation in the *lamb2* gene, newly identified in the zebrafish mutant *softy* (*sof*), could be a candidate gene for genetically uncharacterized human MDs (Jacoby et al. 2009; Maselli et al. 2009). Other types of limb-girdle MDs were studied in zebrafish mutants. Roostalu and colleagues knocked down the *Dysf* gene—that causes dysferlinopathy—and evidenced new steps of the complex sarcolemmal

repair of muscle cells mediated by dysferlin, annexin6 and vesicles (Roostalu and Strahle 2012) while Telfer et al. studied the onset of Ullrich congenital MD (UCMD), that is caused by mutations in the collagen VI (Telfer et al. 2010).

3.2 *Caenorabditis elegans*

Since 1960s, the nematode *C. elegans* was utilized as a precious experimental tool to study development and genetic characteristics of several human diseases. It was largely demonstrated that the large degree of sequence similarity found in genes between *C. elegans* and human genomes resulted in a significant functional similarity of the encoded proteins (Chamberlain and Benian 2000). In particular, as muscle structure and muscle proteins are highly conserved among species, worm was particularly attractive to study muscle. This way, Bessou showed that the nematode was an excellent model to study DMD (Bessou et al. 1998). They identified in the genome of the nematode a gene related to the mammalian dystrophin and utrophin proteins (*dys-1*) whose mutants did not suffer for muscular abnormalities but only hyperactivity. Interestingly, *C. elegans* dystrophin-like transcribed by *dys-1* gene shared the same motifs as the human protein. Moreover, it was found that *dys-1* mutants had increased cholinergic activity, opening the intriguing possibility that dystrophin had a role (direct or indirect) in cholinergic signalling (Bessou et al. 1998). This way, Giuglia et al. found a significant reduced level of acetylcholine activity in *dys-1* mutant (Giuglia et al. 1999).

As in mice model of DMD the combined absence of dystrophin and MyoD caused severe myopathy compared with *mdx* mice (Megeney et al. 1996), Gieseler and collaborators generated a double mutant for *dys-1* and *hlh-1* that is the *C. elegans* homologue of MyoD. As expected, these mutants suffered for impaired locomotion and uncoordinated movements and their body wall muscles were severely disorganized (Gieseler et al. 2000). They also identified a gene called *dyc-1* (dystrophin-like phenotype and Capon related) that reduced the phenotype of double mutants, ameliorating their locomotion. Finally, it was demonstrated that *dyc-1* gene was quite similar to human nNOS-binding protein CAPON leading to the possibility that this kind of protein could be a possible therapeutic target for DMD treatment (Gieseler et al. 2000).

As described before for *Drosophila*, also *C. elegans* was successfully used as model organism to study other forms of muscular disease rather than DMD. The nuclear lamina is a protein structure providing structure and stability to the nucleus: it's formed by lamin and its associated proteins (LAPs) and plays a fundamental role in every nuclear function and in controlling transcription factors and histone deacetylases (Broers et al. 2006). Mutations in genes that constitute the nuclear lamina can cause several diseases, such as limb-girdle muscular dystrophy, *LMNA-related* congenital muscular dystrophy (L-CMD) and Emery–Dreifuss muscular dystrophy (EDMD). By means of studies with knockout and mutant strains of *C. elegans*, it was addressed that these pathologies were caused by defects in muscle itself or in the attachment of the muscle to hypodermis and by mis-regulation of neuromuscular junction (Bank et al. 2012).

3.3 *Murine Models*

3.3.1 **DMD Animal Models**

DMD is the most common form of muscular dystrophy: the discovery of a naturally occurring model, the **mdx mouse** had over 30 years. Mdx mice carried a point mutation in exon 23 of dystrophin, which caused the formation of a premature stop codon and the absence of the protein at the sarcolemma (Sicinski et al. 1989). They showed elevated plasmatic creatine kinase (Cpk) levels, fibres necrosis and calcium overload as observed in DMD patients. Muscle pathology could be evidenced from 2 weeks of age, with a peak in cyclic degeneration and regeneration of myofibres at 4 weeks (Partridge et al. 1989). Mdx myofibres were more prone to contraction-induced damage than wild type mice and showed reduced force per CSA but the observed muscle impairment was milder than in DMD patients. In fact mdx mice conserved quite a normal lifespan. Recently Farini et al. bred the *mdx* mouse with the *scid* immunosuppressed mouse, trying to elucidate the role of immune system in promoting muscle pathology (Farini et al. 2007). Mdx mice, similarly to DMD patients, are characterized by an important inflammatory infiltrates around myofibres. Chronic inflammation is sustained by T lymphocytes and macrophages which promotes collagen deposition and fibrosis. **Scid/mdx mice** lack B and T lymphocyte, so that fibrosis and TGF- β 1 expression were dramatically reduced. Moreover *scid/mdx*, as it is an immuno-permissive system, allowed heterologous cell transplantation, representing a dystrophic model for preclinical studies. The huge problem associated to cell transplantation regards host immune system activation. Although *scid/mdx* and *nude/mdx* mice are available, they do not completely overcome this limitation. In fact both models maintain natural killer (NK) cells so that innate immune function is conserved. It was demonstrated that engraftment of human hematopoietic stem cells in *scid* mice could be improved by the additional deletion of the IL2 receptor common gamma chain (IL2R γ) (Traggiai et al. 2004). This founding was ascribed to lack of host NK cells, especially if donor cells expressed low level of self-MHC class I (Liao et al. 1991; Rideout et al. 2002). As a consequence, Arpke et al. generated the **NSG-mdx^{4Cv} mice** which combined (NOD/SCID; gamma-c) mutation with dystrophin deficiency. Resultant mice were almost identical to mdx mice concerning muscle histology and force assessment. Furthermore they demonstrated the feasibility of xenograft and allograft transplant (Arpke et al. 2013).

The applicability of an animal model correlates with its affinity to the human disease: for this reason, several mutant mice were developed on mdx mice background. Among them double-mutant **mdx/utrophin** mice were generated (Deconinck et al. 1997b). Utrophin, in wild type muscles, is found at the sarcolemma during development, while in mature muscles resides at neuromuscular junctions. In dystrophin-negative myofibres abnormal expression of utrophin was observed at sarcolemma. It has been suggested that mdx mice partially compensate the absence of dystrophin by up-regulating utrophin (a dystrophin homologue)

(Deconinck et al. 1997a). These mice exhibited a severe muscular dystrophy which better recapitulated human disease. As many DMD patients, they developed an important cardiomyopathy and premature death around 3 months of age. Importantly they also developed bone abnormality, scoliosis and kyphosis leading to breathing difficulty (Grady et al. 1997; Isaac et al. 2013).

Similarly, double-mutant **mdx/mTR mice** were generated, which lack both dystrophin and telomerase activity. In fact one hypothesis regarding DMD, postulates that the continuous cycle of fibres degeneration/regeneration leads to exhaustion of stem cell reserve, causing the replacement of lost tissue with fibrosis and adipose tissue. Telomeres are DNA repeats that protect chromosome ends during replication, shortening of telomeres leads to genome instability and cell senescence (Palm and de Lange 2008; Rodier et al. 2005; Sherr and DePinho 2000). It was reported that mdx mouse maintain greater murine satellite cells (MuSC) reserve than DMD patients, thanks to longer telomeres, while a 14-fold greater shortening of telomeres in DMD patients relative to healthy individuals was noticed (Decary et al. 2000). Mdx/mTR mice showed severe dystrophic phenotype which could be ameliorated by transplantation with murine wild type satellite cells (Sacco et al. 2010).

3.3.2 LMGD2B and Miyoshi Myopathy Animal Models

LMGD2B and Miyoshi myopathy arose from a different pathological mechanism than DMD or BMD: in fact the sarcolemma and the DSG complex are preserved. The specific deficit involves dysferlin, a protein fundamental in membrane repair process and vesicular trafficking. Four mice models of dysferlinopathies were described: two are naturally occurring models: SJL/J and AJ, and 2 Dysf^{-/-} models were developed by Brown and Campbell, respectively (Ho et al. 2004; Han and Campbell 2007).

SJL mice developed a progressive muscular dystrophy caused by a splice site mutation in the C2 domains of dysferlin gene (Bittner et al. 1999a; Vafiadaki et al. 2001). These mice showed an active myopathy but also an important inflammatory myopathy and they were markedly susceptible to autoimmune diseases and viral infections, a condition that was not confirmed in human patients. These characteristics together with the presence of low level of dysferlin make the SJL mice an inappropriate model for human dysferlinopathies (Bittner et al. 1999a).

A progressive muscular dystrophy was observed in **A/J mice** during routinely screening in Jackson Laboratory. A genome-wide analysis revealed a retrotransposon (ETn) insertion within intron 4 of dysferlin gene, causing aberrant splicing and protein deficiency (Ho et al. 2004). Both AJ and SJL mice showed foci of degenerating and regenerating fibres with centrally located nuclei, variation in fibre size and fibre necrosis, together with fibrosis deposition. In A/J mice the onset of muscle pathology appeared at 5 months of age later than in SJL and in dysferlin-deficient Dysf^{-/-} mice and the progression is less severe. Proximal muscle were more

severely affected than the distal muscles, especially abdominal muscle were highly compromised, while, differently from *mdx* mice, the diaphragm were only mild affected.

A new animal model was generated by Bansal et al., by deleting the last three exons of dysferlin: the **Dysf^{-/-} mice** (Bansal et al. 2003). These mice showed the same characteristic of other models of dysferlinopathies. Bansal developed a method to study in vivo dysferlin function. This membrane repair assay allowed in vivo testing of new drugs or cell transplantation efficacy, by dissection of single fibres from treated *Dysf^{-/-}* mice, thanks to a laser-produced damage and analysis of membrane permeability with a fluorescent dye. In another dysferlin-deficient **Dysf^{-/-} mouse**, the dysferlin gene was inactivated by replacing part of the highly conserved C2E domain with a neomycin gene cassette (Han and Campbell 2007). Recently the importance of immune system in muscular diseases has been deeply investigated. Dysferlinopathic patients were characterized by an important inflammatory infiltrates composed of especially monocytes/macrophages but also of T lymphocytes. Inflammatory cells were both attracted by chronic muscular damage and by specific signalling alteration related to dysferlin deficiency. In fact up-regulation of inflammasome and MHC class I cells and reduced phagocytic activity were reported. Bushby demonstrated that muscular regeneration after notexin injection is attenuated in a mouse model of dysferlinopathy caused by a reduced recruitment of neutrophils in the first phase of regeneration. He suggested that this reduction was consequent to a reduced release of chemotactic agents, secondary to the altered vesicular trafficking in dysferlin-null mice (Chiu et al. 2009).

Moreover Han and Campbell reported over-expression of complement factors in *Dysf^{-/-}* muscle. To further determine the role of complement activation, they generated **Dysferlin/C3 double-mutant mice** which lack dysferlin and the central component (C3). They observed an amelioration of muscle pathology in *dysferlin*-deficient mutant mice, but no similar effect in *mdx* mice, proving that complement activation was due primary to dysferlin deficiency, rather than to chronic inflammatory stimuli (Han and Campbell 2007). At the same time they developed **Dysferlin/C5 double-mutant ko mice** but not beneficial effects were obtained, they suggested that complement damage was not only related to the formation of MAC (membrane attack complex) by C5a-9 but also by the inflammatory environment it provoked. Both double knockout mice did not show significant difference in force generation versus *Dysf^{-/-}* mice.

Recently Farini and Sitzia showed that immuno-modulation was effective in ameliorating dystrophic feature in **Scid/AJ mice** (Farini et al. 2012). In these mice the absence of B and T lymphocytes reduced inflammatory infiltrates and promoted a switch M1 to M2 in macrophage phenotypes (anti-inflammatory). Consequently a reduction in complement activation and in IL-6 pro-inflammatory cytokine levels was observed. Furthermore *scid/AJ* mice showed lower levels of plasmatic CpK and increased tetanic force than *A/J* mice. *Scid/AJ* mice as *scid/mdx* were also used in preclinical studies involving cell transplantation.

3.3.3 Facioscapulohumeral Muscular Dystrophy Models

The third most common form of MD is FSHD, which is caused by chromatin relaxation of the polymorphic D4Z4 macrosatellite repeat array on chromosome 4 in a repeat array contraction-dependent (FSHD1) or contraction-independent (FSHD2) fashion (van der Maarel et al. 2011). Differently from DMD, FSHD has benign course with heterogeneous phenotypes between patients, both regarding severity and age of onset. Typical affected muscles are facial, shoulder and upper arm muscles, often in asymmetric manner. Recently Gabellini et al generated **FRG-1 transgenic mouse** by over-expressing FRG-1, as this gene is located upstream D4Z4 macrosatellite repeat, which deletion involves a transcriptional silencer of gene located upstream (Gabellini et al. 2002). A role of FRG-1 in pre-mRNA processing was suggested after its identification as a component of purified spliceosomes (Rappsilber et al. 2002). These mice showed a progressive muscular dystrophy comparable to the human disease, which was not imputable to plasma membrane defects as demonstrated by the absence of Evan Blue uptake and normal creatine kinase serum levels and integrity of the DCG complex (Gabellini et al. 2006). In particular the severity of muscle impairment is proportional to the level of FRG-1 over-expression. FRG-1 transgenic mouse showed loss of specific force (Po/CSA) of single muscle fibres and atrophy, which involved particularly fast fibres (D'Antona et al. 2007).

The dystrophin-associated proteins include the dystroglycans, sarcoglycans (Fassati et al. 1997) and syntrophin subcomplexes and with dystrophin and laminins represent the most important membrane cytoskeleton system. Among them, sarcoglycans are glycosylated transmembrane proteins, α -, β -, γ - and δ -, that function synergistically to form a tetrameric complex that is the core dystrophin-associated proteins. Unfortunately, defects in genes encoding for these proteins cause a severe form of muscular dystrophy, known as limb-girdle muscular dystrophy (LGMD-2) D, E, F and C, according to mutations in the α -, β -, γ - and δ -SG, respectively. Affected patients suffer for progressive muscle weakness that leads to death in the second or third decade of life (Angelini et al. 1999). The first animal model for LGMD-2, the so-called inbred Bio 14.6 hamster, was identified in 1962, showing cardiac and muscular problems, such as necrosis and variable size of muscle fibres (Straub et al. 1998; Holt et al. 1998; Li et al. 1999). Only recently it was demonstrated that its dystrophic phenotype was due to a loss-of-function mutation of the δ -SG gene (Sakamoto et al. 1997). Different mouse strains carrying mutations in the SGs were successfully used as model organisms to better elucidate the role of each component in the pathogenesis of muscular dystrophy (Hack et al. 2000). This way, it was described that SGs not only regulated structural integrity of the myofiber plasma membrane but also exerted an important role in vascular smooth muscle function, in inducing ischemic injury in skeletal muscle and in transmitting force in the transverse direction to the long axis of muscle (Watchko et al. 2002). One of the most utilized models is the α -SG-deficient null mutant transgenic mouse; Duclos and colleagues demonstrated that the absence of α -SG protein caused muscle hypertrophy and muscle contractile defects that lead to a progressive muscular dystrophy (Duclos et al. 1998).

3.4 *Canine Models*

Spontaneous mutations in the dystrophin gene causing X-linked muscular dystrophy similar to DMD were identified the Golden Retriever (Cooper et al. 1988), the Rottweiler (Winand et al. 1994) and the German short-haired pointer (Schatzberg et al. 1999). More recently, Shimatsu and colleagues artificially inseminated a female beagle with spermatozoa from dystrophic Golden Retriever, obtaining the CXMD (Shimatsu et al. 2003). Unfortunately, dogs are not easy to maintain and cannot be genetically manipulable; however, adult dogs have a body mass that is similar to DMD patients and more importantly their muscular pathogenesis resemble human condition (Cooper et al. 1988; Howell et al. 1997). As in humans, Valentine et al. found that young **GRMD** had high concentrations of serum creatine kinase and died for respiratory problem or heart failure, especially in the first 2 weeks after birth (Valentine et al. 1988). In particular, they described various lesions in muscles of GRMD consisting in hyperacidophilic cytoplasm of muscular fibres, enormous accumulation of calcium, signs of muscular regeneration and necrosis of clusters of fibres. As suggested by Hoffman and Gorospe, these kinds of lesions were known as Phase I and were caused by the absence of the dystrophin in every animal species suffering for mutations in this protein. Interestingly, it was noted that muscles of newborn GRMD were affected by maximum two of Phase I lesions while in 2-month-old affected dogs the pathological phenotype was dramatically worsened, with the presence of widespread endomysial fibrosis and myofiber atrophy (Valentine et al. 1988). These lesions, known as Phase II, were only seen in DMD muscles rather than the diaphragm of mdx mice (Gorospe and Hoffman 1992). This way, GRMD dogs were largely used in the last years for preclinical and clinical studies.

As published by Shimatsu, the **CXMDJ** suffered for elevated serum CK level that caused the death of 1–3 of littermates. The first muscle that showed dystrophic phenotype was the diaphragm and only after 3 months of age atrophy and weakness appeared evident in limb muscles. Since 4 months, the clinical course of these dogs rapidly worsen due to macroglossia and joint contracture; however, after 10 months of age, the progression of the disease was retarded (Shimatsu et al. 2005). Similarly to GRMD, these dogs showed fibrosis of the left ventricular wall that was accompanied by degeneration of Purkinje fibres (Valentine et al. 1988; Perloff et al. 1967).

4 **Preclinical Studies**

Different subpopulations of human stem cells were proven for their efficacy in muscular differentiation: they were injected into animal models for MDs and it was observed whether they ameliorated their dystrophic phenotype. The aim of these studies was to select myogenic stem cells and translate them into clinical trials on human subjects.

4.1 *Preclinical Studies for DMD*

Since 1989, the group of Partridge showed that myoblast injection into mdx mice ameliorated dystrophin expression (Partridge et al. 1989). According to these exciting results, several clinical trials on human subjects arose but unfortunately failed, as purified myoblasts were not able to proliferate and fuse in host muscular environment (Skuk et al. 2000, 2002). Similarly, Montarras et al. obtained promising results by transplanting into dystrophic mice a pure population of murine-derived satellite cells: they demonstrated that these cells not only formed a pool of satellite cells expressing Pax7 and Pax3 but also possessed an incredible ability of muscular differentiation (Montarras et al. 2005). However, as it was demonstrated for myoblasts, the growth of freshly isolated satellite cells in vitro significantly reduced their in vivo myogenic potential. More recently, Cerletti and colleagues described a newly isolated subpopulation of muscle satellite cells that transplanted into mdx mice not only allowed the re-expression of dystrophin but also regenerated mature muscle fibres and ameliorated host dystrophic phenotype, reducing muscle inflammation and fibrosis (Cerletti et al. 2008). Unfortunately, in vitro expansion of these cells reduced drastically their myogenic potential so that they were not considered for further clinical utilization. Interesting works from several groups identified a population of multipotent muscle-derived stem cell (MDSC), residing in the skeletal muscle (Sarig et al. 2006; Tamaki et al. 2007). It was demonstrated that these cells were different from satellite cells and contained different sub-populations able to efficiently differentiate into skeletal muscle. This way, our group demonstrated that muscle-derived Sca-1+ CD34+ stem cells interacted to endothelium in mdx muscles and participated into muscle regeneration (Torrente et al. 2001) while Qu-Petersen showed that another subpopulation, expressing also c-kit and CD45 could be efficiently transplanted into murine dystrophic muscle (Qu-Petersen et al. 2002). Starting from their demonstration that, under specific stimuli, adult human synovial membrane-derived mesenchymal stem cells (hSM-MSCs) differentiated into muscle (De Bari et al. 2001), de Bari and colleagues assessed that the injection of these cells restored the expression of dystrophin into mdx mice (De Bari et al. 2003). Following the characterization of the immunodeficient dystrophic scid/mdx (Farini et al. 2007), this model was largely used in preclinical studies for muscular diseases. As published by Benchaouir et al. in 2007, two dystrophic CD133+ cell populations, one from blood and one from skeletal muscle, were transduced with a lentivirus carrying a construct designed to skip exon 51 (Benchaouir et al. 2007). As they expressed a short but still functional isoform of dystrophin, engineered CD133+ stem cells were injected into scid/mdx mice. They differentiated into endothelial and muscular cells, took part into muscular regeneration and ameliorated the muscular force in treated animals (Benchaouir et al. 2007).

Following the pioneering work of Partridge regarding the fusion of donor myoblasts in the mdx mouse (Partridge et al. 1989), Prattis e Nakagaki isolated and fully characterized the myoblasts of DMD canine model but never obtained appreciable results (Nakagaki et al. 1994; Prattis et al. 1993). However, Kornegay and colleagues demonstrated that following autologous transplantation myoblasts differentiated in

muscle (Kornegay et al. 2012). More recently, Pichavant et al. transfected dystrophic dog muscle precursor cells with a lentivirus carrying canine microdystrophin cDNA and transplanted them into normal dogs (Pichavant et al. 2010). Interestingly Parker and co-workers showed that myoblast transplantation efficiency was higher if the dogs were previously injected with hematopoietic cell, to induce tolerance (Parker et al. 2008). As Dell'Agnola showed that bone marrow stem cell transplantation did not allow dystrophin re-expression in GRMD dogs (Dell'Agnola et al. 2004), Cossu and colleagues transplanted some dystrophic dogs using MABs transduced with a lentiviral vector expressing human micro-dystrophin (Sampaolesi et al. 2006). Treated dogs expressed dystrophin and ameliorated their dystrophic phenotype, improving muscle function and mobility; furthermore, the large majority of muscle fibres showed normal force contraction and absence of defective mobility (Sampaolesi et al. 2006). Recently, Rouger et al. described how the intra-arterial injection of allogenic muscle stem cells into GRMDs contributed to myofiber regeneration, satellite cell replenishment and dystrophin expression. Interestingly, following cellular transplantation, dogs showed long-term dystrophin expression and amelioration of muscle damage course, due to increased regeneration activity (Rouger et al. 2011). The group of Mayana Zatz transplanted GRMDs with different types of stem cells. After injection of human immature dental pulp stem cells, they appreciated low expression of dystrophin (Kerkis et al. 2008) while dystrophin was not identified following intra-arterial injection of umbilical cord mesenchymal stromal cells (Zucconi et al. 2011). However, in this study, it was demonstrated that systemic injections of umbilical cord MSCs was a safe technique; moreover, even if they did not participate directly in formation of muscle, these cells allowed the expression of several molecules that activated the proliferation of stem cells resident in host muscles. As the MABs are similar to human ADSCs regarding size, proliferation and differentiation capacity, they decided to implant these cells in non-immunosuppressed GRMDs. They found that ADSCs reached several muscles and allowed the expression of dystrophin; moreover, they suggested that, as a therapeutically point of view, at least two systemic injections were required for the maintenance of injected cells in the host muscles (Vieira et al. 2012). Nitahara-Kasahara isolated CD271+ MSCs and transduced them with MyoD to induce myogenic differentiation and to allow the formation of myotubes. Combining these findings with the anti-inflammatory role exerted by MSCs, they injected these cells into CXMD and described the formation of clusters of muscular fibres and re-expression of dystrophin (Nitahara-Kasahara et al. 2012). Bhagavati and co-workers demonstrated that ES could have important effects in favouring muscular development as after injection into mdx mice allowed the formation of vascularized skeletal muscle (Bhagavati and Xu 2005). Mizuno et al. demonstrated that iPS cells were quite similar to skeletal muscle stem cells so that after transplantation in murine models of DMD they differentiated into myofibres repairing damaged muscle tissues and replenishing the host satellite cells compartment (Mizuno et al. 2010). More recently, Darabi et al. showed that Pax7-expressing EC/iPS cells regenerated muscle *in vivo*, improved muscular force of transplanted mdx mice and formed functional satellite cells (Darabi et al. 2012).

4.2 *Preclinical Studies for Dysferlinopathies*

Thanks to lessons learned from cell transplantation in mdx mice, Guerin et al. injected normal myoblast in SJL mice and demonstrated *in vivo* rescue of dysferlin expression (Leriche-Guerin et al. 2002). However the number of dysferlin-positive myofibres was lower than the number expected following normal myoblast transplant in mdx mice (Partridge et al. 1989; Kinoshita et al. 1994; Morgan et al. 1990; Vilquin et al. 1995). This result was ascribed to SJL inflammatory background rather than to immunosuppressive treatment, but the need of alternative strategies or different animal models became evident. To overcome immunosuppressive limitation, Kong et al. injected human umbilical cord blood (HUCB) cells intravenously in SJL mice, as these cells were known to be poor immunoreactive (Gluckman et al. 1997; Rocha et al. 2000). They demonstrated the presence of human nuclei within host fibres, but they observed very low frequency of myogenic differentiation (Kong et al. 2004). A step forward was moved in 2008 when the group of Zatz demonstrated the feasibility of human adipose-derived stromal cells transplant in SJL mice, without need of immunosuppression (Vieira et al. 2008). Furthermore they obtained a significant functional improvement in the injected animals although the body-wide distribution of hASCs was widespread.

Promising results obtained using MABs in the field of DMD raised hope for the treatment of dysferlinopathies too. In fact Díaz-Manera demonstrated that adult murine MABs isolated from normal mice, both after intramuscular or intra-arterial administration to SCID/BIAJ mice could reach and survive in the host muscle. They demonstrated that partially restored dysferlin expression allowed normal repairing ability of the membrane after laser-induced lesions (Diaz-Manera et al. 2010). Some tentative were made with mini-gene replacement in animal model of dysferlinopathies as dysferlin messenger RNA is far above the natural packaging size of rAAV: they demonstrated that mini-dysferlin could maintain its functionality (Krahn et al. 2010). Alternative strategy combined the injection of two independent AAV vectors which together contained the full length sequence of dysferlin cDNA. After intramuscular injection of both AAV vectors into muscle of A/J mice, they obtained the expression of full-length dysferlin, the restoration of the membrane repair capacity of myofibres and increased locomotor activity of the mice (Lostal et al. 2010). However recently, controversy arose about the predictive value of membrane repair assay in myofibres isolated from treated dysferlin-deficient mice. In particular Lostal et al. demonstrated that different approaches that rescue membrane repair after laser damage assay (as described by Bansal) failed to alleviate the dysferlin-deficient pathology in mice (Lostal et al. 2010).

4.3 *Preclinical Studies for Sarcoglycanopathies*

Differently from DMD no experiment involving myoblast transplantation was conducted in sarcoglycanopathies. However sarcoglycan genes are smaller than dystrophin, so that their delivery with recombinant adenovirus or AAV vector is

possible. This approach was firstly tempted in BIO14.6 cardiomyopathic hamster, by injection of AAV vector, which carried δ -SG (Holt et al. 1998; Li et al. 1999; Greelish et al. 1999). Allamand et al. demonstrated the rescue of the expression of α -SG at the sarcolemma and the long-term reconstitution of DSG complex after α -sg AAV vector intramuscular injection in *Sgca*-null mice (Allamand et al. 2000). Although these promising results, the therapeutic effect was obtained only when the vector injection is performed before the onset of the dystrophic pathology as inflammatory environment and fibrosis disturbed AAV vectors transduction of myofibres (Allamand et al. 2000; Fougerousse et al. 2007). Different experiments with cell therapy were attempted, using either bone marrow, vessel associated, or UCSs giving disappointing results (Lapidos et al. 2004; Salah-Mohellibi et al. 2006). These results were probably due to inefficient delivery of donor cells to dystrophic muscles. These difficulties were overcome by Sampaolesi et al. thanks to the intra-arterial injection of MABs from dystrophic mice and transduced with lentiviral vector expressing α -SG in α -Sg null mice. They observed α -sarcoglycan expression, restoration of DGC complex and increased myofibres strength (Sampaolesi et al. 2003). A new intriguing method to treat sarcoglycanopathies arose from experiments of Bartoli's group. They demonstrated that a particular mutation (R77C in α -sarcoglycan gene) which causes protein retention could be bypassed by blocking the protein quality control system. They demonstrated that in vivo delivery of kifunensine (Mannosidase I inhibitor) in living mice could restore protein localization at the sarcolemma without evidence of toxicity (Bartoli et al. 2008).

4.4 Preclinical Studies for FSHD

Unfortunately, pathological mechanisms, underlying FSHD development, were only recently elucidated. In particular Gabellini et al. was able to reproduce FSHD in mice model only 10 years ago (Gabellini et al. 2002). For this reason, first studies with cell transplantation in FRG-1 mice started, when limits of myoblasts and other cell type transplantation were well known. Darabi et al. demonstrated that Pax3-induced ES-derived myogenic progenitors were able to engraft into dystrophic FRG-1 mice and produced functional improvement (Darabi et al. 2012). Thanks to these mice the role of over-expression of FRG-1, and DUX4 genes appeared (Wallace et al. 2012). Recently strategies for *FRG-1* knockdown have been developed. In particular through RNA interference (Ferrari et al. 1998) expression cassette Bortolanza et al. showed restoration of muscle function in FRG-1 mice. They demonstrated that intravenous delivery of AAV6-mediated *FRG-1* RNAi was safe and permit a long-term knockdown of FRG-1 mRNA, leading to significant amelioration of muscle function (Bortolanza et al. 2011).

5 Conclusions

Many pathological mechanism theories are developed, thanks to animal models suitable for experimental or genetic manipulation. Several aspects have to be evaluated if researchers want to use an animal model for preclinical studies (Willmann et al. 2009). The following criteria are fundamental: (1) genetic basis of disease must be the same both for humans and animal models; (2) the animal models should have the same phenotypic characteristics of humans; (3) disease progression must be well characterized in order to obtain precise, comparable and reproducible outcome analysis. In case of DMD, suitable animal models have to present muscle weakness, respiratory insufficiency, cardiomyopathy, centralized nuclei myofibres and fibre size variations.

Murine models provide important findings for the basic study of pathogenesis and development of therapies, but generally the clinical phenotype is not completely comparable to that human patient. Mdx animal models have many advantages, firstly the large number of papers published on these mice guaranteed a good understanding of muscle, cardiac and respiratory pathology (Partridge et al. 1989); later the reproducibility of the phenotype and the commercial availability are important advantages. Finally, this animal model has been used to demonstrate efficacy of several potential treatment strategies including small molecule pharmaceuticals as well as gene correction (Goyenvalle et al. 2004). Also scid/mdx animal model, as it is an immuno-permissive system, allowed heterologous cell transplantation, representing a dystrophic model for preclinical studies (Torrente et al. 2004; Benchaour et al. 2007). GRMD dog displays an inherited degenerative disorder genetically homologous to DMD patients but it shares with DMD patient progressive clinical signs and severe myopathy with contractures and premature death (Thibaud et al. 2007). In human and canine disorders, skeletal muscle shows early fibre necrosis and regeneration together with endomysial and perimysial connective tissue proliferation and severe cardiac defects. The body size and genetic background of canines are more similar to human beings than the murine models. Furthermore, histopathological changes in skeletal muscle appear at preclinical stages in both DMD patient and GRMD dog. GRMD dog represents, thus, the most relevant animal model for DMD, particularly in regard to potential therapeutic approaches and so investigations of stem cells potential in this model are absolutely necessary. One important problem in using animal models is the variability. Moreover, special care must be taken in choosing the control animals and control experiments must be selected with special attention.

Actually, none of the animal models much used totally mimics all the characteristics of the human diseased; the larger animal models, such as dogs, show high variability in disease severity between breeds and among littermates although they all carry the same genetic mutation or non-human primates do not exist for preclinical studies of muscular dystrophy.

In general, methods finalized to increase dystrophin, utrophin, dysferlin and other proteins involved into the onset of MDs are showing great promise in animal

models and are beginning to be tested in clinic studies (Goyenvalle et al. 2011). Nevertheless, the optimization of all the possible approaches to treat MDs will be needed before a declaration of success and not before a delicate balancing act between preclinical animal models and human clinical trials.

Up against these considerations, it is very important to increase the number of Phase I clinical trials in human patients when basic research shows the safety of promising therapeutic approaches in animal models. We hope that the achievement of positive results in cycles of animal/human studies will permit a gradual implementation of new therapies that will ameliorate lifespan and life quality of patients affected from different forms of muscular dystrophies.

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Chapter 2

Understanding Tissue Repair Through the Activation of Endogenous Resident Stem Cells

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1 The Clinical Need of Stem Cells for Cardiovascular Diseases

Heart failure (HF) has been singled out as an epidemic and is a staggering clinical and public health problem, associated with significant mortality, morbidity, and healthcare expenditures, particularly among those aged ≥ 65 years (Roger 2013). In particular, HF has a prevalence of roughly six million in the United States (a similar number is reached in Europe) and more than 23 million worldwide. After the diagnosis of HF, survival remains quite poor with estimates of 50 % and 10 % at 5 and 10 years, respectively. Despite modest progress in reducing HF-related mortality, hospitalizations for HF remain frequent and rates of readmissions continue to rise (Roger 2013). It is predicted that the constant progress in the primary prevention of

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HF will eventually lead to decreasing incidence of the disease. Accordingly, the constant improvement in modern medical care of the acute cardiac diseases will result in improved survival. However, the latter in turn will anyway increase the prevalence of HF. This is so because modern management for HF is mainly a symptomatic treatment that does not fight against its cause, leave organ transplantation as the only alternative available to restore function, with all the logistic, economic and biological limitations associated with this intervention (Kahan 2011). Indeed, the root problem responsible for the poor outcome of the CHF is a deficit of functional myocardial contractile cells (cardiomyocytes) and adequate coronary circulation to nurture them resulting in pathological cardiac remodelling, which, in turn, triggers the late development of cardiac failure in these patients (Jessup and Brozena 2003). For this reason, it has been a goal of cardiovascular research for the past decade to find methods to replace the cardiomyocytes lost as a consequence of the MI in order to prevent or reverse the pathological cardiac remodelling. Overall, the need to identify new therapies has become a key research area in regenerative cardiovascular medicine and stem cell-based therapies are fast becoming an attractive and highly promising experimental treatment for heart disease and failure (Terzic and Nelson 2010).

2 Adult Heart Self-Renewal and Tissue-Specific Endogenous Cardiac Stem/Progenitor Cells

Out of the limelight and apart from the cultural and philosophical wars, over the past 15 years there has been a slow but steady re-evaluation of the prevalent paradigm about adult mammalian—including human—tissue cellular homeostasis. It has been slowly appreciated that the parenchymal cell population of most, if not all, adult tissues is in a continuous process of self-renewal with cells continuously dying and new ones being born. Once cell turnover was accepted as a widespread phenomenon in the adult organs, it was rapidly surmised that in order to preserve tissue mass, each organ constituted mainly of terminally differentiated cells needed to have a population of tissue-specific regenerating cells. Not surprisingly, this realization was rapidly followed by the progressive identification of stem cells in each of the adult body tissues (Yamanaka 2007; Robinton and Daley 2012; Rountree et al. 2012; Reule and Gupta 2011; Kopp et al. 2011; Kotton 2012; Buckingham and Montarras 2008; Suh et al. 2009).

For a long time, the cardiovascular research community has treated the adult mammalian heart as a post-mitotic organ without intrinsic regenerative capacity. The prevalent notion was that the >20-fold increase in cardiac mass from birth to adulthood and in response to different stimuli in the adult heart, results exclusively from the enlargement of pre-existing myocytes (Hunter and Chien 1999; Soonpaa and Field 1998; Lafflamme and Murry 2011). It was accepted that this myocyte hypertrophy, in turn, was uniquely responsible for the initial physiological adaptation and subsequent deterioration of the overloaded heart. This belief was based on two generally accepted notions: (a) all myocytes in the adult heart were formed during fetal life or shortly thereafter, were terminally

differentiated and could not be recalled into the cell cycle (Nadal-Ginard 1978; Chien and Olson 2002); therefore, all cardiac myocytes have to be of the same chronological age as the individual (Oh et al. 2001); (b) the heart has no intrinsic parenchymal regenerative capacity because it lacks a stem/progenitor cell population able to generate new myocytes. Despite published evidence that this prevalent view was incorrect (Beltrami et al. 2001; Quaini et al. 2002; Urbanek et al. 2003, 2005; Anversa and Nadal-Ginard 2002; Nadal-Ginard et al. 2003), it took the publication of Bergmann et al. in 2009, based on ^{14}C dating in human hearts showing that during a lifetime the human heart renews ~50 % of its myocytes (Bergmann et al. 2009), to produce a significant switch in the prevalent opinion. After this, intensive research and still much controversy on the adult mammalian heart's capacity for self-renewal has finally brought a consensus that new cardiomyocytes are indeed formed throughout adult mammalian life (Bergmann et al. 2009; Hsieh et al. 2007). However, the physiological significance of this myocyte renewal, the origin of the new myocytes as well as the rate of adult myocyte turnover are still highly debated. Indeed, while Bergmann et al. (2009) have calculated a yearly cardiomyocyte turnover of about 1 % (Bergmann et al. 2009) others calculated 4–10 % (Senyo et al. 2013) and some as high as 40%/year (Kajstura et al. 2012). This very high spread on the “measured” values of such an important phenomenon raises questions about the conceptual and methodological approaches used in these studies. If Bergmann's calculations were the one more close to the reality, because this “measured” self-renewal is far from being very robust, its physiological significance would be highly doubtful. However, the conclusion of Bergmann et al. (2009) on the rate of turnover depends on the validity of a complex mathematical formula, whose impact on the results dwarfs that of the measured data. Their calculations identify the highest turnover rate during youth and early adulthood followed by a steady decrease with age. The latter conclusion, which is contrary to most or all the turnover values measured for all other human tissues, including the heart (Nadal-Ginard et al. 2003), has passed without a ripple. Similar discrepancy exists with respect to the origin of the new myocytes and their physiological significance. Three main sources of origin of the new myocytes have been claimed: (a) circulating progenitors, which through the bloodstream home to the myocardium and differentiate into myocytes (Quaini et al. 2002); (b) mitotic division of the pre-existing myocytes (Boström et al. 2010; Bersell et al. 2009; Kühn et al. 2007) and (c) a small population of resident myocardial and/or epicardial multipotent stem cells able to differentiate into the main cell types of the heart: myocytes, smooth and endothelial vascular and connective tissue cells (Torella et al. 2007; Rasmussen et al. 2011). It is clear now that the blood borne precursors, although well documented as a biological phenomenon (Eisenberg et al. 2006), might be limited to very special situation (Orlic et al. 2001) and their direct regenerative import is very limited, if any (Loffredo et al. 2011). Myocyte replacement, particularly after injury, was originally attributed to differentiation of a stem-progenitor cell compartment (Beltrami et al. 2003) a source confirmed by genetic cell fate mapping (Hsieh et al. 2007). However, more recently this last investigator group, using the same genetic tools, claims

that myocytes in the border zone of an infarct are actually replaced by the division of pre-existing post-mitotic myocytes (Senyo et al. 2013). Pre-existing cardiomyocyte division has not been convincingly documented and/or remains to be confirmed by different authors. This result, in addition to being contrary to most known biology of terminally differentiated cells, would shift the target of regenerative therapy towards boosting mature cardiomyocyte cell cycle re-entry. However, Senyo et al. (2013) only document that a very small fraction of cardiomyocyte DNA replication occurs in cells that have already activated the α MHC gene, a well-documented part of myocyte development, which falls short of documenting mature myocyte re-entry into the cell cycle. This evidence is indeed equally compatible with new myocyte formation from the pool of multipotent cardiac progenitor cells because it is a well-documented fact that newly born myocytes are not yet terminally differentiated and are capable of a few rounds of mitosis before irreversibly withdrawing from the cell cycle (Nadalin et al. 2003). Undoubtedly, the best documented source of the regenerating myocardial cells in the adult mammalian heart, including the human, is a small population of cells distributed throughout the atria and ventricles of the young, adult and senescent mammalian myocardium, that have the phenotype, behaviour and regenerative potential of bona fide cardiac stem cells (eCSCs) (Torella et al. 2007; Rasmussen et al. 2011; Srivastava and Ivey 2006). In 2003, we identified the first population of eCSCs in the adult mammalian rat heart (Beltrami et al. 2003). These cells express the stem cell marker c-kit (c-kit^{pos}), are positive for Sca-1 and MDR-1 (ABCG2), yet are negative for markers of the blood cell lineage, CD31, CD34 and CD45 (described as Lin^{neg}). They are self-renewing, clonogenic and multipotent and exhibit significant regenerative potential when injected into the adult rat heart following a myocardial infarction (MI), forming new myocytes and vasculature and restoring cardiac function (Beltrami et al. 2003). c-kit^{pos} eCSCs with similar properties to those originally identified in the rat have been identified and characterized in the mouse (Messina et al. 2004; Fransioli et al. 2008), dog (Linke et al. 2005), pig (Ellison et al. 2011) and human (Messina et al. 2004; Torella et al. 2006a, b; Bearzi et al. 2007; Arsalan et al. 2012). These cells are present at a similar density in all species (~1 eCSC per 1,000 cardiomyocytes or 45,000 human eCSCs per gram of tissue) (Torella et al. 2007). Similar to the rodent heart, the distribution of c-kit^{pos} eCSCs in the pig and human heart varies with cardiac chamber. Although a variety of markers have been proposed to identify eCSCs in different species and throughout development (Messina et al. 2004; Oh et al. 2003; Matsuura et al. 2004; Martin et al. 2004; Laugwitz et al. 2005; Moretti et al. 2006; Kattman et al. 2006; Wu et al. 2006; Smart et al. 2011), it still remains to be determined whether these markers identify different populations of eCSCs or, more likely, different developmental and/or physiological stages of the same cell type (Ellison et al. 2010). Recently, another multipotent cell type, present in the epicardium and derived from the pro-epicardial organ has been described (Ellison et al. 2007a). The role of these cells in normal or pathological myocyte turnover remains to be elucidated.

The progeny of a single eCSC is able to differentiate into cardiac myocytes, smooth muscle and endothelial vascular cells and when transplanted into the

border zone of an infarct regenerates functional contractile muscle and the microvasculature of the tissue (Beltrami et al. 2003). In a normal adult myocardium, at any given time, most of the eCSCs are quiescent and only a small fraction is active to replace the myocytes and vascular cells lost by wear and tear. In response to stress (hypoxia, exercise, work overload, or other damage), however, a proportion of the resident eCSCs are rapidly activated, they multiply and generate new muscle and vascular cells (Ellison et al. 2007a, 2012a), contributing to cardiac remodelling. Recently, two studies have questioned the *in situ* myogenic potential of c-kit^(pos) cardiac cells in adult life as being significantly reduced compared to their neonatal counterparts (Zaruba et al. 2010), and in a model of myocardial cryo-injury (Jesty et al. 2012). Thus, whether the c-kit^{pos} eCSCs are necessary and/or sufficient for the adult cardiac regenerative response to damage/injury remained unproven. However, recently, using mouse and rat experimental protocols of severe diffuse myocardial damage which unlike an experimental infarct spares the eCSCs (Ellison et al. 2007b) combined with several genetic murine models and cell transplantation approaches, we have shown that the eCSCs, in the presence of a patent coronary circulation, fulfil the criteria as the cell type necessary and sufficient for myocyte regeneration, leading to complete cellular, anatomical and functional myocardial recovery (Fig. 2.1) (Ellison et al. 2013). To follow c-kit^{pos}eCSC physiological response to cardiac injury, we induced severe diffuse myocardial damage in adult rats with a single high dose of isoproterenol (ISO) (Ellison et al. 2007b). This treatment—in the presence of a patent coronary circulation—produces a Takotsubo-like cardiomyopathy (Akashi et al. 2008) (a clinical syndrome affecting up to 2 % of patients with symptoms and signs of acute myocardial infarction (AMI), characterized by catecholamine overdrive and reversible cardiomyopathy) killing-10 % of the LV myocytes and resulting in overt acute heart failure (Ellison et al. 2007b). Interestingly, the myocardial damage and heart failure spontaneously reverses anatomically and functionally by 28 days. The anatomical and functional recovery is met through a robust c-kit^(pos) eCSC activation and ensuing new myocyte formation, the latter completely balancing the myocyte loss by ISO injury. Using inducible double-transgenic reporter mice to track the fate of adult cardiomyocytes in a “pulse-chase” fashion we compellingly show that new myocytes after diffuse myocardial injury are not generated through the division of pre-existing terminally differentiated myocytes but rather from non-myocyte cells, with the characteristics of a stem-progenitor compartment. Then, to directly identify whether c-kit^{pos} eCSCs replenish cardiomyocytes lost by myocardial damage, we genetically tagged *in situ* the resident c-kit^{pos}eCSCs and their committed progeny. Through these *in vivo* genetic cell-fate mapping experiments, we have eventually proved that new myocytes after myocardial injury in the adult mammalian heart originate from resident c-kit^{pos}eCSCs. Furthermore, we have shown in a rat model of severe cardiomyopathy induced by ISO injury and 5-Fluoro Uracil that the ablation of the eCSCs abolishes regeneration and functional recovery. The regenerative process however is completely restored by replacing the ablated eCSCs with the tagged progeny of one eCSC. These eCSCs recovered from the primary host, and re-cloned, retain

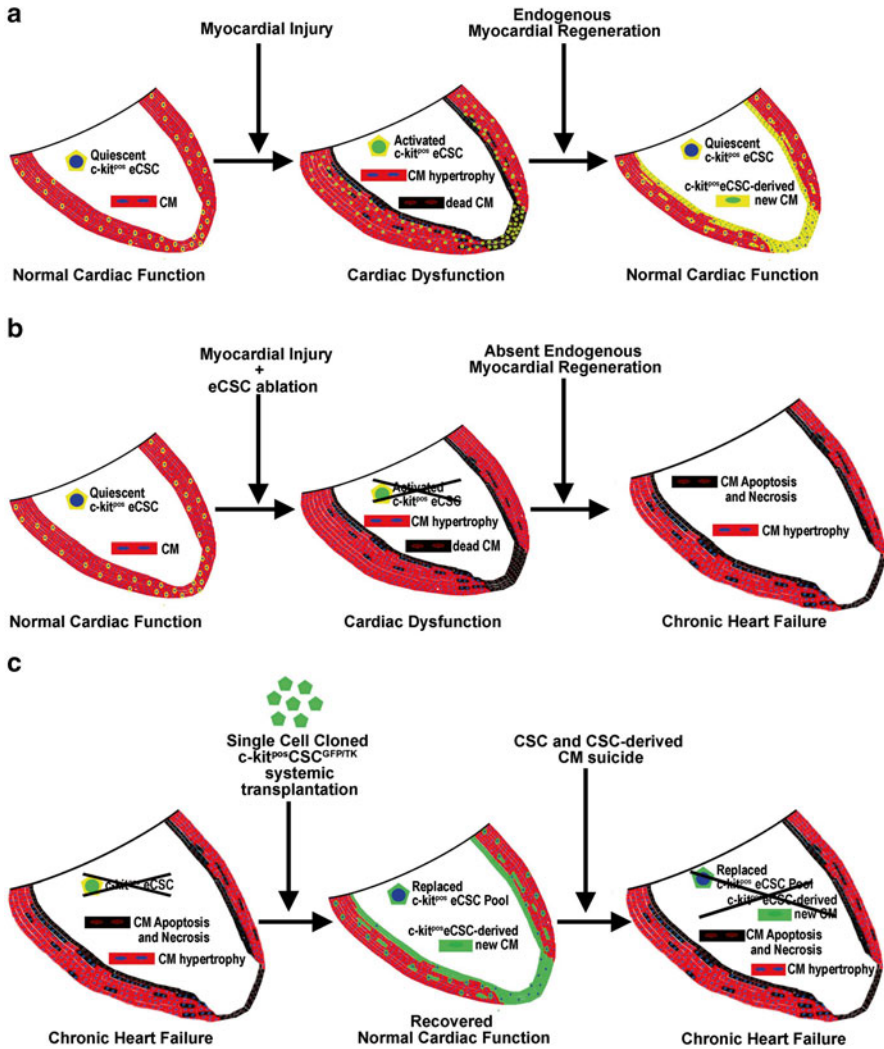


Fig. 2.1 eCSCs are necessary and sufficient for myocardial repair and regeneration. (a) Schematic of myocyte damage and cardiac recovery through eCSC activation and new myocyte formation. (b) When eCSC are ablated, cardiac regeneration is absent with the development of a severe cardiomyopathy. (c) If CSCs are exogenously transplanted, cardiac anatomy and function is restored. However, the selective suicide of the transplanted CSCs and their progeny sets back the animal in heart failure

their regenerative potential *in vivo* and *in vitro*. Finally, after regeneration, selective suicide of these exogenous eCSCs and their progeny abolishes the regeneration, severely impairing ventricular performance. Thus, overall these data have provided the ultimate and solid documentation that the resident tissue-specific eCSCs are necessary and sufficient for the regeneration of the adult myocardium and establish these cells as true cardiac regenerative agents.

3 Autologous Cardiac Stem Cell Therapy for Heart Failure

Many questions about eCSC basic biology still remain unanswered, particularly their long-term effectiveness and regenerative potential as well as their origin. It is imperative that such issues be addressed quickly if the full potential of these cells is to be realized, manipulated and applied clinically. In particular, it is imperative to document whether the teratogenic and neoplastic potential of the *in vitro* expanded eCSCs is low enough to make their use in humans safe. However, clinical trials using autologous cardiac stem/progenitor cells are already underway (Bolli et al. 2011; Makkar et al. 2012). In the SCIPIO (Stem Cell Infusion in Patients with Ischemic Cardiomyopathy; NCT00474461) trial, 16 patients with ischaemic cardiomyopathy with post-infarction LV dysfunction (ejection fraction $\leq 40\%$) who had undergone coronary artery bypass grafting, had 500,000–1 million of autologous c-kit positive, lineage negative, cardiac progenitor cells infused intracoronary, ~4 months after surgery (Bolli et al. 2011). The control group was not given any treatment. LVEF increased by 8 EF points at 4 months after infusion, whereas the LVEF did not change in the control patients, during the corresponding time interval. Moreover, LVEF increased by 12 EF points in eight of the treated patients at 1-year follow-up. cMRI of seven of the treated patients showed that infarct size decreased at 4 and 12 months (Bolli et al. 2011). Furthermore, the interim analysis of myocardial function and viability by magnetic resonance in SCIPIO on a total of 33 patients (20 CSC-treated and 13 control subjects) confirmed the improvement in both global and regional LV function, and a reduction in infarct size at 1 year (Chugh et al. 2012). In the prospective, randomized cardiosphere-derived autologous stem cells to reverse ventricular dysfunction (CADUCEUS) trial, 17 patients (with left ventricular ejection fraction of 25–45%) were infused into the infarct-related artery with up to 25 million, CD105-positive, autologous cardiosphere-derived cells (CDCs), 1.5–3 months after myocardial infarction (Makkar et al. 2012). Eight patients received standard care and acted as the control group. Compared with controls at 6 months, MRI analysis of patients treated with CDCs showed significant reductions in scar size and mass, increased viable heart mass, regional contractility and systolic wall thickening. However, changes in end-diastolic volume, end-systolic volume, and LVEF did not differ between groups at 6 months (Makkar et al. 2012). Recently, the ALCADIA clinical trial was initiated, which will focus on a hybrid biotherapy approach for treating chronic ischemic cardiomyopathy. This translational study is focusing on the safety and efficacy of autologous clonally amplified CSCs, which have shown to be enriched for embryonic stem cell markers and have mesenchymal cell characteristics (Matsubara and Kyoto Prefectural University School of Medicine 2012). This trial is also investigating cell therapy with the controlled release of basic fibroblast growth factor (bFGF) from a gelatin hydrogel sheet. While this innovative work has proved promising in the respect that transplantation of autologous CSCs has not resulted in any adverse health effects, we now await further studies, which focus on the efficacy of eCSC-based therapies and compare these to results obtained with BMDCs. Indeed, because of the high cost and the long wait for the availability of the cells for autologous cardiac stem/

precursor cell therapy, it will become imperative to compare the beneficial effects of this approach to that obtained with BMDCs because of their easier availability, accessibility and lower cost of the procedure. Furthermore, the widespread use and applicability of autologous cardiac stem cell therapy is highly debatable. Firstly, the procedure for cell acquisition, scale-up and transplantation is complex, time consuming and very expensive. The isolation and expansion of eCSCs to the number needed from catheter and surgical biopsies takes 1–3 months. Therefore, the cells are not available to be administered when they would be most effective, that is when a patient with an AMI in progress arrives at the hospital. Furthermore, the cost of the procedure in human and material resources would make it unavailable to patients beyond those few required to establish proof-of-concept for the therapy and to a small group of individuals with abundant economical resources. Finally, eCSCs undergo senescence with severe pathological consequences (Torella et al. 2004, 2006a; Matsubara and Kyoto Prefectural University School of Medicine 2012; Chimenti et al. 2003). Accordingly, for the cohort of patients (the aged population) most likely candidates for the regenerative therapy, >50 % of their eCSCs can be senescent and unable to participate in the regenerative process (Torella et al. 2004, 2006a; Matsubara and Kyoto Prefectural University School of Medicine 2012; Chimenti et al. 2003). Thus, if eCSC “aging” is an age or cell cycle dependent process, which affects all or most of the eCSC population, most or all regenerative therapies based on eCSC isolation and expansion will likely result in further exhaustion of the self-renewal capability of these cells with an accelerated loss of their regenerative capacity.

4 Stimulation of the Myocardial Endogenous Capacity for Repair and Regeneration

As above mentioned, while autologous eCSCs undoubtedly hold great promise for cardiac repair, their isolation and expansion prior to cell transplantation can be complex, time consuming and costly. This has raised the question of whether it may be advantageous to target the activation and regenerative capacity of the resident eCSCs to reconstitute damaged myocardium in the absence of cell therapy. One potential therapeutic mechanism of action by which the different forms of transplantation cell therapy are now receiving a lot of attention, is the activation, through a paracrine mechanism, of survival pathways in the cohort of cells at risk together with the endogenous regeneration compartment, represented by the eCSCs. A corollary of this hypothesis is that the identification of the molecules secreted by the transplanted cells should make possible the design of therapies, which eliminate the use of the cells and concentrate on the administration of the principal effector molecules these cells had identified. Myocardial regenerative cell-free therapies effective on the in situ activation, multiplication and differentiation of the resident eCSCs should have many advantages over those based on cell transplantation. First, therapeutic components should be available as “off-the-shelf” and ready to use at all

times without the lag time required for the cell therapy approaches; second, they should be affordable, in terms of the production costs of the medicinal product; third, such a therapy should be easy to apply and compatible with current clinical standard of care for AMI, including the widespread use of percutaneous coronary interventions (PCI); and fourth, because of the robustness of the regenerative response produced it should be able to produce and/or recover ~50–60 g of functional myocardial tissue, which is the minimum needed to change the course of the disease in a seriously ill patient. In an attempt to move towards cell-free, protein-based therapies, various growth factors and cytokines have been identified as potential candidates for therapeutic cardiac regeneration and as this list expands so too does our awareness of growth factor-mediated regenerative potential. Vascular endothelial factor (VEGF) is one such factor, which has been identified as central in promoting neo-vascularization post-MI (Crottogini et al. 2003). Initially phase II clinical trials suggested that limited functional benefits were observed upon direct administration of VEGF post-MI (Henry et al. 2003). However, this is now known to be due to the short half-life of VEGF and goes some way to demonstrate how important initial experimental studies are when designing clinical trials. Recent studies have focused on delivering VEGF in combination with various scaffolds and have achieved much greater success in stimulating angiogenesis and restoring cardiac function (Wu et al. 2011; Formiga et al. 2010). Neuregulin 1 (NRG-1) is another key factor implicated in stimulating cardiac repair and regeneration (Wadugu and Kühn 2012; Waring et al. 2012). An Ig-domain containing form of NRG-1 β , also known as glial growth factor 2 (GG2) has been shown to improve LVEF and remodelling in pigs post-MI, compared to controls (Kasasbeh et al. 2011). It is thought that NRG-1 imparts functional benefits by activating and increasing c-kit^{pos} eCSC proliferation (Waring et al. 2012), inducing cardiomyocyte replacement (Bersell et al. 2009), protecting cardiomyocytes from apoptosis and improving mitochondrial function (Kasasbeh et al. 2011). Testing regenerative therapies in mouse models of human diseases, although a necessary step in pre-clinical assays, is not an accurate predictor of their human effectiveness. This is so not only because of the potential biological differences between the two species but because of the three order of magnitude difference in mass between the two organisms, which make the challenges not only quantitatively but qualitatively different. Therefore, it is necessary that pre-clinical testing of therapies be carried out in a model, which is more similar in tissue biology, size and physiology to the human than the rodent models commonly used. The pig, because of its size, rapid growth rate, well-known physiology and availability, has proven a very useful and frequently used pre-clinical large animal model for many pathologies, particularly those involving tissue regeneration. Thus, we have recently tested the regenerative effects of intracoronary administration of two growth factors known to be involved in the paracrine effect of the transplanted cells (Ellison et al. 2011). Insulin-like growth factor I (IGF-1) and hepatocyte growth factor (HGF), in doses ranging from 0.5 to 2 μ g HGF and 2 to 8 μ g IGF-1, were intracoronary administered, just below the site of left anterior descendent occlusion, 30 min after AMI during coronary reperfusion in the pig. This growth factor cocktail triggers a regenerative response

from the c-kit^{pos} eCSCs, which is potent and able to produce anatomically, histologically and physiologically significant regeneration of the damaged myocardium without the need for cell transplantation (Ellison et al. 2011). IGF-1 and HGF induced eCSC migration, proliferation and functional cardiomyogenic and microvasculature differentiation. Furthermore, IGF-1/HGF, in a dose-dependent manner, improved cardiomyocyte survival, and reduced fibrosis and cardiomyocyte reactive hypertrophy. Interestingly, the effects of a single administration of IGF-1/HGF are still measurable 2 months after its application, suggesting the existence of a feedback loop triggered by the external stimuli that activates the production of growth and survival factors by the targeted cells, which explains the persistence and long duration of the regenerative myocardial response. These histological changes were correlated with a reduced infarct size and an improved ventricular segmental contractility and ejection fraction at the end of the follow-up assessed by cMRI (Ellison et al. 2011). Despite their effectiveness, the administration of IGF-1 and HGF has a significant drawback. Although it is very effective in the regeneration of the myocytes and micro-vessels lost, the rate of maturation of the newly formed myocytes is heterogeneous and quite slow. While the newly formed myocytes which are in contact with spared ones mature rapidly and can reach a diameter close to a normal pig cardiomyocyte, there is an inverse correlation between new myocyte size and their distance from the small islands of spared myocardium scattered within the ischemic zone (Ellison et al. 2011). With the exception of those new myocytes in close proximity to spared micro-islands of surviving pre-existing myocytes within the ischemic tissue or those in the border region, at 3 weeks after treatment the length and diameter of the remaining new myocytes (~85 % of those regenerated) is between 1/2 and 1/5, respectively, of an adult myocyte, which means that their volume is significantly less than 1/10th of their mature counterparts (Ellison et al. 2011). Because of this slow maturation process, although the therapy is very effective in restoring the number of myocytes lost by the AMI this is not the case as to the regeneration of the lost ventricular mass which lags behind very significantly. In consequence, the myocardial generation of force capacity, that is the meaningful functional recovery, also lags significantly behind the regeneration of the cell numbers to the pre-AMI state. Despite the beneficial effect of the therapy in reducing the scar area, pathological remodelling and partial recovery of ventricular function, there is little doubt that it would be desirable to obtain a more rapid recovery of the ventricular mass and the capacity to generate force. All the currently proposed autologous cell approaches are very attractive from the theoretical and biological standpoint. For those rare diseases with chronic and long-term evolution affecting hundreds or even thousands of potential patients to be treated, these personalized therapies, despite their high cost in medical and material resources, might even make sense from an economic standpoint. Unfortunately, this is not the case for diseases of high prevalence, such as the consequences of ischaemic heart disease, with millions of patients/candidates for regenerative therapy. Not even the developed world has the resources needed to start a program of personalized regenerative medicine for the patients already in CHF who presently are left with heart transplantation as the only realistic option for recovery. Therefore, although the cell

transplantation approaches outlined are very valuable as proof-of-concept and as research tools with the possibility of greatly improving a narrow subset of patients in need of therapy, we believe that all of the autologous cell strategies taken together, now and in the foreseeable future, are and will continue to be ineffective to favourably impact the societal healthcare problem posed by the consequences of CHF post-AMI (Ellison et al. 2012b). Moreover, as outlined above, a consensus is gaining ground that most of the favourable effects of cell transplantation protocols used until now exert their beneficial effect by a paracrine mechanism of the transplanted cells over the surviving myocardial cells at risk and/or through the activation of the endogenous myocardial regenerative capacity represented by the eCSCs. If this is correct, then there seems to be little advantage in the use of autologous cells because a similar, and perhaps enhanced, effect can be obtained by the administration of the proper cell type isolated from allogeneic sources. These can be produced in large amounts beforehand, kept stored frozen before their use, and remain available at all times, which would allow their use not only for the treatment of the pathological remodelling once it has developed but soon after the acute insult in order to induce early regeneration of the cells lost in order to prevent or diminish the pathological remodelling. Mesenchymal stem cells (MSC) have a broad repertoire of secreted trophic and immunodulatory cytokines, however they also secrete factors that negatively modulate cardiomyocyte apoptosis, inflammation, scar formation and pathological remodelling (Ranganath et al. 2012). Medicetty and colleagues (2012) used a porcine model of AMI and delivered 20–200 million allogeneic, multipotent, adult BMDCs (MultiStem; that are nonimmunogenic and can suppress activated T-cell proliferation and have anti-inflammatory and angiogenic properties as well), directly to the myocardium via the infarct related vessel using a transarterial micro-syringe catheter-based delivery system, 2 days after AMI. Echocardiography showed significant improvements in regional and global LV function and remodelling at 30 and 90 days after myocardial injury (Medicetty et al. 2012). Rapidly following on from this pre-clinical study, Penn et al. (2012) conducted a multi-centre phase I trial of the effects of adventitial delivery of MultiStem in patients 2–5 days after primary PCI. In patients with EF determined to be <45 % before the MultiStem injection, at 4 months after AMI, a 1, 4, 14, and 11 % absolute increase in EF was observed following injection of 20, 50, and 100 million cells, respectively (Penn et al. 2012). Recently, Marban and colleagues (2012) have tested the safety and efficacy of using allogeneic, mismatched cardiosphere-derived Cells (CDCs) in infarcted rats. Rats underwent permanent ligation of the LAD coronary artery and two million CDCs or vehicle were intramyocardially injected at four sites in the peri-infarct zone. Three weeks post-MI, animals that received allogeneic CDCs exhibited smaller scar size, increased infarcted wall thickness and attenuation of LV remodelling. Allogeneic CDC transplantation resulted in a robust improvement of fractional area change (~12 %), ejection fraction (~20 %), and fractional shortening (~10 %), and this was sustained for at least 6 months. Furthermore, allogeneic CDCs stimulated endogenous regenerative mechanisms (cardiomyocyte cycling, recruitment of c-kit^{pos} eCSCs, angiogenesis) and increased myocardial VEGF, IGF-1 and HGF (Malliaras et al. 2012). Unlike other cell types (Janssens 2010;

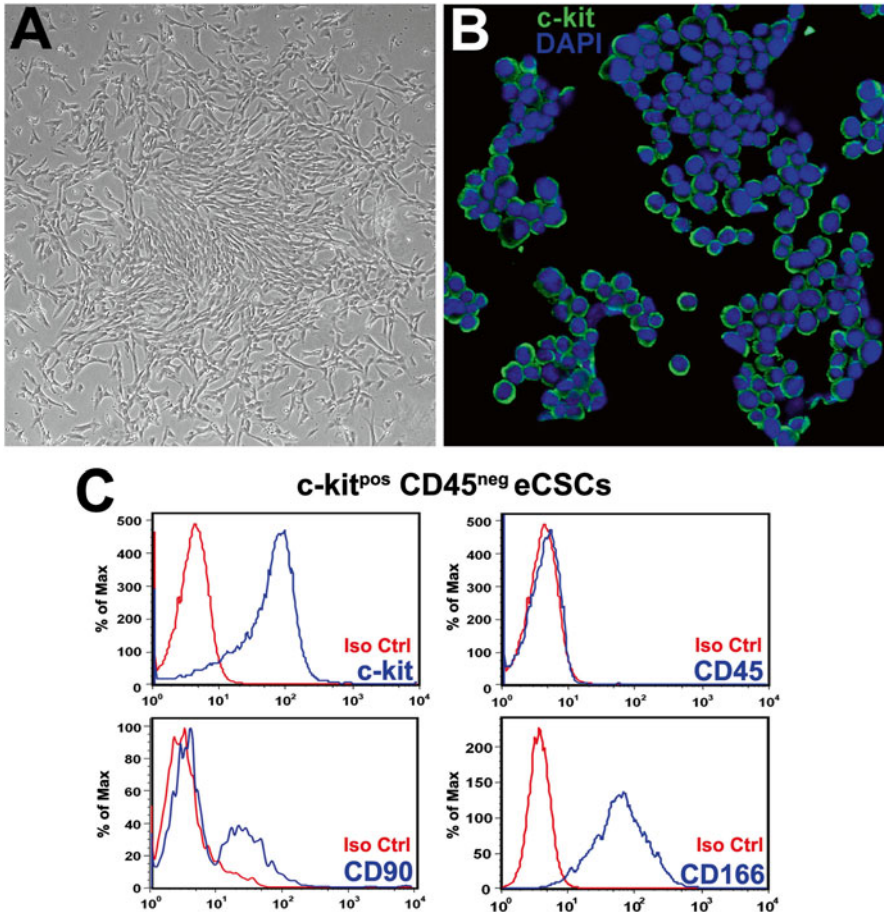


Fig. 2.2 Endogenous cardiac stem cell essential phenotype. (a) Light microscopy representative image of long-term cultured pig eCSCs. (b) Cytospin preparation and c-kit immunofluorescence of cloned eCSCs. (c) Essential CD phenotype of a typical CSC preparation (b, c) are adapted from Ellison et al. (45)

Hofmann et al. 2005), eCSCs have a very high tropism for the myocardium (Ellison et al. 2013). Under proper culture conditions it is possible to clone and expand a single rodent, porcine or human eCSC to up to 1×10^{10} cells without detectable alteration of karyotype, loss of differentiating properties or the phenotype of the differentiated progeny (Fig. 2.2) (Ellison et al. 2011). These cloned cells produce a repertoire of pro-survival and cardiovascular regenerative growth factors [Our unpublished data]. For this reason, we decided to test whether these in vitro expanded cells, when administered into allogeneic animals, would be the source of a more complex and physiologic mixture of growth and differentiating factors which, through a paracrine effect would produce a robust activation of the eCSCs

with more rapid maturation of their progeny. It was expected that once their short-term effect had been produced and the auto/paracrine feedback loop of growth factor production has been activated in the eCSCs, the allogeneic cells would be eliminated (presumably by apoptosis) and that the regeneration triggered by activated eCSCs would be completely autologous. *c-kit*^{pos} eCSCs do not express either MHC-I locus or co-activator molecules and have strong immunomodulatory properties *in vitro* when tested in the mixed lymphocyte reaction [Our unpublished Data]. We therefore expected the expanded cells to survive long enough in the allogeneic host to produce their paracrine effect before being eliminated by the host immune system. Allogeneic, non-matched, cloned male EGFP-transduced porcine eCSCs, were administered intracoronary in white Yorkshire female pigs, 30 min after MI and coronary reperfusion (Ellison et al. 2009). Pig serum was injected to control pigs after MI (CTRL). The cells or sera were injected through a percutaneous catheter into the anterior descending coronary artery just below the site of balloon occlusion used to produce the AMI. We found a high degree of EGFP^{pos}/*c-kit*^{pos} heterologous HLA non-matched allogeneic porcine CSCs nesting in the damaged pig myocardium at 30 min through to 1 day after MI. At 3 weeks post-AMI, all the injected allogeneic cells had disappeared from the myocardium and peripheral tissues (i.e. spleen). There was significant activation of the endogenous GFP^{neg} *c-kit*^{pos} CSCs (eCSCs) following allogeneic CSC treatment (Fig. 2.3), so that by 3 weeks after MI, there was increased new cardiomyocyte and capillary formation, which was not evident in the control hearts (Fig. 2.3). Moreover, through paracrine mechanisms, *c-kit*^{pos} heterologous HLA non-matched allogeneic CSC treatment preserved myocardial wall structure and attenuated remodelling by reducing myocyte hypertrophy, apoptosis and scar formation (fibrosis) (Ellison et al. 2009). In summary, intracoronary injection of allogeneic CSCs after MI in pigs, which is a clinically relevant MI model, activates the eCSCs through a paracrine mechanism resulting in improved myocardial cell survival, function, remodelling and regeneration. A possible risk of using large numbers of *in vitro* expanded CSCs is the appearance of transformed cells with the potential to form abnormal growths. This risk is completely eliminated by the use of allogeneic cells, with a different HLA allele from the recipient, because they all get eliminated by the immune system without immunosuppression. Claims that some of the transplanted allogeneic cells have a long-term survival in the host, have not been reproduced or thoroughly documented (Malliaras et al. 2012; Quevedo et al. 2009; Huang et al. 2010). If their survival proves to be correct, many of the immunology concepts, which have ruled transplant biology until now, will need to be revised. Furthermore, despite thorough pathological examination and contrary to many iPS- and ECS-derived cell lines, the adult tissue-specific eCSCs have a very low or non-existent capacity to form tumours and/or teratomas in syngeneic or immunodeficient animals [our unpublished data and (Chong et al. 2011)]. Allogeneic CSC therapy is conceptually and practically different from any presently in clinical use. The proposed cell therapy is only a different form of growth factor therapy able to deliver a more complex mixture of growth factors than our present knowledge permits us to prepare. The factors

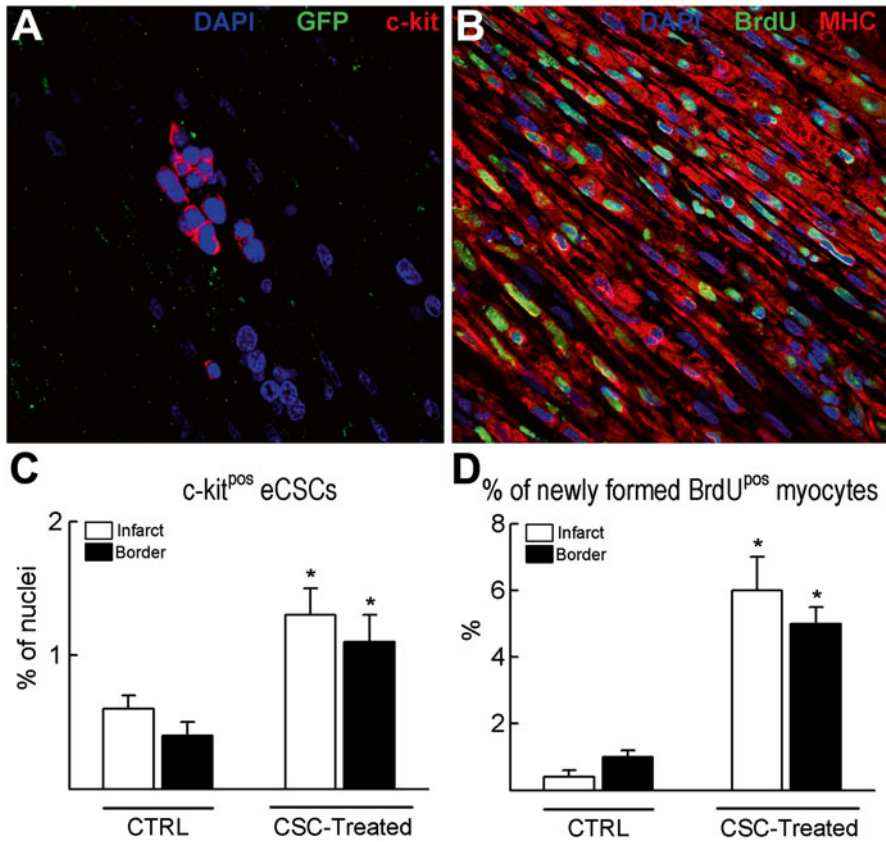


Fig. 2.3 Activation of tissue-specific endogenous resident $c\text{-kit}^{\text{pos}}$ CSCs following intracoronary injection of $c\text{-kit}^{\text{pos}}$ allogeneic porcine CSCs after acute myocardial infarction in pigs. **(a)** A cluster of activated GFP^{neg} , $c\text{-kit}^{\text{pos}}$ (red) endogenous CSCs in the 3-week-old infarcted region of the allogeneic CSC-treated porcine myocardium. Nuclei are stained by DAPI in blue. **(b)** Regenerating band of newly formed BrdU^{pos} (green) cardiomyocytes (red, MHC) in the infarct region, 3 weeks following allogeneic CSC treatment. Nuclei are stained by DAPI in blue. **(c)** The number of $c\text{-kit}^{\text{pos}}$ endogenous CSCs significantly increased following intracoronary allogeneic CSC treatment. $*P < 0.05$ vs. CTRL. **(d)** New BrdU^{pos} myocyte formation significantly increased following allogeneic CSC therapy. $*P < 0.05$ vs. CTRL. Adapted from Ellison et al. *J Cardiovasc Transl Res.* 2012;5:667–77

produced by the allogeneic cells are designed to stimulate the endogenous stem cells of the target tissue but the transplanted cells themselves survive only transiently and do not directly participate in the production of progeny that contributes to the regenerated tissue. Once more information is available, the allogeneic cells could be used either alone or in combination with the available factor therapy to improve the activation of the eCSCs and the maturation of their progeny.

5 Summary and Conclusions

The findings that the adult heart harbours a regenerative multipotent cell population composed by eCSCs and that mammalian, including human, cardiomyocytes are replaced throughout adulthood represents a paradigm shift in cardiovascular biology. The presence of this regenerative agent within the adult heart supports the view that the heart has the potential to repair itself if the eCSCs can be properly stimulated. Indeed, it is predicted that in the near future it should be possible to replace cell transplantation-based myocardial regeneration protocols with an “off-the-shelf”, readily available, unlimited and effective regenerative/reparative therapy based on specific growth factor administration or on the paracrine secretion by allogeneic CSC transplantation able to produce the activation in situ of the resident eCSCs. However, before reaching this optimistic clinical scenario, it is mandatory to obtain a better understanding of eCSC biology in order to fully exploit their regeneration potential. The latter will ultimately lead to developing realistic and clinically applicable myocardial regeneration strategies. Cardiac regenerative medicine is set to revolutionize the treatment of cardiac diseases and such research will have significant and long-term impact on socio-economics and patient well-being. Indeed, therapies which are based on findings from high quality research will undoubtedly cut deaths from cardiovascular disease, reduce recovery times, increase life expectancy and quality of care and save money.

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Chapter 3

Large Animal Induced Pluripotent Stem Cells as Models of Human Diseases

Anjali Nandal and Bhanu Prakash V.L. Telugu

Abbreviations

EpiSC	Epiblast stem cells
ESC	Embryonic stem cells
FGF	Fibroblast growth factor
iPSC	Induced pluripotent stem cells
LIF	Leukemia inhibiting factor

1 Pluripotent Stem Cells

Pluripotency is defined by the ability of cells to self renew indefinitely, and differentiate into cells representing all the three germ layers: ectoderm, mesoderm, and endoderm. In mouse, pluripotent stem cells have been established either from the inner cell mass (ICM) of early blastocyst-the embryonic stem cells (ESC); a more mature epiblast-the epiblast stem cells (EpiSC); germline tumors or teratocarcinomas-the embryonic carcinoma cells (ECC); or the primordial germ cells (PGC) of the fetus-the embryonic germ cells (EGC). Among the different pluripotent cell types, ESC are of particular interest because they are relatively more stable in culture and can be modified with desired mutations, and transferred to the next generation. To be considered as a genuine ESC, the stem cells have to pass standard pluripotency tests and be capable of: (1) forming embryoid bodies in vitro; (2) giving

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rise to teratomas upon transplantation into an immunocompromised animal; (3) derivation of chimeric offspring *in vivo* with a demonstration that the ESC contributed to the tissues derived from the endoderm, mesoderm, and ectoderm, in addition to the germline; and (4) succeeding in the most stringent of the pluripotency tests, which is tetraploid complementation, where the ESC alone gives rise to the embryo proper (Nagy et al. 1993).

Among the ESC established from the preimplantation embryos so far, the ESC from mouse (m) is the only cell type that has met all the stringent measures of pluripotency outlined above, indicating that all pluripotent stem cells from the early embryos are not identical. This is evident from the human and primate ESC that differ in morphology, growth factor requirements, and other aspects of their phenotype from mESC. The existence of at least two, if not more distinct pluripotent stem cells was further corroborated by the establishment of a different kind of ESC, also from the mouse, the EpiSC (Brons et al. 2007; Tesar et al. 2007). The mEpiSC are similar to the primate ESC in colony morphology and require activin A and FGF2 for pluripotency, which is in stark contrast to mESC that are dependent on LIF. Because the EpiSC are established from an advanced differentiated epiblast—stage of mouse conceptuses in comparison to the “naïve” ESC from the ICM, they are classified as “primed” ESC. Besides the obvious differences in phenotype and growth factor requirements, the mEpiSC also differ from mESC in their inability to tolerate dispersion into single cells, responsiveness to BMP4, and relatively poor contribution to chimerism, confirming their distinctiveness from the naïve ESC. It can therefore be hypothesized that primate ESC that share properties of EpiSC, presumably originated from a naïve epiblast, but spontaneously progressed to the primed epiblast stage *in vitro* before giving rise to a stable cell line. Arguably, the primate ESC are actually EpiSC though they have been classified under ESC. Regardless, at least in mouse, the primed cells can be converted into naïve cells by incorporating small molecule compounds activating WNT signaling pathway, and by using kinase inhibitors inhibiting the MEK signaling pathway (Rodriguez et al. 2012). This interchangeability is of particular interest to stem cell biologists because, the naïve stemness, a hallmark of mESC is coveted for their ease of passage, cryopreservation, rapid proliferation, stability in culture, and high competence for producing germline chimeras (Brons et al. 2007; Tesar et al. 2007).

A little less than a decade after the first publication of mESC, reports of ESC/ESC-like lines from *in vivo*- or *in vitro*-derived embryos were reported from pigs (Notarianni et al. 1990; Piedrahita et al. 1990) and sheep (Notarianni et al. 1991). These ESC-like cells bore characteristics of primed ESC in their morphology but failed to maintain self-renewal, or remain in an undifferentiated state, or differentiate efficiently into various cell types of the three germ layers *in vitro* or *in vivo*. Although chimera formation was reported, tissue analysis was minimal or ESC-derived tissue types were limited. Similarly, the origin of the differentiated teratoma tissue was assumed to be ESC-derived, but it was not proven. Despite two decades of additional effort, proven ESC lines from any domestic large animal species has yet to be reported. This failure could largely be attributable to differences in the developmental biology of ungulate (u) species. In human and mouse embryos, morula stage is generally similar to ungulates but one significant difference is that

human and mouse embryos reach blastocyst stage 24–48 h earlier than cattle and swine. In human and mouse, the epiblast develops *in vivo* following implantation but ungulates have an extended peri-implantation period following hatching during which, epiblast is formed (Vejlsted et al. 2006), extraembryonic membranes expand (Geisert et al. 1982); gastrulation begins and Rauber's Layer is shed, exposing underlying epiblast to the uterine luminal fluid (Flechon et al. 2004). These distinct phenomena suggest that the ungulate embryos are possibly exposed to unique developmental cues and hence, the optimal time to establish uESC lines could be different from human and mouse. Analysis of recently established putative bovine (Lim et al. 2011) and porcine ESC (Alberio et al. 2010) showed that although they expressed OCT4, SOX2, and NANOG; only the bovine ESC were AP and REX1 positive. It is noteworthy to point out that both AP and REX1 expressions were observed in the initial culture of the porcine epiblasts but were lost during cell line establishment (Alberio et al. 2010). This indicates that the expression of some other crucial factors may have been compromised with *in vitro* culture as the three core pluripotency factors were present in both bovine and porcine ESC. Other reasons could be the non-availability of factors that define pluripotency in uESC, and the lack of appropriate *in vitro* culture system(s) to maintain pluripotent and self-renewing uESC. Since robust uESC are not available, one possibility of bypassing the need of uESC is the derivation of induced pluripotent stem cells (iPSC) as described below.

2 Induced Pluripotent Cells

Six years ago, in the initial landmark publication, Takahashi and Yamanaka (2006) reported reprogramming of somatic cells to iPSC by the ectopic expression of four transcription factors Oct4, Sox2, Klf4, and c-Myc. The mouse iPSC that was reported in this and subsequent studies had properties common with ESC, including their ability to form teratomas, generate germline chimeras and live offspring (Kang et al. 2009; Zhao et al. 2009). The technology has since revolutionized the field and has been adopted to successfully establish iPSC from humans (Takahashi et al. 2007) and several other primate and non-primate species. Genome-integrating lentiviral (Winkler et al. 2010) and retroviral vectors (Okita et al. 2007) were widely used initially for the generation of iPSC. However, a spate of recent publications were aimed at creating iPSC lines by either modifying the vectors, including adenoviruses (Stadtfeld et al. 2008), plasmid vectors (Okita et al. 2010), episomal vectors (Yu et al. 2009), minicircle DNA (Jia et al. 2010); or modes of delivery, such as those required for delivery of small molecules/chemicals (Despots and Ding 2010; Hou et al. 2013), mRNA (Tavernier et al. 2012) and recombinant proteins (Zhou et al. 2009). Various mechanisms to generate iPSC are briefly discussed in Fig. 3.2. Nevertheless, the aim is to improve reprogramming efficiency of the somatic cells by using minimal or no reprogramming factors as chemical reprogramming could efficiently generate cells with the least genomic variations. The realization that

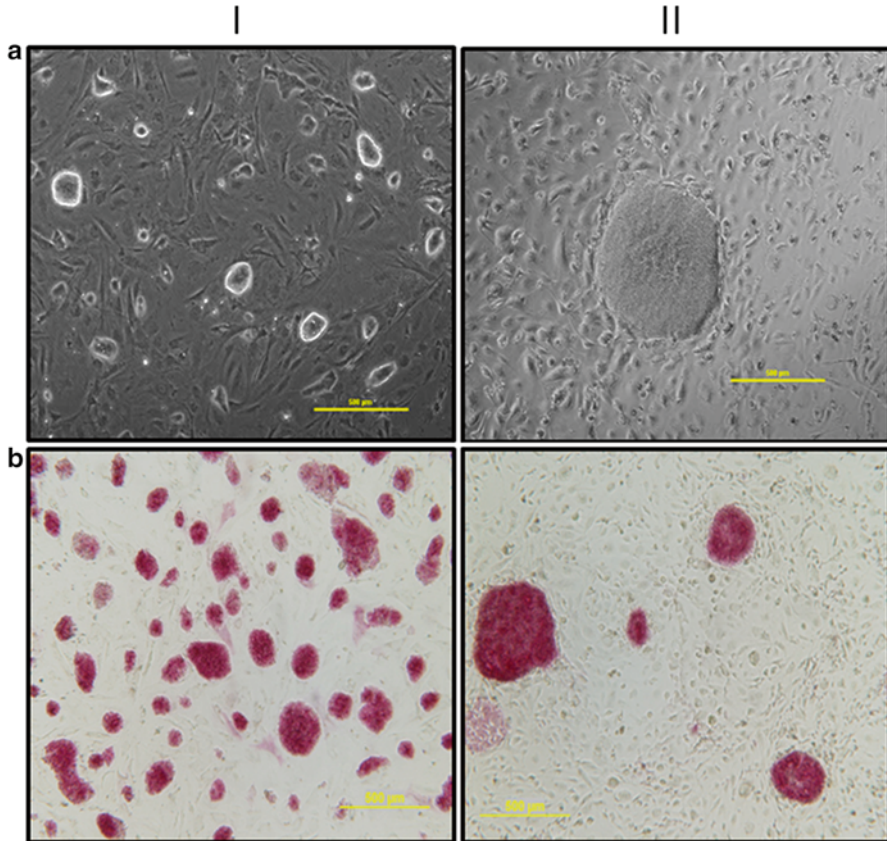


Fig. 3.1 Typical morphological features of 'naïve' and 'primed' induced pluripotent cells (a). (I) The colonies of naïve-type caprine iPSC reprogrammed from fetal fibroblasts with six factors (OSKMLN) through lentiviral transduction and 2i/LIF (Leukemia Inhibitory Factor) supplemented medium on a feeder layer of mouse embryonic fibroblasts (MEF) at day 3. (II) Colonies of primed-type goat iPSC cells derived from fibroblasts by the delivery of four factors and addition of bFGF to the medium on a feeder layer of MEF at 3–4 days. (b) Alkaline phosphatase (AP) stained naïve and primed caprine colonies to further emphasize the size and morphology difference between the colonies (unpublished data). The brightfield and AP stained images were magnified using 4X and all the iPSC were maintained on feeders

iPSC could be readily created from these animal models prompted investigation into the establishment of similar lines from other important animal models such as the dog and pig, and from endangered species and disappearing breeds (Roberts et al. 2009; Telugu et al. 2010b). It is believed that somatic cells transformed into iPSC maintain the culture requirements typical of the species of origin with mouse cells giving rise to the naïve type of stem cells, and human cells generating cell lines with the characteristics of the primed state (Telugu et al. 2010a). This indicates that once somatic cells are reprogrammed to pluripotency, it follows the

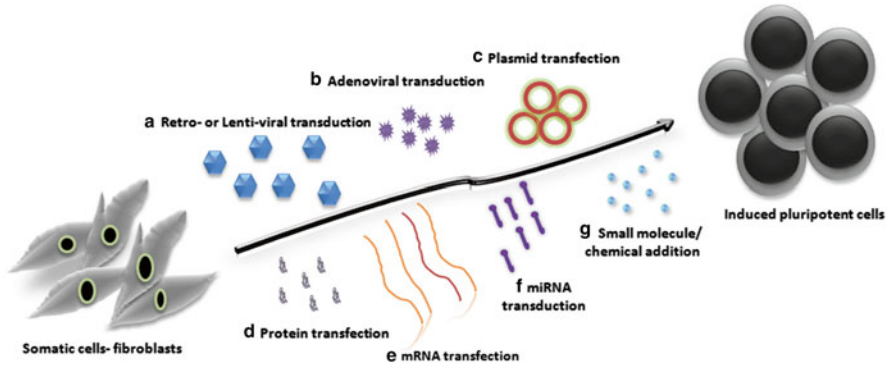


Fig. 3.2 Various reprogramming strategies to induce pluripotent stem cells from adult somatic cells. Earlier methods generally involved the use of efficient viral delivery system by (A) retroviruses and lentiviruses, which integrate into the genome at high frequency or (B) adenoviral transduction, where virus does not integrate. (C) Transfection of comparatively less efficient but non-viral and non-integrating DNA-based methods like plasmids, minicircles, and transposons have been successfully utilized, albeit with less frequency. Similarly, non-integrating (D) reprogramming factor proteins and (E) *in vitro* transcribed RNA (also capped and poly A-tailed) can be transfected in the cells for efficient reprogramming. Additionally, (F) miRNA-mediated reprogramming and (G) efficient induction of pluripotency by small molecules/chemicals has also been recently reported

default behavior that is typical of the epiblast of its species. It would be therefore very useful to dissect the properties of ungulate iPSC and, as a consequence, their typical default stemness state. Since ESC and iPSC are very similar, iPSC also either have a naïve or primed stemness state. The differences between naïve and primed iPSC are illustrated by brightfield and Alkaline Phosphatase (AP) stained images for goats (unpublished data) in Fig. 3.1.

In many studies with pig, sheep, and bovine iPSC, it is difficult to classify the cells either as naïve or primed as they were propagated in media supplemented with both LIF and FGF2 (Montserrat et al. 2012; Sumer et al. 2011; Liu et al. 2012). However, in two of these studies, morphology was described as similar to that of the human ESC which suggests that they could be primed cells. In some other studies, the iPSC generated were possibly wrongly classified as naïve as the morphological characteristics described were similar to the human primed cells (Han et al. 2011). In another study, true pluripotency was observed and chimera formation at least at the blastocyst stage, was reported for sheep iPSC (Montserrat et al. 2011). Therefore, the whole picture is unclear but suggests that currently in most cases, ungulate iPSC belong mainly to the primed category. It would be a significant study in the future to develop novel methods to reprogram ungulate primed iPSC into naïve iPSC. The establishment of naïve iPSC by Telugu et al. (2010a, b) is certainly a good beginning. In spite of various advances in generating iPSC from ungulates and other large animals, there are still many issues that need to be resolved before we can efficiently generate robust iPSC from these animals.

3 Difficulties in Making Completely Reprogrammed Large Animal iPSC

In large animals, iPSC have been from the somatic cells of nonhuman primates (Liu et al. 2008), dogs (Shimada et al. 2010), pigs (Esteban et al. 2009, 2010; Ezashi et al. 2009; Wu et al. 2009; Telugu et al. 2010a, b; West et al. 2010; Montserrat et al. 2012; Hall et al. 2012), cattle (Huang et al. 2011), buffalo (Deng et al. 2012), sheep (Liu et al. 2012), goat (Ren et al. 2011) and horse (Nagy et al. 2011). A summary of the different studies that reported the establishment of iPSC from various farm animals is outlined in Table 3.1. In majority of these studies, however, the established iPSCs were plagued by deficiencies such as the failure to turn-off expression of exogenous genes or factors responsible for reprogramming, failure to self-sustain for a longer period of time, or more importantly meet all the criteria for pluripotency, specifically the *in vivo* tests.

One major challenge in establishing robust farm animal iPSC from the outset has been the difficulty in identifying the right set of conditions and factors required for each large animal species to propagate and sustain iPSC in culture. This has been a rather arduous task, since some experiments to replicate the culture conditions that are successful with the mouse or human have failed to yield self-sustaining large animal iPSC lines. The search has been made complex by the species-specific requirements of ungulates, which led many investigators down an unsuccessful path of trial and error to identify the “missing” factors. Different combinations of genes or factors have been introduced in somatic cells with varying efficiencies, although OCT4 is usually included (Montserrat et al. 2011) as a reprogramming factor in most animal species, and NANOG is necessary for complete reprogramming in bovines (Malaver-Ortega et al. 2012). In some species like bovine, it is imperative that the reprogramming factors should be from the same species as the somatic cells but in others like ovine, the origin of the reprogramming genes does not seem critical. It could be due to conservation of reprogramming factors in various animal species, although a comparison of efficiencies of these factors from different species for reprogramming a particular somatic cell would be beneficial. It is also very important, according to some studies, to use plasmid-based vectors rather than ones based on a virus (Huang et al. 2011) as the use of integrating retroviral vectors leads to the continued expression of the transgenes in the reprogrammed cells. In addition to the risk of mutagenesis due to the random integration of transgenes, continued expression of pluripotent genes could also limit the differentiation potential of iPSC. For iPSC to have a better utility, the inserted transgenes should either be deleted or effectively silenced after the cells have been reprogrammed. Alternatively, reprogramming must be achieved through the use of non-integrating vectors or plasmids (Okita et al. 2008; Yu et al. 2009), introduction of “stemness” proteins rather than genes (Zhou et al. 2009), or pharmaceutically with a suitable combination of small molecules or chemicals. The disadvantage of using non-integration methods of iPSC generation is that their induction efficiencies are quite low and as a result, they inefficiently generate completely reprogrammed iPSC.

Table 3.1 Summary of large animal iPSC reports

Animal species	Year/author	Parental cells	Culture conditions	Tests	Markers	Factors	iPSC type
Porcine	Fan et al. (2013)	Ear fibroblasts, Bone marrow cells, fetal fibroblasts	DMEM with LIF, bFGF	Embryoid bodies, Teratomas	Oct4, Nanog, Sox2, c-Myc, Klf4	Human OSKM	Naive
-	Fujishiro et al. (2013)	Embryo fibroblasts	bFGF, LIF, pLIF, FK	Embryoid bodies, chimera	Oct4, Nanog, ERas, Sox2, Lin28, SSEA-1, SSEA-3, SSEA-4	Human OSKM	Naive
-	Park et al. (2013)	Embryo fibroblasts	DMEM, FBS	Embryoid bodies, in vitro differentiation	Oct4, Sox2, Nanog, TDGF1, REX1, bFGF, FGFR1, FGFR2, Nodal	Mouse OSKM	Primed
-	Cheng et al. (2012)	Embryo fibroblasts	KSR, KO-DMEM, LIF, bFGF	Embryoid bodies, Teratomas	Oct4, Sox2, Nanog, Klf4, SSEA-1 and SSEA-4, Tra-1-60, Tra-1-81, TERT	Mouse OSKM	Naive
-	Hall et al. (2012)	Fetal fibroblasts	KO-DMEM, KSR, bFGF	Embryoid bodies	OSKM, Nanog, SSEA-1, SSEA-4	Human OSKM, Nanog	Primed
-	Montserrat et al. (2012)	Ear fibroblasts	hbFGF, LIF	Embryoid bodies, Teratomas	Nanog, SSEA-4, TRA-1-60	Human SKM	Primed
-	Telugu et al. (2010a)	Fetal fibroblasts	KSR, hLIF, 2 inhibitors PD03259 CHIR99021	Teratomas	Oct4, Sox2, Nanog, Cdh1, Lin28, Gcnf, Gnl3, Zfp42, Utf1, Tdgl1, Acv2b, Lin28	Human OSKM	Naive

(continued)

Table 3.1 (continued)

Animal species	Year/author	Parental cells	Culture conditions	Tests	Markers	Factors	iPSC type
-	West et al. (2011)	Mesenchymal stem cells	KSR, hbFGF, mTeSR1 medium	Embryoid bodies, Chimera	AP, Oct4, Sox2	Human OSKM	Primed
-	Ezashi et al. (2009)	Porcine fetal fibroblasts	FGF2	Embryoid bodies, Teratomas	AP, SSEA-1, Oct4, Nanog, Sox2,	Human OSKM	Primed
-	Esteban et al. (2009)	Miniature pig fibroblast	FGF2, LIF	Teratomas	AP, Nanog, Sox2, Klf4, Oct4, Rex1, Lin28, SSEA4	Mouse and Human OSKM	Primed
-	Wu et al. (2009)	Ear fibroblast and bone marrow cells	hESC medium with doxycycline	Embryoid bodies, Teratomas	AP, SSEA3, SSEA4, Tra-1-60, Tra-1-81, Oct4, Nanog, Sox2, Rex1 and Cdh1.	Human OSKM	Primed
Cattle	Cao et al. (2012)	Fetal fibroblasts	LIF, bFGF	Embryoid bodies, Teratomas	Oct4, Nanog, AP, SSEA-1	Human Oct4, Porcine SKM	Primed
-	Huang et al. (2011)	Fetal fibroblasts	2i/LIF medium, PD0325901, CHIR99021	Embryoid bodies, Teratomas	Oct4, Sox2, Cdh1, DPPA-3, Nanog, ZFP42, Tra-1-60, Tra-1-81, SSEA3 and SSEA4	Bovine OSKM	Not clear
-	Han et al. (2011)	Fetal fibroblasts	KSR, bFGF	Embryoid bodies, Teratomas	DPPA-3, DPPA-4, Esrrb, TERT, E-Cadherin	Bovine OSKM, Lin 28, Nanog Human OSKM	Naive?

-	Sumer et al. (2011)	Adult fibroblasts	bFGF, LIF	Embryoid bodies, Teratomas	OSKM, Nanog, Rex1, ALP1, AP, SSEA1, SSEA4	Human OSKM, Nanog	Not clear
Buffalo	Deng et al. (2012)	Embryonic fibroblasts	Pfifithrin	Embryoid bodies, Teratomas	AP, Oct4, SOX2, Nanog, SSEA1, SSEA4, Tra-1-81, E-Cadherin	Buffalo OSKM	Primed
Sheep	Liu et al. (2012)	Fetal fibroblasts	bFGF, LIF	Embryoid bodies, Teratomas, tetraploid embryo	AP, Oct4, Nanog	Human OSKM	Naive
-	Sartori et al. (2012)	Fetal fibroblasts	Human and mouse ESC media	Embryoid bodies, Chimera, Teratomas	Nanog, SSEA1, SSEA4	Mouse OSKM	Not clear
-	Bao et al. (2011)	Primary ear fibroblasts	KSR	Embryoid bodies, Teratomas	AP, Oct4, Sox2, Nanog, SSEA1, DPPA4, Tra-1-81, Rex1, E-Cadherin	Human OSKM, Nanog, Lin28, SV40	Naive
-	Li et al. (2011)	Fetal fibroblasts	hFGF2, LIF, KO-DMEM	Embryoid bodies, Teratomas	AP, Oct4, Sox2, Nanog, SSEA4	Mouse OSKM	Primed
Goat	Ren et al. (2011)	Primary ear fibroblasts	ES media	Embryoid bodies, Teratomas	AP, Oct4, Sox2, Nanog, SSEA1, DPPA4, Tra-1-60, TRA-1-81, Rex1, SALLA, E-Cadherin	Mouse OSKM, SV40, Large T, hTERT	Naive
Horse	Breton et al. (2013)	Fetal and adult fibroblasts	KSR, Knockout DMEM, bFGF, LIF	Embryoid bodies, Teratomas	Oct4, Sox2, Nanog, Rex1, Lin28, SSEA1, SSEA4, Tra1-60	Mouse OSKM	Primed

(continued)

Table 3.1 (continued)

Animal species	Year/author	Parental cells	Culture conditions	Tests	Markers	Factors	iPSC type
-	Khodadadi et al. (2012)	Adult fibroblasts	LIF, bFGF, EGF	Embryoid bodies, Teratomas	AP, Oct4, Sox2, Nanog, SSEA1, SSEA 4, STAT3	Human OSK	Primed
-	Nagy et al. (2011)	Fetal fibroblasts	bFGF, LIF, GSK; MEK; TGF; ALK receptor inhibitor, Thiazovivin	Embryoid bodies, Teratomas	AP, Oct4, Klf4, Nanog, SSEA1, SSEA4, DPPA4, Tra-1-60, Tra-1-81	Mouse OSKM	Primed
Dog	Whitworth et al. (2012)	Dermal fibroblasts	Knockout DMEM, KSR, LIF, bFGF, GSK; MEK and TGF- β antagonist	Embryoid bodies	AP, Nanog, Oct4, Telomerase, SSEA1, SSEA4, TRAI-60, TRAI-81, Rex1.	Human OSKM, Lin28, Nanog	Naive
-	Lee et al. (2011)	Fibroblasts, adipose stromal cells	hbFGF, hLIF	Embryoid bodies, Teratomas	AP, Oct4, Sox2, Nanog, SSEA4, Tra-1-60	Human OSKM	Primed
-	Shimada et al. (2010)	Embryonic fibroblasts	hbFGF, hLIF, VPA, PD0325901, CHIR99021, A83-01	Embryoid bodies, Teratomas	AP, Oct4	Canine OSKM	Primed
Nonhuman primates	Emborg et al. (2013)	Skin fibroblasts from female macaca	Primate ES cell medium, bFGF	Teratoma	SOX2, Nanog	Human OSKM	Primed
-	Deleidi et al. (2011)	Macaque skin fibroblasts	Human stem cell media with KSR	Neurons, Teratomas	AP, OSKM Nanog, SSEA 4, Tra-1-60, Tra-1-81	Human OSKM	Primed

-	Zhong et al. (2011)	Macaca oral fibroblasts	hbFGF, PD0325901, VPA, Y-27632	Embryoid bodies, neural precursors, cardiomyocytes, hepatocytes, Teratomas	Oct4, Nanog, SSEA3 and 4, Tra-1-60, Tra-1-81	Human OSKM	Primed
-	Liu et al. (2008)	Rhesus macaque ear fibroblast	KSR	Embryoid bodies, Teratomas	AP, Oct4, Sox2, Nanog, SSEA4, Tra-1-60, Tra-1-81	Monkey OSKM	Primed

The culture media used also has a significant effect on the quality of iPSC generated and should be standardized for each animal species. The presence of FBS in the culture medium used in the reprogramming stage was implicated in generating pre-iPSC in mice (Chen et al. 2012), because it contains growth factors of the BMP family that cause alterations of H3K9 methyltransferase and demethylase activities. The alternative use of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with knockout serum replacement (KSR) medium (Chen et al. 2010) improved the efficiency of "true" iPSC generation (Chen et al. 2012). These findings from mouse cells may not apply to improving reprogramming condition for porcine iPSC, but it seems possible that the differences of DNA methylation status of the endogenous *OCT4* promoter observed between porcine iPSC reprogrammed in KSR (Wu et al. 2009) and FBS-supplemented media is due to FBS interference with reprogramming of the latter (Fujishiro et al. 2013). Secondary to the choices of the growth factor(s) and culture media, another technical consideration is the concentration of the supplement as the commercially available mouse or human ligands do not necessarily carry similar potency in farm animals. Another unresolved issue which could affect the quality of iPSC generated is the choice of cell lines for use as feeders, as well as their density in culture. Yet another cause could be incomplete resetting of epigenetic profile. Variations in fidelity of epigenetic reprogramming, which can be different in different cells, can be quite significant even in iPSC that otherwise meet established pluripotency criteria. The donor cell-type-specific epigenetic patterns ("somatic memory" of the cells) and other unknown factors can affect complete reprogramming into, and later differentiation of iPSCs.

Although human and mouse iPSC are well defined, most other animal models also suffer from a general lack of reliable iPSC markers. For instance, reports of canine pluripotent cell derivation have shown that in different studies the surface marker expression (such as SSEA-1, SSEA-3, SSEA-4) varies, despite the common expression of OCT4, SOX2, and NANOG (Shimada et al. 2010; Whitworth et al. 2012). Ungulates, such as pig, sheep, and cow, also show inconsistencies in surface marker expression (Huang et al. 2011; Li et al. 2011), and there is a further complication in that porcine, and bovine blastocysts express primary pluripotency genes and surface markers, such as OCT4 and SSEA-4, in both the ICM and the trophectoderm. Furthermore, Ezashi et al. (2009) reported that the process that results in pig iPSC also produces the by-product of trophectoderm-like cells. Like iPSC, these cells can grow in iPSC culture conditions, have high expression of telomerase, and a subset of pluripotency genes, making it difficult to distinguish them from iPSC following reprogramming. The lack of appropriate markers for different cells in different animals makes it impossible to distinguish animal-specific iPSC from other cells and judge the extent of their reprogramming.

In addition to problems with characterization, another consideration is the accumulation of chromosomal abnormalities following routine passages of human and mouse iPSC within a few generations (Ben-David et al. 2010; Mayshar et al. 2010; Ronen and Benvenisty 2012). This finding suggests that the long-term culture of large animal iPSCs may result in similar abnormalities, and therefore should be monitored carefully for culture-induced genetic changes. Another limitation has been the limited number of studies demonstrating that the uiPSC can be directed

in vitro to transform into more specialized sub-lineages that might be tested for their ability to form functional grafts in large animals like pigs. In one example, primed iPSC derived from pig fetal fibroblasts (Ezashi et al. 2009) were directed along the ectoderm lineage to form a mixture of cells that included rod photoreceptor lineage cells (Zhou et al. 2011). These cells when injected into the eye were able to integrate into the retina, differentiate into photoreceptors, and generate outer segment-like projections (Zhou et al. 2011). In other studies, due to lack of further differentiation, the state of reprogramming could never be tested experimentally. A limited number of other papers have demonstrated analogous directed differentiation in vitro from pig iPSC (Bui et al. 2012; Yang et al. 2013) but the work carried out in other species has so far been very limited in scope.

Bonafide iPSC from ungulates are not here yet, but we are closer than ever before in making this a reality. Above all, a defined conceptual framework is beginning to emerge due to the recognition of the difference between naïve and primed epiblast iPSC and signaling networks involved in maintaining them. More in vivo studies on the factors, signaling pathways, and their interactions for the generation of iPSC would make the generation of fully reprogrammed cells easy and possible. The best strategy for future iPSCs derivation would be to use chemicals or small molecules for reprogramming, reduce the number of transcription factors used, and use non-genome-integrating methods of DNA or protein delivery. Since integration-free methods are very inefficient, they can be improved by better combination of factors, selection of an easily reprogrammable parental cell type, and by determining optimal culture conditions. After production of robust iPSC from large animal species, an important step for the translation of basic research into clinical applications is the testing of these cells using large animal models that are intermediate between laboratory rodents and humans.

4 Potential Utility of Large Animal iPSC to Human Disease Therapy

The ability to propagate in an undifferentiated state and differentiate into target cell types under defined conditions make the ESC and iPSC valuable tools for investigating early embryonic developmental events and regenerative medicine applications. For example, miPSC lines have been generated from animals with a specific genetic disease, the genetic defect was corrected in vitro, and the cells were transplanted back into a preclinical mouse model (Hanna et al. 2007; Wu et al. 2011). Likewise, hematopoietic progenitors derived from genetically corrected iPSC were transplanted into the mouse model and this led to the improved production of human β -globin (Wang et al. 2012). Another recent and more significant observation was the establishment of functional liver buds from iPSC-derived human hepatic progenitors. The liver buds had confirmed functionality as determined by detoxification of drugs, and secretion of human albumin (Takebe et al. 2013). These significant albeit proof-of-principle studies in mice need to be thoroughly evaluated for their safety and efficacy on a larger recipient host prior to

clinical application in humans. Although, nonhuman primates are the best candidates for these studies, they are also incredibly expensive to maintain and perform research on due to various ethical concerns. This precludes their use for promising “upcoming” therapies. Conceivably, in this regard, large animal species specifically ungulates are better suited to bridge the gap. Although relatively more expensive to maintain than the mouse, ungulates enable long-term evaluation of the safety and efficacy of potential stem cell therapies (Dall et al. 2002; Vodicka et al. 2005). Specifically, pig has several potential advantages over mouse for predicting whether or not stem-cell-based therapy is likely to be safe when considering outcomes, such as toxicity, immune responses, and tumorigenicity. Their large size facilitates the expansion of implanted human or animal cells to numbers sufficient for biochemical analysis, standardization of dosage, the decision on the type of surgical equipment required for humans, and also allows multiple samplings over time. Additional key characteristics such as the similarity in physiology, anatomy, and pathology to humans as compared to mice, and above all similarity in their perceived primed stemness to humans make them attractive model systems (Ourednik et al. 2001; Brevini et al. 2008).

Pigs and other ungulates are genetically well standardized, and their genomes are sequenced, making transgenic approaches feasible (Campbell et al. 1996; Wilmut et al. 1997). At the cellular level, many of the transcriptional regulators, genes and proteins share a high degree of similarity to humans and can be used interchangeably (Zanjani et al. 1994a, b; Verfaillie 2000) in various species. For these reasons, many of the domestic animals have already made inroads into transplantation and preclinical research. Sheep and goats have been used extensively in preclinical studies of bone marrow transplantation, and for the xenotransplantation of human stem cells into sheep and goat fetuses for more than 50 years (Zanjani et al. 1991a, b; Zeng et al. 2005; Narayan et al. 2006; Almeida-Porada et al. 2007; Michelini et al. 2008). Similar to sheep and goats, pigs have also been widely recognized for their potential as donors for xenotransplantation research (Klymiuk et al. 2010). They are also used as models for studying the pathophysiology of human diseases, such as cystic fibrosis (Rogers et al. 2008), where mice fail to develop the relevant symptoms encountered in human patients. Pigs also have an important role in studying cardiovascular disease, lipoprotein metabolism, diabetes, wound, burn repair, intestine and immune system development (Swindle 2007). They already play a significant role in developing surgical techniques and cell delivery procedures, and for optimizing the number and type of cells to be used for a particular type of graft. For instance, pigs have already been treated with a variety of “adult” stem cells to determine whether cardiac function can be improved after induced ischemia (Amado et al. 2006; Gandolfi et al. 2011; Mazhari and Hare 2012). Pig iPSC, after conversion to endothelial cell precursors, have been successfully transplanted into mice with myocardial infarctions that appear to promote neovascularization in the ischemic regions (Gu et al. 2012). Presumably, the next step will be to use iPSC to test disease therapies and mechanisms involved in different disease conditions to confirm the regenerative potential of the iPSC approach. Porcine iPSC, for example, could be matched to specific pigs, making them essentially “patient-specific,” and then could be differentiated into

specific lineages. These qualities make it possible to use pigs to test transplantation therapies with iPSC for safety and efficacy before applying the procedures to human patients. Another ungulate, equine, can serve as an excellent model for a range of human degenerative diseases, especially those involving joints, bones, tendons and ligaments, such as arthritis. Another large animal, dog, can serve as a clinically relevant model of human hereditary disease as compared to rodents. Canine iPSC have also been used to test the relevance of human heart therapies as dog's heart is more similar to humans as compared to the mouse (Lee et al. 2011).

Currently, many groups are using large animal iPSC for transplantation in the more traditional small animal mouse model. Zhu et al. (2011) reported the generation of insulin-producing pancreatic cells from rhesus monkey iPSC but tested their efficacy in a diabetic mouse model. Similarly, Zhong et al. (2011) used genetically modified nonhuman primate iPSC in a mouse model to show that suicide genes can be conditionally activated to eliminate pluripotent cells *in vivo*. Although such studies provide clues regarding how iPSC behave following transplantation in humans, transplantation of autologous iPSC or their derivatives using large animal models would be more insightful by providing better preclinical safety data needed to progress towards human clinical trials. Since no single animal model is ideal, a rational approach to their use for chimeric studies of human stem cells would arguably involve related studies in more than one model. Initial experiments in small, relatively inexpensive and versatile animal models provide a good platform for moving to larger animal models with greater relevance for preclinical studies. Clinical researchers generally gravitate toward the use of large animal models due to their similarity to humans, whereas basic researchers lean towards the small diverse animal models as they are comparatively inexpensive and easy to maintain. Regenerative medicine can benefit enormously from joining of these two schools of thought and in designing studies that would use many different models to accelerate the translation of animal research to human applications. As the use and efficiency of iPSC generation increases, so would the range of applications for these diverse animal models. It is likely that the increasingly sophisticated applications involving human/animal chimeric models will emerge through advances in genetic manipulation, naïve iPSC generation and differentiation, efficient cell delivery, and *in vivo* imaging, all immensely significant developments in their own right. In the context of human/animal chimeric studies, they represent a means to a greater end: the eventual use of human-induced pluripotent cells to treat human diseases.

Besides the promise of stem cell therapies, large animal iPSC also offer greater potential for use in basic research, biotechnology, agriculture, pharmacy, and medicine. In ungulates where the ESC are hard to isolate and maintain, iPSC have a much greater role to play in genetic engineering applications as well, such as the generation of transgenic animals with special production characteristics or resistance against diseases, biopharming human recombinant proteins (Nowak-Imialek et al. 2011), and reviving endangered or extinct breeds and species (Ben-Nun et al. 2011). As the field of large animal iPSC advances, it is anticipated that the findings will enable new stem-cell based regenerative therapies in human and veterinary medicine, and aid in the development of preclinical models leading to human applications.

5 Conclusions

Numerous studies have revealed that iPSC can be established using a variety of methods, cell sources, induction methods, reprogramming factors, chemicals, and culture conditions. The iPSC are quite similar to ESC in morphology, proliferative capacity, surface antigens, overall gene expression, epigenetic status of pluripotent cell-specific genes and specific gene loci. Therefore, the recent establishment of iPSC from medically relevant large animals such as pigs, cattle and sheep, for which authentic ESC are not currently available was a valuable step forward for testing the potential utility of stem cells in human medicine and agriculture. In addition to the advantages associated with ESC, iPSC will also allow the establishment of “personalized” stem cells for transplantation. If iPSC can be created from the desired animal and successfully directed along particular pathways of differentiation, it will be possible to transplant the cells into the same animal from which the founder cells originated. This would protect against the immune-rejection and promote better survival of the graft. The ability of these transplanted cells to be incorporated into damaged organs or tissues can be easily measured and the functionality and stability of these grafts can be assessed over a longer life span of large animals. Of equal importance, a study on the large animals will provide confidence that stem cell transplantation can be performed safely without the risk of cancer occurring over a period of years from adolescence to more mature age.

Despite the rapid progress of the field, several key concerns remain. Completely reprogrammed iPSCs from most large animals or humans are difficult to derive on a routine basis, and there is a general lack of effective and reproducible reprogramming protocols. Therefore, the need of the hour is to develop reliable differentiation protocols and reagents capable of driving differentiation into targeted lineages such as neuronal, cardiac, endothelial and hepatic cells. Although no animal study can truly compare to a human study, every effort should be made to ensure that the model system is as close to the human system as possible, particularly when translational medical research is the goal. With this in mind, large animal models should and can play a more significant role in translational research, but they are often overlooked due to perceived difficulties and/or costs.

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Chapter 4

Fetal Adnexa-Derived Stem Cells Application in Horse Model of Tendon Disease

Anna Lange-Consiglio and Fausto Cremonesi

1 Structure of Tendon

Healthy tendon is a highly specialized tissue designed to resist enormous unidirectional forces. Tendons are responsible not only for transmitting mechanical forces from skeletal muscle to bone but also as joint stabilizers and as “shock absorbers” to limit muscle damage. The functional properties of the tissue are derived from its structure and its components.

Tendon is composed predominantly of water (approximately 70 %). Of the remaining 30 % dry matter, the major constituents are collagen and a noncollagenous matrix representing the extracellular matrix (ECM) that is synthesized by a few residing tendon fibroblasts. The main component of the ECM is *type I* collagen (Williams et al. 1980). Other collagens (for example: collagen *-II*, *-III*, *-IV*, and *-V*) are present although in smaller quantities and in specific locations. *Type II* collagen is found within enthesious insertions and regions where the tendon changes direction around a bony prominence, reflecting the fibrocartilage-like nature of the matrix in this region designed to withstand compressional as well as tensional forces. *Types III*, *IV*, and *V* are confined to basement membranes and endotendon (Goodship et al. 1994; Perez-Castro and Vogel 1999).

Collagen molecules are arranged hierarchically into fibrils, fibers, and fascicles, according to the direction of the force application (Fig. 4.1). Endotendinous connective tissue (endotenon) surrounds the fiber bundles and contains blood vessels, lymphatics, and nerves. The whole tendon, composed of multiple bundles and endotenon, is surrounded by the epitenon, a thin layer of connective tissue that is contiguous with the endotenon. Intermolecular cross-links between the *type I* collagen molecules reinforce the tensile strength of the tendon matrix (Thorpe et al. 2010).

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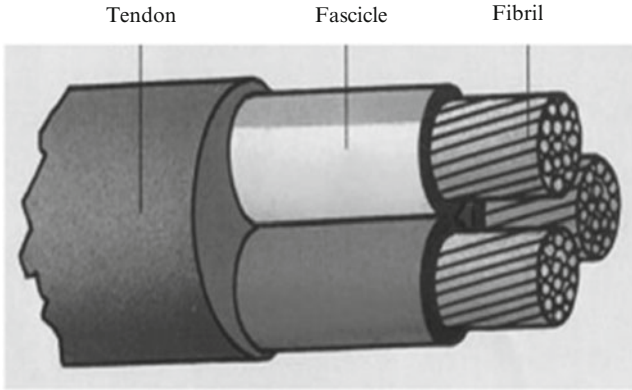


Fig. 4.1 Schematic diagram representing the architectural hierarchy of the tendon

The noncollagenous substance is composed of tenocytes and glycoproteins. Three distinct cell types (*I*, *II*, and *III*), have been identified on light microscopy within the fascicles of normal equine tendon, based on the morphology of their nuclei. The distribution of cell types varies with age, between tendons and within tendons (Goodship et al. 1994; Smith and Webbon 1996). The functions of these different cell types are unclear but it is postulated that *type II* and *III* cells have higher metabolic activity because their nuclei are larger and contain nucleoli. These cells may be concerned primarily with ECM synthesis (Smith and Webbon 1996) but considerably more information is needed on the nature of these different cell types and their function. There is a further population of cells resident within the endotenon septa of the tendon whose functions are largely unknown.

Of the glycoproteins, cartilage oligomeric matrix protein (COMP) is one of the most abundant (Smith et al. 1997). Proteoglycans consist of glycosaminoglycan side-chain(s) attached to a protein core. Various glycosaminoglycans have been demonstrated within the equine superficial digital flexor tendon (SDFT) including chondroitin sulfate, dermatan sulfate, keratan sulfate, heparin, heparin sulfate, and hyaluronic acid (Smith and Webbon 1996), but this knowledge is only of limited benefit as this does not indicate the nature of the proteins to which they are attached. The metacarpophalangeal regions of normal SDFT contain more of the larger, cartilage-like proteoglycans, and experience higher rates of proteoglycan synthesis compared to the midmetacarpal region. These differences probably reflect functional and metabolic variance that exists between regions of tension and compression (Smith and Webbon 1996; Perez-Castro and Vogel 1999). The small proteoglycans decorin, fibromodulin, and biglycan have been thought to influence tenocyte functions, collagen fibrillogenesis, and the spatial organization of fibers, thereby influencing tendon strength (Gu and Wada 1996).

Tendons have high mechanical strength and elasticity necessary to perform their function. Tendons also exhibit viscoelasticity as they display properties of stress relaxation and creep (Sharma and Maffulli 2005a).

2 Mechanical Properties of Tendon

Biomechanical properties of tendons during their repair and regeneration have been studied extensively and their properties compared with normal tendon. Mechanical testing involves separate clamps to grip the muscle–tendon complex at one end and the bone at the other end ensuring that the ends are held firmly without slippage, the tendon is loaded along its longitudinal axis, and the force and displacement are recorded until the tissue fails (Riemersa and Schamhardt 1982). The in vitro mechanical properties of normal tendon have been characterized and shown to approximate a sigmoidal curve when force is plotted against elongation (Goodship et al. 1994) (Fig. 4.2).

There is an initial lax phase, termed the “toe” region, where the crimp is eliminated followed by a linear phase due to progressive stretching of the straightened collagen fibers, until tendon failure occurs at extreme forces. The methodology of in vitro biomechanical testing has been shown to be repeatable, and, to provide objective data on the biomechanical properties of tendons in response to various treatments. The mechanical properties of tendon may be correlated at the molecular level with the mechanical characteristics of the collagen fibers (Cohen et al. 1974). Tendon viscoelastic behavior (Kastelic and Baer 1980; Hooley et al. 1980), which is the result of complex interactions between various components, is dependent on age and activity. Tendons do not usually fail or rupture under normal conditions; therefore, it is more appropriate to quantify the physical properties within the linear region, including stress relaxation, creep, hysteresis loop, and viscoelasticity.

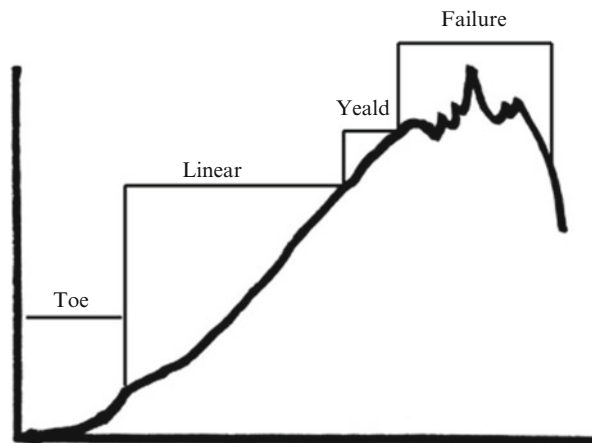


Fig. 4.2 Simplified stress–strain curve for the superficial digital flexor tendon. (1) “Toe” region; (2) linear deformation; (3) yield; (4) rupture

3 Tendons and Their Diseases in Man

A variety of tendons in man may be affected by overstrain injury, including elements of the rotator cuff and supraspinatus tendon in the shoulder, the forearm extensors, biceps brachii, the Achilles tendon in the lower limb, the tibialis posterior tendon in the foot, and the patella tendon in the knee (Kader et al. 2002; Rees et al. 2006). Tendon injuries, clinically appearing either as acute or chronic disease, can be caused by both extrinsic (trauma) and intrinsic (overstrain) factors (Rees et al. 2006). Whilst spontaneous acute injuries do occur in tendon diseases, there is evidence that such injuries are often preceded by degeneration of the tendon matrix in the period prior to onset of clinical signs (Kannus and Jozsa 1991; Astrom and Rausing 1995). There is a growing body of evidence that proteolytic enzymes, such as matrix metalloproteinases, in part mediate such degeneration (Jones et al. 2006). In recent decades, the incidence of tendon injury has risen due to both an increase in the elderly population, and a rise in participation to recreational and competitive sporting activities (Kader et al. 2002).

In man, loading of a tendon up to 2 % strain leads to flattening of the crimp pattern in tendon. Between 2 and 4 % strain, tendons deform in a linear fashion as a result of intramolecular sliding of collagen triple helices and fibers become more parallel. At strains less than 4 % tendons behave in an elastic fashion and return to their original length when unloaded. Microscopic failure occurs when the strain exceeds 4 %, and between 8 and 10 % strain, macroscopic failure occurs (Sharma and Maffulli 2005b).

4 The Importance of Injuries to Energy-Storing Tendons in Large Athletic Animals

It is widely accepted that there is no model in which all potential therapies may be tested appropriately; however, it has become essential to have an animal model matched to the clinical pathology prior to initiating a clinical trial. When selecting an appropriate animal model, the salient criteria to consider are a closely matched anatomic structure and the ease with which the treatment can be translated to a clinical setting (Smith et al. 2005). A study about adhesions formed after injury to the flexor tendon in the primate was found to have the closest resemblance to humans in both surgical technique and physical attributes of the matching tissues and organs (Pruzansky 1987). Use of dogs and chickens has been ruled out primarily because their physical and anatomic features do not match the human anatomy. This is essential for research translation from an animal model to the human situation. In short, testing materials, biological factors, and surgical techniques in an animal model that mimics the pathogenic condition in humans is ideal because it will facilitate research translation to humans. Murine models that simulate various disease conditions, such as genetic disorders, growth factor deficiencies, cancers, and

immunodeficiency are available to many investigators. Animal models have given a major boost to the design of research tools and are available for the development and validation of novel therapies prior to human trials. These models are used to test procedures quantitatively and to determine the ease and effectiveness of surgical procedures, and they allow for statistically significant studies in mice, rats, and rabbits. In addition, larger animals, such as goats, sheep, as well as porcine and equine models, have been utilized in appropriate circumstances. The choice of the animal model is also determined by the outcomes sought by the investigators. Most animal models are appropriate for the determination of histology, cell proliferation, and biochemical analysis in order to determine the effectiveness of surgical or therapeutic treatment procedures. The need to have a technically reproducible and mechanically stable tendon injury model is dominant to the ultimate utility of the animal model. In addition, the availability of techniques that will measure the strength of the regenerate tissue is necessary within most musculoskeletal components. These considerations set the lower limit to the anatomical size and morphological features within the experimental model. In this context a large animal as the equine is a good model for the study of human tendon disease (Roshan et al. 2008).

As in human, among equine there are athletes, too. Tendon injuries associated with athletic activity and aging are most frequent and important both in people and horses. Due to its clinical importance and to the easier attainability of tissue specimens not achievable in the medical field, the most completely investigated pathologies of tendons are those of the distal limb of the horse, in particular the injury to SDFT. The SDFT in the horse is a weight bearing tendon and has many similarities to the human Achilles tendon in both its structure and matrix composition. This tendon acts as a spring, absorbing and releasing elastic energy during different phases of the stride, contributing to both the high efficiency of locomotion and, along with its associated muscle, also acting as a shock absorber for the limb (Wilson et al. 2001). The horse has maximized its ability to store energy for efficient locomotion by having maximal strains in the SDFT far higher than in humans, recorded at 16 % at the gallop in horses (Minetti et al. 1999). This value is in close agreement with measured in vitro strains at rupture of 12–21 %. These findings suggest that at maximal exercise the SDFT operates close to its physiological limits with a relatively narrow safety margin. Consequently, minor disruption of the tendon matrix composition and arrangement dramatically increase the incidence of tendonitis. Conversely, improving the “quality,” or strength, of the tendon matrix reduces incidence.

The specialized nature of equine digital tendons and certain human tendons essential for the efficiency of high-speed locomotion, and the large size of both species, bring the clinical relevance of laboratory rodent and rabbit models of exercise-induced tendon damage into question.

The risk of SDFT injury is one of the most frequent causes of lameness of thoroughbred horses internationally (Kasashima et al. 2004; Ely et al. 2009) and it has been shown to increase with horse age in a number of surveys (Kasashima et al. 2004; Perkins et al. 2005). Re-injury rates of 23–67 % have been reported in various equine sports disciplines; 19–70 % are ultimately retired due to the original or the

subsequent re-injury (Dyson 2004; Perkins et al. 2005; O'Meara et al. 2010). All of these factors cause significant economic loss and animal welfare concerns.

The risk of Achilles tendon (AT) injury in people, is associated with elite athletic activity and with age, as for SDFT injury in horses; incidences of up to 29 %, 18 %, and 52 % have been reported in elite male runners, elite gymnasts, and running athletes, respectively, with the latter figure correlated with hours spent training (Knobloch et al. 2008; Emerson et al. 2010). Up to 29 % of patients with AT injuries may require surgery (Zafar et al. 2009); time for a return to activity varies from 6 weeks to almost 10 months depending on the complexity and chronicity of the injury, and re-rupture rates of 2–12 % have been reported (Saxena et al. 2011).

The functional equivalence of the SDFT and AT is largely due to their pivotal role in saving energy during high-speed locomotion, by reducing muscular work (Malvankar and Khan 2011). Most tendons (“positional” tendons) simply connect skeletal muscle and bone, stabilizing and moving joints. In the horse, recovery of mechanical work during tendon elastic recoil (i.e., with the SDFT acting as a “biological spring”) has been estimated at 36 % at gallop; in the human AT, energy returns contribute to over 50 % of positive work done at the ankle (Farris et al. 2011). Energy-storing tendons must undergo high strains (i.e., changes in tendon length as a percentage of initial length) to store sufficient amounts of energy.

5 Evidence of Microdamage and Aetiological Factors in Tendons Diseases

The majority of clinical tendonitis lesions have, as a precursor, some undetectable subclinical change that precedes clinical tendonitis and this change is age-related and accelerated by exercise. Degenerative changes described in human tendons are similar (but not identical) to those found in equine exercise study and include evidence of new collagen synthesis with increased *type III* collagen and reduced *type I* collagen, increases in levels of fibronectin, tenascin C, GAGs, and proteoglycans and histological observations of apparently irreversible matrix change including irregular arrangement, altered crimping, disruption, and reductions in density of collagen (Riley et al. 1994a, b; Jarvinen et al. 1997; Cook et al. 2004). Fibronectin and tenascin C are glycoproteins that are thought to modulate cellular activity and migration (Riley 2005). Increased amounts of *type III* collagen at AT rupture sites in one study were not associated with evidence of recent synthesis (cleaved propeptides), supporting the theory that this accumulates prior to clinical injury (Eriksen et al. 2002). Histological degenerative lesions seen in human AT, but not noted in horses, include mucoid degeneration, fatty degeneration (accumulation of adipocytes), calcification, and neovascularization; this may to some extent relate to the fact that they are not identical anatomical structures. In both asymptomatic and painful degenerate human tendons, various histological changes have been noted in tenocyte populations including increased or decreased cellularity, plump ovoid or rounded nuclei with ultrastructural evidence of increased production of collagen and

proteoglycans, and chondroid change in more extreme cases; tendon cells cultured from lesions have maintained a higher proliferation rate and produced greater quantities of *type III* collagen (Rolf et al. 2001; Cook et al. 2004). Ultrastructural changes in the tenocytes have been interpreted as indicative of hypoxic degeneration, including alterations in sizes and shapes of mitochondria and nuclei, increased numbers and sizes of lysosomal vacuoles, “hypoxic” and lipid vacuolation and intracytoplasmic or mitochondrial calcification (Kannus and Jozsa 1991; Leadbetter 1992).

In equine, when the surface of a tendon is viewed at an angle, the presence of a wave form or crimp within the fascicles can be detected (Goodship and Birch 1996). This crimp pattern plays a role in imparting elasticity to the tendon during the early stages of loading (up to 2–3 % of strain). Crimp angles and lengths were found to be 19–20° and 17–19 μm respectively in the midmetacarpal region of the young SDFT (Wilmlink et al. 1992) in equine species, reducing to 12–17° and 11–15 μm with aging. The elimination of the crimp with age is thought to contribute partly to the increased stiffness of tendon with age.

Most tendons in mature horses exhibit patchy acellularity, although there is no overall decrease in cell number with age in the equine SDFT (Birch et al. 1999). Further degenerative signs become more evident in all horses age >3 years (Pool 1996) and these include matrix fibrillation, chondroid metaplasia, chondrone formation, neovascularization, and fibroplasia (Pool 1996). All these changes have the effect of inducing relative tendon weakness and, although an association between elastic modulus and age has been demonstrated, the correlation between decreasing mechanical strength and age has not been proven (Gillis et al. 1997).

Recent investigations have indicated that the equine SDFT attains maturity at approximately 2 years of age. Collagen fibril diameter, mature collagen cross-links, and crimp morphology have stabilized by this age (Patterson-Kane et al. 1997a, b). Tendons from horses over 2 years of age have significantly stiffer mechanical properties (Gillis et al. 1995b). This increased stiffness is thought to be associated with the reduction of crimp, the presence of increasing numbers of nonreducible cross-links and decreasing fascicle size in older tendons (Gillis et al. 1997).

Exercise also causes a reduction in the mass average diameter of collagen fibrils within the central region of the SDFT compared to the peripheral region fibrils (Patterson-Kane et al. 1997c). As glycation levels are unaltered, which indicates that the smaller fibrils are not new collagen, these results suggest that microdamage, with splitting of the collagen fibers, is occurring.

In the human being, Dowling et al. (2000) report about investigations of specific pathogenetic mechanisms of tendonitis. These can be classified into two broad categories: (a) physical and (b) vascular.

(a) Physical mechanisms

Fatigue, poor conformation, lack of fitness, incoordinate muscle activity (McIlwraith 1987) in nonathletic people will all act to produce excessive biomechanical forces on the tendon (Silver et al. 1983; Crevier-Denoix and Pourcelot 1997) which may accelerate the degenerative change by physically disrupting the matrix, or are sufficient to induce full clinical tendonitis by exceeding the

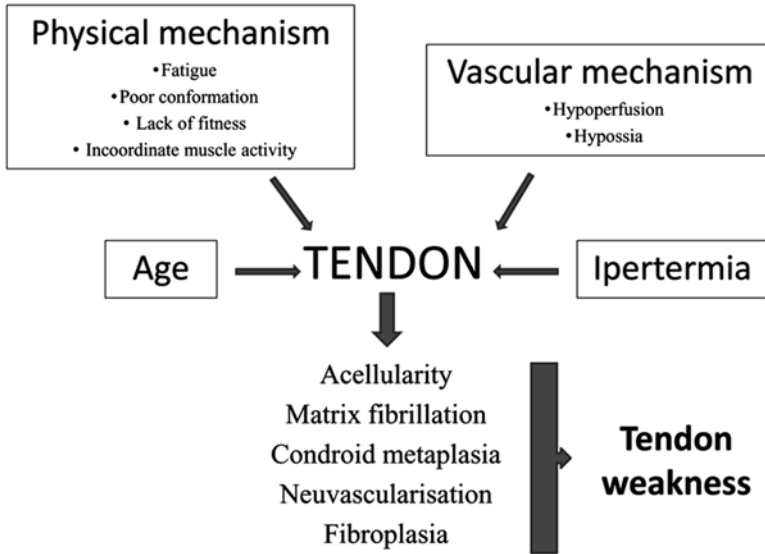


Fig. 4.3 Aetiological factors in tendons diseases

mechanical properties of the tendon. On the other hand, it is generally agreed that the levels and most likely also the patterns, types, and durations of physical stress on tendons are the most important aetiological factors in athletic injuries (Riley 2005). In addition to the mechanical environment, the only other directly measured or calculated stress factor specific to energy-storing tendons is hyperthermia. Due to loss of some strain energy as heat (hysteresis), which is not effectively dispersed by the vascular system, temperatures of at least 43–45 °C have been measured in the SDFT core of the galloping horse (Wilson and Goodship 1994). Conservative estimates of hyperthermia in the human AT, calculated by mathematical modeling, predicted a core temperature of at least 41 °C during 30 min of treadmill running, with the potential to rise to up to 44 °C in some individuals (Farris et al. 2011). Such temperatures would be expected to be lethal or at the very least highly stressful to fibroblastic cells.

(b) Vascular mechanisms

Other damaging conditions that may be experienced during and/or following exercise within the tendon tissue include hypoxia due to tendon hypoperfusion and increased levels of reactive oxygen species (ROS) induced by ischaemia-reperfusion and/or hyperthermia (Millar et al. 2012).

However, the equine SDFT has good vascular network (Kraus-Hansen et al. 1992) and blood flow in this tendon has been recorded at a level similar to that of resting skeletal muscle and is increased by exercise (Jones 1993). At present there is little experimental evidence to support or refute these potential mechanisms, but it seems logical that each may interact or summate to cause tendon injury (Fig. 4.3).

6 Tendon Healing

The restoration of normal tendon function after injury requires reestablishment of tendon fibers and the gliding mechanism between tendon and its surrounding structures (Abrahamsson and Gelberman 1994). The initial stage of repair involves formation of scar tissue that provides continuity at the injury site (Dunphy 1967) however, lack of mechanical stimulus on the tendon will cause proliferation of scar tissue and subsequent adhesions that are undesirable and harmful because they impede normal tendon function, particularly in the hand. Although stability to the injury site is necessary, mobility is critical, and mechanical loading that is associated with motion of the healing tendon decreases the formation of postoperative adhesions and increases the strength. After tendon injury, the body initiates a cascade of distinct events or phases distinguishable by the cellular and biochemical processes that occur. The sequence of repair involves a progression through three stages: tissue inflammation, cell proliferation, and remodeling (Ingraham et al. 2003; Sharma and Maffulli 2005b) (Fig. 4.4).

During inflammatory stage, vasodilators and proinflammatory molecules attract inflammatory cells from the surrounding tissue. Erythrocytes, platelets, neutrophils, monocytes, and macrophages migrate to the wound site where the clot, cellular debris, and foreign body matter are engulfed and resorbed by phagocytosis. Fibroblasts recruited to the site begin to synthesize various components of the ECM (Lindsay and Birch 1964) Angiogenic factors are released during this phase and initiate the formation of a vascular network (Myers and Wolf 1974) These processes include an increase in DNA and in ECM, which establishes continuity and partial stability at the site of injury.

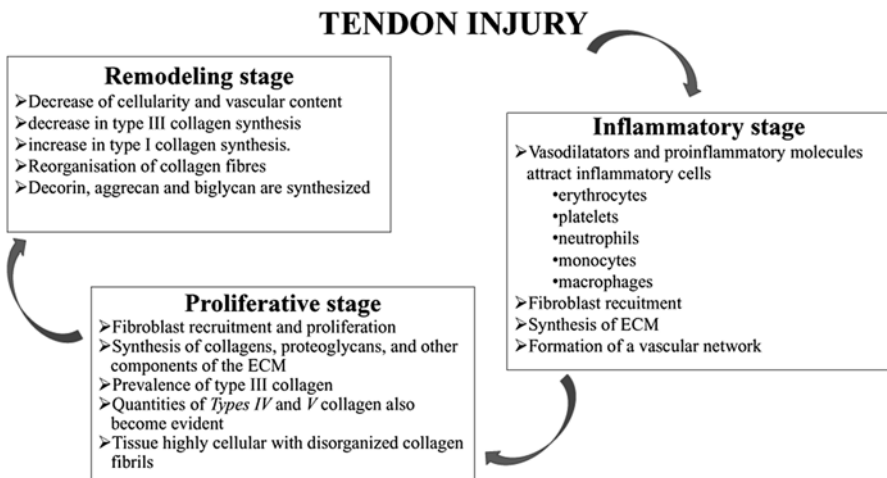


Fig. 4.4 Stages of tendon healing

In the proliferative stage, fibroblasts and their rapid proliferation at the wound site are responsible for the synthesis of collagens, proteoglycans, and other components of the ECM. These components are initially arranged in a random manner within the ECM, which at this point is composed largely of type III collagen (Garner et al. 1989). *Type III* collagen forms interfibrillar cross-links conferring early stability and mechanical strength to the site of injury (Williams et al. 1980; Cheung et al. 1983). Increased quantities of *Types IV* and *V* collagen also become evident (Williams et al. 1980; Silver et al. 1983). At the end of the proliferative stage, the repair tissue is highly cellular and contains relatively large amounts of water and an abundance of ECM components.

The remodeling stage begins 6–8 weeks after injury. This phase is characterized by a decrease in cellularity, reduced matrix synthesis, decrease in *type III* collagen, and an increase in *type I* collagen synthesis (Roshan et al. 2008). Loose fibrils of *Type I* and *III* predominate until 6 months post injury. After this period, linearly arranged *Type I* collagen fibrils prevail, indicating progressive remodeling and normalization of the healing tissue (Watkins et al. 1985). *Type I* collagen fibers are organized longitudinally along the tendon axis and are responsible for the mechanical strength of the regenerate tissue (Liu et al. 1995). During the later phases of remodeling, interactions between the collagen structural units lead to higher tendon stiffness and consequently greater tensile strength; however, the repair tissue never achieves the characteristics of normal tendon. Indeed, abnormal quantities of *Type III* collagen, smaller collagen fibrils, and lack of fiber bundles and linear arrangement may still be detected up to 14 months post injury (Silver et al. 1983) and probably persist even longer. It is the abnormal composition and arrangement of matrix in fibrous scar tissue, which has poor biomechanical properties in comparison to normal tendon, and the slow rate of healing that are believed to be responsible for the high incidence of reinjury (Pool 1996).

7 Diagnosis of Tendonitis

Ultrasonography is presently the most common technique used to diagnose tendonitis and monitor healing. The commonly measured variables include tendon and lesion cross-sectional area (CSA), lesion type and location, and fiber alignment (Smith et al. 1994; Gillis et al. 1995a, b; Genovese et al. 1996). An increase in tendon CSA is reportedly the most sensitive indicator of fiber damage (Genovese et al. 1996) and for detecting re-injury during the convalescent period when the reintroduction of exercise may be excessive. Additional measurement includes changes in echogenicity. Also magnetic resonance could be applied for diagnosis of tendinitis. Kasashima et al. (2002) demonstrated that magnetic resonance imaging is the better imaging modality for the objective detection of chronic scar tissue in live horses when the chronic scar tissue is difficult to discern by ultrasonography.

8 Classical Therapies in Tendon Diseases

Injury initiates several signaling events that recruit fibroblasts and stimulate the local tenocyte population to synthesize collagen and other extracellular components, establishing physical continuity at the site. In most cases of tendon laceration or rupture, surgical intervention is required to direct the natural process of healing, and occasionally the damage exceeds the natural ability to repair even with existing treatment modalities. Tendon repair is a slow process and characterized by a scar with mechanically inferior tissue, at least initially and it is complicated by the need to provide appropriate and timely tension to the repair tissue (Roshan et al. 2008).

In the past, repair of tendon injuries were performed by different methods. In particular, clinical therapy was based on ice application, bandage, box rest, and controlled exercise. An alternative approach consisted on the use of corticosteroid (inflammation reduction) and other drugs (sodium hyaluronate, polysulphated glycosaminoglycans, beta aminopropionitrile fumarate). Furthermore, surgical treatments like accessory ligament desmotomy, local irritation by line firing or pin firing were commonly used. More recently ultrasound, laser therapy, and electromagnetic field therapy have been considered. Unfortunately, they did not allow complete tissue healing and quite often people or animals did not regain competitiveness.

9 New Treatments in Tendinopathies: Regenerative Medicine

During rehabilitation with controlled exercise, there is an ideal mechanical stimulation allowing the newly created tissue to organize itself in the direction of the force application, hence this approach can be referred to as “in vivo tissue engineering.” But a better understanding of the mechanisms that regulate the function and the differentiation of tendon cells is essential to developing new treatments for tendinopathy such as tendon rupture or ectopic ossification resulting from injury caused by overuse or trauma. In recent years, research on tendinopathy therapies has focused on regenerative medicine and tissue engineering approaches, which are considered to be promising tools to prospect normal restore, or close to normal, structure and function to an injured organ. Regenerative medicine requires an exogenous cell source and this approach comes from the knowledge that most tissues have a sub- or side-population of precursor cells (tissue-specific progenitor cells) used to replenish cells due to natural turnover and aid in repair post injury (da Silva Meirelles et al. 2006). Several lines of evidence suggest that multipotential stem cells are present also in tendons and ligaments. First, both human and mouse tendons develop fibrocartilage and ossification in response to injury (Fenwick et al. 2002). Second, tendon-derived immortalized cell lines or human tendon-derived fibroblasts express genes of adipogenic, osteogenic, and chondrogenic differentiation pathways, suggesting that they possess multiple differentiation capacities in vitro (Salingcarnboriboon et al. 2003; de Mos et al. 2007). Finally, postnatal stem cells

capable of differentiating into adipocytes and osteoblastic cells have been identified in human periodontal ligaments (Seo et al. 2004) while human and mouse tendons harbor a unique cell population, termed tendon stem/progenitor cells (TSPCs), that has universal stem cell characteristics such as clonogenicity, multipotency, and self-renewal capacity (Bi et al. 2007). Recently, Lovati et al. (2011) identified TSPCs specifically in the horse SDFT with the ability to be highly clonogenic, to grow fast and to differentiate in different induced-cell lineages as well as bone marrow-derived progenitor cells (BM-MSCs). The hypothesis that TSPCs possess a mesenchymal stem cell (MSC) behavior opens a new prospective for tendon regenerative medicine approaches because TSPCs could represent an important tool to study basic tendon biology. The exact site for TSPCs cells within tendon is not known, but they are most likely to reside in the endotenon tissue between the collagen fascicles and adjacent to the vasculature (da Silva Meirelles et al. 2006). Although this might be true in young growing tendon, mature equine tendon, however, does not appear to possess a substantial population of these cells capable of differentiating into multiple cell lines and this may explain why this component of the repair process is limited and hence natural repair is inferior to normal tendon.

During the repair process, there is a large influx of cells into the lesion. Kajikawa et al. (2007) showed that at 24 h after the injury, the wound contained circulation-derived cells but not tendon-derived cells. Tendon-derived cells appeared in the injured area at 3 days after the wound, and significantly increased in number with time and maintained a high level of proliferative activity until 7 days after the injury, whereas the circulation-derived cells decreased in number and are replaced by the tendon-derived cells. These findings suggest that circulation-derived and tendon-derived cells contribute to the healing of tendons in different periods as part of a biphasic process but that the cells mainly involved in the synthesis of new tissue are believed to be tendon-derived cells (Kajikawa et al. 2007). For these reasons some authors hypothesized that the implantation of far greater numbers of progenitor stem cells, than are present normally within tendon tissue, would have the potential of regenerating or improving the repair of the tendon. Fibroblasts derived from tendon or other sources could be used (Kryger et al. 2007), but the removal of sections of tendon to recover cells leads to the formation of a secondary lesion in the horse that is unacceptable. Alternative cell sources under investigation include dermal fibroblasts, which were shown to be capable of functionally bridging a tendon defect and to have similar histological and tensile properties to the tenocyte-seeded scaffold (Liu et al. 2006) although *in vitro* these cells behave differently from tenocytes (Evans and Trail 2001). By contrast, the implantation of MSCs of different sources in far greater numbers than are present normally within tendon tissue would have the potential for regenerating or repairing tendon. MSCs have been implanted into surgical defects in tendons in multiple *in vivo* experiments in laboratory animals with mostly positive outcomes. Most of these models used surgically created defects in rabbit or rat tendons and have variously shown some improvement in structure and strength of defects implanted with MSCs in a biodegradable scaffold (collagen gel, Vicryl knitted mesh or fibrin glue) over controls implanted with just the scaffold, as assessed by histology or simple biochemical assays (Awad et al. 2003;

Juncosa-Melvin et al. 2007; Butler et al. 2008). In other studies using a rat patellar defect model, MSCs implantation has been associated with both greater ultimate tensile stress and improved quality of reparative tissue determined by an increased collagen *I/III* ratio (Hankemeier et al. 2005, 2007). Thus, MSCs-seeded constructs implanted in vivo have shown the ability to integrate into the tissue and induce the synthesis of tissue-specific ECM. Implantation has been associated with both greater ultimate tensile stress and improved quality of reparative tissue determined by an increased collagen *I/III* ratio (Hankemeier et al. 2005, 2007).

Unfortunately, it is still unclear whether the major contribution of the MSCs to the healing process is to differentiate into tenocytes and thus produce ECM molecules, whether it is rather to supply growth factors and thus stimulate the residing cells within the tendon (Richardson et al. 2007; Chong et al. 2009) or whether a combination of the two mechanisms occurs (Yagi et al. 2010; Fortier and Travis 2011). In addition, recent studies have suggested an anti-inflammatory role of implanted stem cells. Animal model studies have demonstrated that MSCs are hypo-immunogenic and inhibit the activation of T and B lymphocytes and natural killer cells (Ren et al. 2008; Herrero and Pérez-Simón 2010). The precise mechanism of the anti-inflammatory effect of these cells is largely unknown, although a combination of this activity along with an antiapoptotic effect, additional recruitment of local multipotent stem cells, stimulation of vascular ingrowths, and the liberation of growth factors could all contribute to tissue repair (Alves et al. 2011). It is not known which of these actions occur after stem cell implantation, although current opinion favors the paracrine actions as being most important (Murphy et al. 2003).

The clinical nature of the employment of MSCs for horse tendinopathies preclude the routine *post mortem* analyses that can answer to these questions, but some experimental works has been carried out to monitor the fate of injected MSCs in horses and the structural aspect of the healing. Guest et al. (2008) studied the fate of autologous and allogeneic MSCs transfected with green fluorescent protein (GFP) following injection into the SDFT and revealed that GFP labeled cells located mainly within injected lesions, but with a small proportion integrated into healthy tendon. Furthermore, the authors showed that both autologous and allogeneic MSCs may be used without stimulating an undesirable cell-mediated immune response from the host. Other postmortem examinations have shown that MSCs application improved the ECM structure of damaged tendons. In histological sections of MSCs-treated tendon lesions, compared to non-treated tendon lesions, increased tendon fiber densities, increased organization of the collagen fibers, and a reduced vascularity have been found (Smith et al. 2003, 2009; Nixon et al. 2008). However, statistically significant differences have not been detected either in DNA, proteoglycan, or total collagen content, or in the expressions of *type I* collagen, *type III* collagen, or tissue remodeling enzymes such as matrix metalloproteinases (Schnabel et al. 2009; Guest et al. 2008), suggesting that the beneficial effect of MSCs is due to improvement of structural organization rather than of matrix composition. However, it has been shown that MSCs treatment can enhance expression levels of COMP (Guest et al. 2008; Crovace et al. 2010), a glycoprotein that is known to

be important for tendon elasticity and stiffness. Ultrasonographic follow-up examinations showed significant improvements in fiber alignment and echogenicity scores at 1, 3, and 6 months after MSCs treatment (Leppänen et al. 2009), supporting the histological findings in the above-mentioned studies.

The optimal *in vivo* regenerative response due to MSCs could be achieved by the combination of three key factors (Butler et al. 2008):

1. A cell source capable of the formation of an optimal matrix.
2. A scaffold capable of promoting the survival of implanted cells by mechanical protection and/or nutritional support.
3. An anabolic stimulus usually combining, for musculoskeletal tissues, growth factors, and appropriate mechanical load to promote optimal ECM synthesis and organization.

9.1 Cell Source

Today, there are multiple choices for the selection of a cell source for regenerative medicine and at this moment it is not clear which source will prove to be therapeutically optimal (Table 4.1). The cells carrying the greatest hope for effective different cell lines are MSCs that have been isolated from bone marrow and fat but, recently, other sources of MSCs potentially useful for tendon and ligament therapies include extra-fetal stem cells that can be easily recovered at birth and stored for future use (Cremonesi et al. 2011; Lange-Consiglio et al. 2012) and embryonic or fetal embryonic-like stem cells (ESC or fdESC, respectively) (Guest et al. 2010; Watts et al. 2011).

9.1.1 Adult MSCs

The stromal compartment of bone marrow was the first source reported to contain multipotent progenitor cells (Fortier et al. 1998; Pittenger et al. 1999). For this reason, bone marrow is currently the best investigated origin of MSC. Bone marrow is generally collected from the sternum and it is favored for cell-based therapies in equine regenerative medicine probably to the reliable isolation success of bone marrow-derived MSC following an easy preparation procedure and separation of MSC via plastic adherence and cell culture (Vidal et al. 2006) Looking at differentiation capacities, bone marrow-derived MSC can be directed towards the osteogenic, chondrogenic, and adipogenic lineage (Pittenger et al. 1999), with differences in the potency of differentiation compared to MSCs derived from other sources. For equine MSC, Kisiday et al. (2008) reported a higher chondrogenic differentiation potential in response to TGF- β 1 compared to MSC derived from adipose tissue. In contrast, compared to umbilical cord blood-derived MSC, chondrogenic differentiation of bone marrow MSC was inferior (Berg et al. 2009). Osteogenic

Table 4.1 Sources for cell therapy of tendinopathies

Cell source	Advantages	Disadvantages	References	
Embryo	Embryonic stem cells (ESC)	– Pluripotent	Paris and Stout (2010)	
Extra-fetal tissues	Amnion-derived cells	– No invasive collection – High plasticity and proliferative capacity – High number of immediately available cells for therapy	Lange-Consiglio et al. (2012, 2013a)	
	MSCs from umbilical cord tissue	– Well-tolerated by horses		
		– No invasive collection		
		– Greater multipotent than BM-MSCs	– Strict surveillance of parturition	Corradetti et al. (2011) Carrade et al. (2011)
	Adult tissues	Concentrated bone marrow aspirate (BMC)	– Possibility to obtain more rapidly proliferating cells by cell sorting – No immune response – Minimal manipulation	Fortier et al. (2010)
Stromal vascular fraction from adipose tissue		– No cell expansion	– Invasive aspiration procedure with risk of pneumopericardium – No reports on the use of BMC on tendinitis	
		– Minimal manipulation – No cell expansion – Well-tolerated by horse	– Invasive collection	Alexander (2012)

(continued)

Table 4.1 (continued)

Cell source	Advantages	Disadvantages	References	
Adult stem/progenitor cells	MSCs from bone marrow (BM-MSCs)	<ul style="list-style-type: none"> - Multipotent - No immune response 	<ul style="list-style-type: none"> - Invasive aspiration procedure with risk of pneumopericardium - Limited potential than ESC in terms of expansion (delay of 2–4 weeks to obtain a sufficient number of cells to in vivo implant) 	<ul style="list-style-type: none"> Durando et al. (2006), Vidal et al. (2012) Butler et al. (2008)
	MSCs from adipose tissue	<ul style="list-style-type: none"> - Higher proliferative potential and less senescence of BM-MSCs 	<ul style="list-style-type: none"> - Invasive collection 	Vidal et al. (2007)
	Tendon stem/progenitor cells	<ul style="list-style-type: none"> - Multipotent - Possible activation of this endogenous population - Multipotent 	<ul style="list-style-type: none"> - Invasive collection (removal of sections of tendons leads to the formation of secondary lesion) - Mature equine tendon do not possess a substantial population of these cells 	<ul style="list-style-type: none"> Colleoni et al. (2009) Lovati et al. (2011)
Adult differentiated cells	Tenocytes	<ul style="list-style-type: none"> - Appropriate tendon matrix synthesis 	<ul style="list-style-type: none"> - Invasive collection - Age-related reduction in synthesis of matrix ability 	Kryger et al. (2007)
	Fibroblasts derived from tendon	<ul style="list-style-type: none"> - Appropriate tendon matrix synthesis 	<ul style="list-style-type: none"> - Invasive collections 	Kryger et al. (2007)
	Dermal fibroblasts	<ul style="list-style-type: none"> - Easy to recover, with acceptable donor site lesion - Similar histological and tensile properties than tenocyte 	<ul style="list-style-type: none"> - Different protein-matrix synthesis than tenocytes 	Liu et al. (2006)

differentiation potential of bone marrow MSC was reported to be highest among MSC derived from several other sources (Toupadakis et al. 2010) but lowest compared to amniotic-derived cells (Lange-Consiglio et al. 2012, 2013a). Although BM-MSCs represent the most widely investigated cells for application in veterinary regenerative medicine to date, it is important to underline that procurement of BM from horses requires an invasive aspiration procedure, which occasionally has been associated with pneumopericardium (Durando et al 2006). In addition, BM-MSCs represent a very small fraction (0.001–0.01 %) of the total population of mononuclear cells isolated from a Ficoll density gradient of bone marrow aspirate, so, any clinical effect of bone marrow aspirate might be attributed to the numerous bioactive substances in the acellular fraction such as growth factors produced by cells or platelets. The BM-MSCs, moreover, have a more limited potential than embryonic stem cells in terms of *in vitro* proliferation ability (about 32 days for expansion between isolation and implantation) and this is a true limitation to use autologous BM-MSCs because there is a delay of 2–4 weeks while MSCs are multiplied *in vitro* before sufficient numbers are available for use (Guest et al. 2010; Lange-Consiglio et al. 2013a). Furthermore, BM-MSCs show reduced plasticity and growth with increasing donor age and *in vitro* passage number (Vidal et al. 2012) and do not appear to noticeably improve long-term functionality (Paris and Stout 2010).

The concentrated bone marrow aspirate (BMC) can overcome these problems avoiding the lag time from diagnosis to treatment when culture-expanded BM-MSCs are used. In addition to the concentration of stem cells, the concentrations of platelets and therefore anabolic growth factors are increased (Fortier et al. 2010), in addition it allows the concentration of the relatively few stem cells, and the production of a cell pool with minimal cell manipulation and no risk of cell transformation during the growth *in vitro*. When combined with thrombin, the fibrinogen present in BMC is converted to fibrin and a solid scaffold forms to retain the cells and growth factors in a given location. However, the precursors of bone and fat cells contained into BM may be detrimental to the healing by triggering the formation of dystrophic mineralization or metaplasia at the site of inoculation.

No peer-reviewed preclinical or clinical reports on the use of BMC for tendonitis have been published while an acute cartilage injury (15-mm-diameter lesions) was treated with BMC (Fortier et al. 2010). At 8 months, all macroscopic, histologic, and magnetic resonance imaging data indicated sustained improvement in BMC-grafted repair tissue compared with the control.

Another source of MSC commercially used in humans and animals is adipose tissue (Vidal et al. 2007; Del Bue et al. 2008; Jezierska-Woźniak et al. 2010; Martinello et al. 2011). The quick and successful recovery of adipose-derived MSC via lipectomy (horses) (Vidal et al. 2007) or collection of visceral fat during ovariohysterectomies (dogs) (Martinello et al. 2011), followed by enzymatic digestion, makes adipose tissue a promising MSC source for clinical applications. Adipose-derived MSCs seem to display a higher proliferation potential and less senescence compared to MSCs from other sources (Wagner et al. 2005; Colleoni et al. 2009; Vidal and Lopez 2011). Several studies report a good differentiation potential into mesodermal tissue lineages (Jezierska-Woźniak et al. 2010; Martinello et al. 2011),

whereas other groups observed a limited chondrogenic and osteogenic potential in rat or horse adipose-derived MSC (Yoshimura et al. 2007; Vidal et al. 2008). However, the collection of this tissue in the horse remains invasive.

In any case, since MSCs treatment regime was first published by Hertel (2001) there have been several experimental and clinical studies with encouraging results, giving evidence of the benefit and safety of adult MSCs application for tendon regeneration.

In these studies, autologous adult progenitor cells have been used, either expanded bone marrow-derived MSCs (Pacini et al. 2007; Smith 2008, 2009; Burk and Brehm 2011), or adipose-derived MSCs (Del Bue et al. 2008; Schnabel et al. 2009) or adipose-derived nuclear cells (Nixon et al. 2008; Dahlgren 2009; Leppänen et al. 2009). Furthermore, the effects of autologous bone marrow-derived expanded MSCs and bone marrow-derived mononuclear cells on tendon healing have been compared, revealing a similar improvement in both treatment groups compared to the control group, which was demonstrated by significantly improved ultrasonography and histology scores, higher COMP expressions and relatively lower *type III* collagen contents (Crovace et al. 2007, 2010; Lacitignola et al. 2008).

9.1.2 Extra-Fetal MSCs

To overcome the invasive collection of BM and adipose tissue, progenitor cells derived from extra-fetal sources could represent attractive alternative candidates, with the further potential to circumvent many of the limitations of BM-MSCs and to open new perspectives for developmental biology and regenerative medicine (Lange-Consiglio et al. 2012).

Extra-fetal sources of MSCs are the umbilical cord (both, matrix and blood) and amnion.

Use of adnexal tissue has many potential advantages including the noninvasive nature of the isolation procedure and the large tissue mass from which cells can be harvested. Moreover, the fact that the placenta is fundamental for maintaining fetomaternal tolerance during pregnancy suggests that cells present in placental tissue may have immunomodulatory characteristics (Evangelista et al. 2008) that could decrease the risk of the recipient rejecting transplanted stem cells immunologically.

With regard to amnion, this membrane is the innermost layer of the fetal adnexa and consists of a thin epithelial layer, a thick basement membrane, and an avascular stroma. Since the amniotic epithelium develops prior to gastrulation, which is a “tipping point” at which cell fate is specified, it has been suggested that pluripotent stem cells, derived from the contiguity of embryonic epiblast (Fig. 4.5), may be retained in the amnion even at full term pregnancy. The fibroblast layer of the amnion originates from the extraembryonic mesoderm that develops from the hypoblast around 2–3 weeks post-fertilization.

In equine species, and for the first time in veterinary medicine, amnion-derived mesenchymal cells (AMCs) were characterized (Lange-Consiglio et al. 2012) and afterward compared to equine BM-MSCs (Lange-Consiglio et al. 2013a). Results of

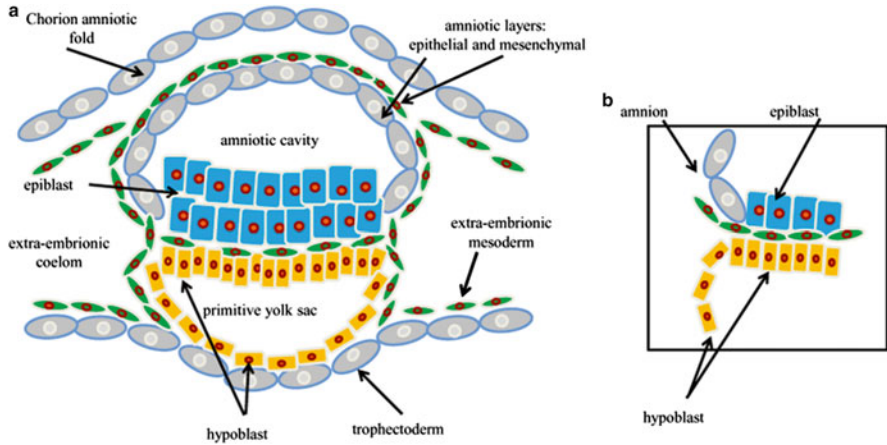


Fig. 4.5 Illustration of embryo at early gastrulation. (a) During gastrulation, an early amniotic cavity appears as chorion amniotic folds develop. The epithelial layer of the amnion, at the embryonic disk, is continuous with the epiblast. The mesenchymal layer is composed of extraembryonic mesoderm. (b) Detail of the amnion that is continuous with epiblast, outlined in (a)

these studies demonstrated that AMSCs and BM-MSCs both exhibited adult stromal cell-specific gene expression and immunocytochemical properties but showed substantial differences in the density of collection, and in their proliferative and differentiation potential.

BM-MSCs were collected at low density: on average, 8×10^6 MSCs were obtained from 30 mL of sternal aspirated from each analyzed sample, with a maximum of 25×10^6 and a minimum of 5.2×10^6 MSCs, while 100×10^6 stromal cells from about 24 g of tissue were obtained after digestion of amnion tissue.

For AMCs the mean doubling time (DT) was significantly lower ($P < 0.05$) than that observed for BM-MSCs (1.17 ± 0.15 vs. 3.27 ± 0.19 days, respectively). The average DT value of AMCs was 2 days shorter than the value observed in BM-MSCs (1.17 vs. 3.27 days). We calculated that when 5×10^5 AMCs are plated, it is possible to obtain an average of 8×10^6 cells in 6 days, while, by plating 5×10^5 of BM-MSCs, it takes 14 days to obtain the same number of cells. Also Fortier and Smith (2008) reported that in the equine species at least 2–3 weeks are necessary to expand cells isolated from BM and to get 10×10^6 cells available for implantation into a tendon injury, even if this therapeutic dose is empirically derived and other doses could potentially be efficacious. Moreover, BM-MSCs, compared to AMCs, also demonstrated a significantly ($P < 0.05$) lower clonogenic capability expresses as colony forming unit (CFU), with a mean value of 1 CFU-F each 590.15 cells seeded for BM-MSCs and 1 CFU-F each 242.73 cells seeded for AMCs.

About differentiation potential, after differentiation in osteogenic, adipogenic, chondrogenic, and neurogenic lines, BM-MSCs did not differentiate into glial cells and the osteogenic differentiation process was longer compared to AMCs (Table 4.2). In addition, Lange-Consiglio et al. (2012) showed that equine amnion-derived cells

Table 4.2 Characteristics of the MSCs isolated from bone marrow and amniotic membrane

MSCs-source	Phenotype	Efficiency of isolation	Differentiation potential	Doubling time	Clonogenic capability	In vivo results % re-injuries
Bone marrow	Mesenchymal and hematopoietic markers: CD44+, CD29+, CD105+,CD34-HLA-ABC+HLA-DR- Pluripotent markers: Oct-4+, SSEA-4+, TRA-1-60+	From 30 ml about 8×10^6 MSCs	Osteogenic Adipogenic Chondrogenic	3.27 ± 0.19 days	1 CFU-F each 590.15 cells seeded	23.08
Amnion membrane	Mesenchymal and hematopoietic markers: CD44+, CD29+, CD105+,CD34-HLA-ABC+HLA-DR- Pluripotent markers: Oct-4+, SSEA-4+, TRA-1-60+	From 24 g about 100×10^6 MSCs	Osteogenic Adipogenic Chondrogenic Neurogenic	1.17 ± 0.15 days	1 CFU-F each 242.73 cells seeded	4

+ and - signs respectively indicate presence or absence of gene expression

can also be frozen and recovered without loss of their functional integrity in terms of morphology, presence of specific stemness markers, and differentiation ability.

The noninvasive nature and low cost of collection, the rapid proliferation along with a greater differentiation potential, and the “off the shelf” preparation potential could make AMCs useful for cell therapy.

Cells obtained from other extra-fetal adnexa in domestic animal share a relatively consistent set of surface markers, which appears to support the hypothesis that they are MSC-like (for review see Cremonesi et al. 2011).

Extra-Fetal MSCs and Clinical Application

In a preclinical study, Carrade et al. (2011) injected autologous and allogeneic equine MSCs derived from either umbilical cord blood or umbilical cord tissue in non-injured horse joints to test the immunological properties of these cells. Their data suggest that there are no differences in the type or degree of inflammation elicited by self, related, or nonself cells. Moreover, the inflammation was clinically mild, not associated with gait abnormalities, self-limiting, allowing to conclude that, with the mild to moderate self-limiting inflammation and the absence of a systemic immune response, placental-derived MSCs do not elicit any hyperacute or acute (MHC-mediated) immune response.

To date, in the horse, the only data about the first clinical approaches for the treatment of horse spontaneous tendon diseases employing equine amniotic cells are from our laboratory (Lange-Consiglio et al. 2012, 2013b). After comparative study employing cryopreserved heterologous AMCs and fresh autologous BM-MSCs in spontaneous tendon diseases *in vivo*, our results showed that AMCs were well-tolerated by horses, and all of the clinical findings reported (i.e., the quick reduction in gross tendon size, palpation sensitivity, and ultrasonographic CSA measurements), provide compelling evidence to support the exertion of beneficial effects by the injected cells. Moreover, the ultrasonographic evolution showed for tendon and ligament architecture is similar to that reported after injecting autologous BM-MSCs. Comparing this innovative therapy with the conventional treatment with BM-MSCs, we observed that, although the recovery time is very similar (only a minimum period of about 4 months is required in both groups), the rate of re-injury in amniotic-treated horse was lower (4.00 % vs. 23.08 % for amniotic and BM-MSCs, respectively). The good result with AMSCs may be due to the opportunity to inject these cells in real time, before any ultrastructural change occurs within the injured tendon. Indeed, BM-MSCs, used as fresh autologous cells, required prolonged *in vitro* culture, limiting the time frame for implantation and probably, in this situation, animals were prone to re-injury due to a not optimal regeneration. The regenerated tissue could be less elastic and therefore functionally inferior to normal or native tendon. The AMCs, instead, were used as cryopreserved cells, then immediately available. The timing of the implantation should be carefully considered as well. Using AMSCs it could be hypothesized that the earlier implantation would offer greater benefits to the healing tendon by reducing inflammation, recruiting

native stem cells, and promoting production of collagen and other ECM proteins (Alves et al. 2011). The good time of implantation, together with the higher plasticity and proliferative capacity of the amniotic-derived cells compared to BM-MSCs (Lange-Consiglio et al. 2013a), represent the main features of interest for this novel approach for the treatment of equine tendon diseases. In addition, recent studies of *in vivo* transplantation of ovine amniotic membrane-derived epithelial cells into ovine Achilles tendon (Barboni et al. 2012) or into spontaneous equine tendon injuries (Muttini et al. 2013), demonstrated production of ovine collagen supporting the possibility to apply amniotic epithelial cells for tenocytes regeneration. Moreover, according to previous works (Smith et al. 2003; Dyson 2004) for equine embryonic stem cells and fetal-derived equine embryonic-like stem cells, it is possible to speculate that AMSCs, being most primitive compared to adult cells, exhibit higher level of engraftment with respect to BM-MSCs.

9.1.3 Embryonic or Fetal Embryonic-Like Stem Cells

To overcome the problem related to the use of adult stem cells, in addition to amniotic-derived cells, very recently, the first results concerning the use of ESC or fdESC, respectively for treatment of equine tendinopathy have been published (Guest et al. 2010; Watts et al. 2011). Guest et al. (2010) injected labeled ESC or labeled MSCs into artificially created tendon lesions, and horses were subjected to euthanasia after 20, 30, 60, or 90 days. ECS showed a high survival rate *in vivo*, which remained constant over 90 days and also migrated to other damaged areas within the tendon, whereas MSCs showed a lower and decreasing survival rate and did not migrate. Furthermore, there was no evidence of any immune response or tumor formation following cell application. Watts et al. (2011) reported that, over a period of 8 weeks after fdESC application, no differences in tendon-specific gene expression, collagen, or DNA content could be detected compared to the controls, while a significant improvement in tissue architecture, tendon size, lesion size, and tendon linear fiber pattern was shown. However, while these results are very encouraging, the long-term success and safety of ESC treatment remain to be proven.

9.2 Scaffold

Apart from blood cells, most, if not all other, normal cells in human and animal tissues are anchorage-dependent residing in a solid matrix called ECM. There are numerous types of ECM in human and animal tissues, which usually have multiple components and tissue-specific composition.

ECM provides structural support and physical environment for cells residing in that tissue to attach, grow, migrate, and respond to signals. Moreover, ECM gives the tissue its structural and therefore mechanical properties, such as rigidity and elasticity that is associated with the tissue functions. For example, well-organized

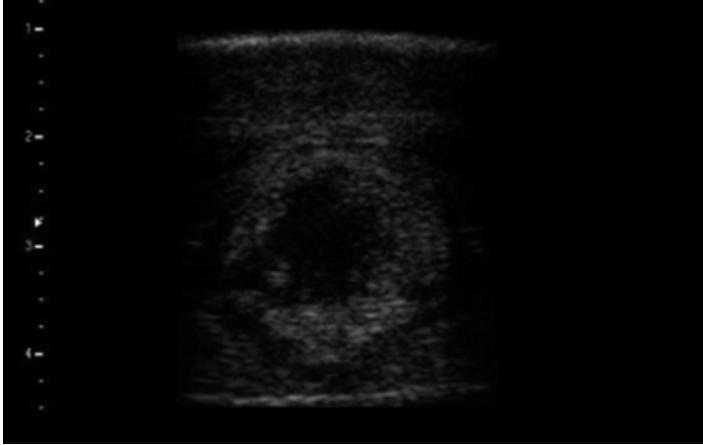


Fig. 4.6 Tendon lesion manifests within the central core of the tissue

thick bundles of collagen type I in tendon are highly resistant to stretching and are responsible for the high tensile strength of tendons. On the other hand, randomly distributed collagen fibrils and elastin fibers of skin are responsible for its toughness and elasticity. In addition, ECM may act as reservoir of growth factors and potentiates their bioactivities and provides a degradable physical environment so as to allow neovascularization and remodeling in response to developmental, physiological, and pathological challenges during tissue dynamic processes namely morphogenesis, homeostasis, and wound healing, respectively.

Intuitively, the best scaffold for an engineered tissue should be the ECM of the target tissue in its native state. Nevertheless, the multiple functions, the complex composition and the dynamic nature of ECM in native tissues make it difficult to mimic exactly (Chan and Leong 2008).

Scaffolds, typically made of polymeric biomaterials, provide the structural support for cell attachment and subsequent tissue development. As pointed out previously, in the horse, tendon injuries are mostly located in the SDFT, which represent the strongest tendon in the equine body. The SDFT displays several similarities to the human Achilles tendon concerning anatomy, biomechanics, and pathogenesis of tendinopathy. Yet, in most cases, pathomorphology of tendinopathy differs in lesion size. In the horse, one typical so-called “core lesion” is usually centrally located within the tendon, extended in length and still surrounded by intact tendon tissue. The equine SDFT injury lends itself to cell therapy because it provides many of the additional elements required for tendon tissue engineering. The lesion manifests within the central core of the tissue provides a natural enclosure for implantation that, at the time of stem cell implantation, is filled of granulation tissue which acts as a scaffold (Alves et al. 2011). This enables the application of MSCs without any artificial scaffold material, merely by injecting a cell suspension directly into the lesion; thereby the MSCs are exposed to a natural environment providing collagen fibers and growth factors (Fig. 4.6).

Table 4.3 Some growth factors involved during tendon repair

Growth factor	Activity	Repair phase
PDGF	<ul style="list-style-type: none"> • Induces expression and attraction of other growth factors (e.g., IGF-1) • Increases cell proliferation • Stimulates the synthesis of collagen and other ECM components 	Inflammatory
IGF-1	<ul style="list-style-type: none"> • Stimulates recruitment of fibroblasts and inflammatory cells to the injury site • Cell proliferation (DNA synthesis) • ECM remodeling 	Inflammatory Proliferative Remodeling
TGF- β	<ul style="list-style-type: none"> • Regulation of cell migration • Cell proliferation (DNA synthesis) • Stimulates cell–matrix interactions • Collagen type III synthesis 	Inflammatory Proliferative

9.3 Growth Factors

Growth factors are peptide signaling molecules that regulate many aspects of cellular metabolism including the cell cycle, cell growth, and differentiation, and the production and destruction of ECM products. Their effects are mediated primarily via autocrine and paracrine mechanisms, which provide the rationale for local administration of exogenous growth factors to influence cellular metabolism (Schnabel et al. 2009). Of the growth factors influencing tendon metabolism, platelet-derived growth factor (PDGF), insulin-like growth factor-I (IGF-I), and transforming growth factor β (TGF- β) show the most promise for enhancing tendon healing (Dahlgren et al. 2002). In Table 4.3 are summarized some effects of these factors involved during tendon repair.

PDGF induces the expression of other growth factors such as IGF-1 during the initial repair phase. In addition, the delivery of PDGF to tendon injuries in animal models increases cell proliferation and stimulates the synthesis of collagen and other ECM components in a dose-dependent manner during the remodeling phase (Wang et al. 2004; Thomopoulos et al. 2005). During the initial repair process and the inflammatory phase, upregulation of growth factors and cytokines such as IGF-1 stimulates the migration and proliferation of fibroblasts and inflammatory cells to the wound site (Kurtz et al. 1999; Tsuzaki et al. 2000). IGF-1 may be stored as an inactive precursor protein in normal tendon and, upon injury, enzymes release the growth factor to exert its biological activity. During the later phases such as remodeling, IGF-1 stimulates synthesis of collagen and other ECM components; studies in vitro have shown that the effects of IGF-1 on matrix metabolism are dose dependent (Abrahamsson et al. 1991; Abrahamsson and Lohmander 1996). Investigations with equine flexor tendon injury models have shown that both cell proliferation and collagen content increase on treatment with IGF-1. These changes are accompanied by increased stiffness in the treated tendon (Dahlgren et al. 2002).

Transforming growth factor- β (TGF- β) is a product of most cells that are involved in the healing process; During the initial inflammatory phase after trauma, TGF- β expression is elevated and stimulates cellular migration and proliferation, as well as interactions within the repair zone (Kashiwagi et al. 2004). Synthesis of collagen type I and collagen type III is increased greatly during the later phases. One of the isoforms of the growth factor, TGF- β 1, is responsible for the initial scar tissue that forms to establish tissue continuity at the wound site. In the later phases of wound healing, increased expression of TGF- β 1 leads to scar proliferation and reduced functionality. Transforming growth factor- β 3 acts as a negative regulator of scarring at the wound site (Klein et al. 2002; Campbell et al. 2004). TGF- β 1 also serves to regulate the synthesis of collagen in tendon mechanically during physical exercise (Heinemeier et al. 2003).

Although exogenous IGF-I has been shown to stimulate tendon healing in vivo in an equine model (Dahlgren et al. 2002) it has a short half-life, which necessitates repeated dosing, making clinical application challenging and costly. For this reason Schnabel et al. (2009) examined the effects of MSCs, as well as IGF-I gene-enhanced MSCs (AdIGF-MSC) on tendon healing in vivo finding that both MSC and AdIGF-MSC injection resulted in significant histological tendon healing and a trend towards improved biomechanical characteristics of healing tendon in the early period after injury, with minimal added value of IGF-I gene-enhanced MSC implantation compared to native MSCs injection. This minimal added value underlines the possibility that the MSCs can act mainly to supply immunomodulatory and trophic factors.

Current knowledge of the biological characteristics of MSCs also focus much attention on the role of paracrine factors released from these cells, on the mechanisms of their regenerative/repairative potential and on their ability to control the inflammatory process.

To test the hypothesis that the beneficial effects observed with transplantation of AMCs to promote horse tendon repair (Lange-Consiglio et al. 2012, 2013b) are mediated via AMC-secreted paracrine-acting molecules and by their immunomodulatory properties, we evaluated the immunomodulatory capacity of horse amniotic membrane-derived cells and of the conditioned medium derived from the culture of these cells (AMCs-CM), and we investigated the effects of injecting AMCs-CM in spontaneous horse tendon and ligament injuries in vivo (Lange-Consiglio et al. 2013c Stem Cell Development accepted in press).

To our knowledge for the first time in veterinary medicine, we have demonstrated that horse AMCs are capable of inhibiting peripheral blood mononuclear cells (PBMC) proliferation not only when cultured in cell-cell contact with responder cells, but also when separated from them by a transwell membrane. The inhibitory effects were more pronounced when increasing numbers of AMCs were added to the cultures, suggesting a dose-dependent effect. The finding that inhibition of T-cell proliferation was induced by AMCs in the transwell system suggests that soluble factors are implicated in this phenomenon. This hypothesis was further supported by our findings that PBMC proliferation was inhibited by the conditioned medium derived from AMCs (AMCs-CM). Our results are similar to findings that

have been reported using human term placenta, which clearly demonstrate that cells isolated from the mesenchymal portion of human amniotic membrane, as well as CM derived from the culture of these cells, could both inhibit lymphocyte proliferation (Wolbank et al. 2007; Magatti et al. 2008; Rossi et al. 2012).

Even though it is generally accepted that MSC from amniotic membrane or other sources act in a paracrine fashion, mechanisms underlying the immunosuppressive actions of these cells remain to be fully elucidated (for review see: Nauta and Fibbe 2007; Herrero and Pérez-Simón 2010; Engela et al. 2012). The identity of these soluble factors remains unknown, even though TGF- β 1, hepatocyte growth factor, prostaglandin E2, interleukin-10, heme oxygenase-1, interleukin-6, and human leukocyte antigen-G5 are all known to be constitutively produced by MSC (Aggarwal and Pittenger 2005; Chabannes et al. 2007; Selmani et al. 2008). Recently Rossi et al. (2012) suggested prostaglandins as one of the key effector molecules of the antiproliferative activity of human amniotic membrane-derived cells.

An important role for soluble factors in stem/progenitor cell-mediated reparative effects has been supported by animal experiments using CM as an effective treatment for different tissue injuries. Indeed, in a porcine model of myocardial ischemia and reperfusion, intravenous and intracoronary injection of CM obtained from human MSC cultures reduced infarct size and improved cardiac performance (Timmers et al. 2007). Furthermore, systemic infusion of human MSC-CM has been shown to reduce apoptosis and stimulate proliferation of hepatocytes in a rat model of acute liver injury (van Poll et al. 2008). In support of a key role for soluble factors in the suppressive action of human amniotic membrane-derived cells (Cargnoni et al. 2009, 2012), used a mouse model of lung fibrosis to show that a reduction in severity and progression of the disease could be obtained both after transplantation of amniotic membrane-derived cells and also after the injection of their conditioned medium, thereby underlining the anti-inflammatory effect of these media.

All of the important results described above were obtained using CM in induced disease, while in our study, for the first time, CM derived from cultured horse amniotic membrane-derived cells was used to treat spontaneous horse tendon and ligament injuries (unpublished data). We treated 13 sports horses with different tendon or ligament injuries that were treated with 2 mL of AMCs-CM ultrasonographically injected in the lesions and followed up for 2 years. Clinical and ultrasonographical evaluation of the involved tendon or ligament did not reveal evidence of inappropriate tissue or tumor formation, independently of the type and severity of diseases. In the absence of any perioperative collateral treatment, it was possible to attest that no local or focal reaction has occurred. These results are suggestive of a correlated anti-inflammatory effect of AMCs-CM. Indeed, besides a demonstrated lack of worsening of the lesion straight after treatment, a marked reduction in swelling and CSA in the very early postoperative period was also noticed. Moreover, interestingly, using power doppler US, neo-vessels were imaged within the treated area as soon as 1 month after AMCs-CM injection. Vessel size and quantity decreased and eventually disappeared by approximately 2 months after treatment, while improvement in echogenicity and fiber architecture were also observed.

This is clearly correlated to the positive tissue healing process, probably induced by angio-acting cytokines, and must be considered as an important timing predictor in the rehabilitation program. Clinical outcomes after AMCs-CM injection can be considered favorable after 2 years of follow-up where the rate of re-injuries was 9 %. Considering that increasing evidence has recently highlighted the ability of MSC isolated from various sources to produce bioactive molecules, which are potentially able to exert several types of paracrine effects (e.g., anti-scarring, anti-inflammatory, antiapoptotic) on target cells (Meirelles et al. 2009), our observations lead us to hypothesize that soluble factors released by AMCs may interact with and stimulate tendon-resident cells to initiate an anti-inflammatory and angiogenic response that leads to a regenerative/repairative response. In particular, factors derived from AMCs-CM may counteract the action of inflammatory cells accumulated at the site of injury, and may also exert trophic effects, as documented by Tang et al. (2004) in ischemic myocardium and in bleomycin-induced lung fibrosis (Cargnoni et al. 2012). These results are consistent and overlapping (in terms of time interval needed to reach complete healing) with the beneficial effects of transplanted horse amniotic membrane-derived cells that we have previously observed in the same animal model (Lange-Consiglio et al. 2012, 2013b), and therefore suggest that cell treatment could be substituted by the use of CM derived from the culture of these cells.

Our results may open the way to consideration of cell-free treatment approaches for tendon and ligament diseases, offering a series of added advantages with respect to other current treatments. In particular, AMCs-CM can be produced easily and in large quantities; it can be stored efficiently because it maintains its efficacy after the lyophilization process; as a cell-free treatment, it can drastically reduce the risk of adverse immunological reactions, infectious risks, and other potential long-term negative effects caused by the presence of exogenous cells; finally, it is also conceivable that AMCs-CM could be administered safely via intravenous injection, avoiding clot formation and lung capillary entrapment (Cargnoni et al. 2009). Even though this study identifies AMCs-CM as a novel therapeutic biological cell-free product in spontaneous tendon and ligament diseases, elucidation of the nature and characteristics of the paracrine soluble molecules underlying the efficacy of AMCs-CM therapy, and an understanding of the mechanisms responsible for the beneficial effects of the paracrine actions performed by stem cells in general, remain ongoing challenges for all researchers in the field.

10 Debate

Although our understanding of tendon biology and the biological processes that regulate repair have progressed tremendously, many challenges need to be addressed to bring about a successful treatment strategy. Recently, amniotic-derived stromal cells have been shown to differentiate into multiple lineages under appropriate chemical cues and to heal tendon diseases. In addition, growth factors derived from these cells (amniotic cells) can improve the healing of tendon injured lowering the

recurrence rate. Treatment options involving growth factors, alone or in combination with a stromal population, may form functional repair or regenerate tendon faster than existing treatment modalities.

In any case, irrespective of the type of stem cell transplanted or of injected CM, there was no evidence of any significant adverse effects after MSCs treatment in the horses studied in different trials. Evaluation of tendons clinically, ultrasonographically, scintigraphically, and histologically showed no evidence of inappropriate tissue or tumor formation and clinical outcomes after MSCs treatment were favorable. Whereas high re-injury rates of 23 % up to 80 % following conventional treatment regimes have been reported (Dowling et al. 2000; Dyson 2004), re-injury rates following MSCs treatment are decreased. After a period variable between 48 weeks and 3 years of adult stem cell transplantation, the average of re-injured varied between 13 and 36 % (Smith 2008; Leppänen et al. 2009; Burk and Brehm 2011; Godwin et al. 2012), while with amniotic-derived cells, varied between 0 % at the beginning of the study on three treated cases (Lange-Consiglio et al. 2012) and 4.0 % on 48 cases treated after a follow-up of 2 years (Lange-Consiglio et al. 2013b) and increased to 9 % with conditioned medium, but in this study the clinical record was very limited.

Even in spite of the best results obtained with the AMSCs, it is very difficult to demonstrate improved healing in treated animals and the comparison between different sources of cells because no control animals, generally, are included. Difficulties to determine a control population are correlated to the treatments given in the equine industry. Moreover, the interpretation of the results can be misled by the variety of athletic sports in which the horses were involved, the nature of injury, the severity of the initial injury, and the age of the horse. In addition, the statistical analysis is difficult to standardize considering the treatment of different equine tendons that are highly variable. All these factors are influencing the prognosis. The optimal time of treatment and the optimal dose and route of administration of stem cells have not been determined yet for any stem cell type, lesion, or animal species. Many authors agree with Richardson (2007) suggesting that the optimal timing of cell implantation is after the initial inflammatory phase but before fibrous-tissue formation. It has been hypothesized that the presence of mature fibrous tissue within the tendon would make implantation more difficult and reduce the benefits of the stem cell therapy because of its persistence. Other issues are debated and concern the amount of stem cells needed to stimulate regeneration or the period of follow-up to allow a direct comparison. To date, there are no dose response-based studies: in literature the range varies between 1 and 50×10^6 cells depending on the extent of the lesion while the follow-up ranges from 1 month to 3 years.

Further research studies will be required to answer these questions.

It is hoped that experience gained from treating naturally occurring tendon injury in horses will provide sufficient supportive data to encourage the translation of this technology into the human field where large randomized controlled trials will lead to a higher level of clinical evidence (Godwin et al. 2012).

11 Conclusion

Exercise-induced injury to energy-storing tendons of horses and people occurs at a similarly high frequency, with failure of tissue regeneration contributing to high re-injury rates or retirement from athletic activity. There is now increasing research focus on the role of the tenocyte in accumulation of the matrix and cellular micro-damage that predisposes to tendon rupture. Access to equine tissues and the ability to make measurements *in vivo* have already significantly improved our knowledge of the composition, structure, and mechanical environment of injury-prone and non-injury-prone digital tendons over a wide age range. The imperative now is to develop tractable cell culture models using this species in order to study stress-induced damage and dysfunction; these models will facilitate a more authentic replication of the *in vivo* microenvironment (Patterson-Kane et al. 2012).

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Part II
Stem Cells in “Non-conventional” Species

Chapter 5

Using Stem Cells to Study and Preserve Biodiversity in Endangered Big Cats

Rajneesh Verma and Paul John Verma

1 Introduction

One of every four animal species on the planet is threatened by extinction. Historically, strategies for preserving biodiversity have focused on saving habitat and, by default, species in these native environments (in situ) (Ben-Nun et al. 2011).

One support strategy is managing ‘assurance’ populations ex situ for hundreds of mammals, birds, reptiles, amphibians and fish. This preserves genetic integrity, allows basic research to be conducted and is a source of animals for reintroduction programmes. Ex situ species propagation approaches are complex and demand expertise and resources, including specialized animal space and facilities. Even then, achieving reproduction is challenging. Animals can and often do have preferences for sexual partners, and it is expensive and complicated to transfer wild animals between locations to make ideal genetic matches to retain maximal heterozygosity. As a result, assisted reproductive technologies (ART) have been explored for helping manage ex situ wildlife populations.

Much progress has been made during the last decade in the development of assisted reproductive techniques, not only in the economically driven fields of human infertility and domestic animal breeding, but also in the area of animal conservation. Endangered felids are often difficult to breed either in captivity or even

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under natural conditions. One of the most important reasons for infertility or sub-fertility in this group is decreased genetic diversity caused by inbreeding, due to genetic bottlenecks as a consequence of geographical isolation and population contraction. Because of this, there has been increasing interest in maintaining genetic diversity for the conservation of wild felids and preservation of valuable cat breeds. ART comprising of techniques, such as artificial insemination (AI), intracytoplasmic sperm injection (ICSI), in vitro fertilization (IVF) and somatic cell nuclear transfer (SCNT), has been promoted over the past two decades as a potential means to conserve and manage threatened wildlife populations. But one of the major problems with the implementation of in situ and ex situ conservation programmes is the lack of availability of suitable biological material, which is required for a better understanding of reproductive patterns as well to maximize reproductive efficiency. This constraint arises from the strict procedures adopted for restraining or anaesthetizing free-living animals for collection of biological/reproductive samples due to inherent risks (Verma et al. 2012).

2 SCNT, an Alternative to ART

SCNT also called cloning represents an alternative for the production of animals genetically (chromosomally) identical to the donor somatic cell, albeit with mitochondria being contributed by the recipient oocyte, and offers the possibility of preventing the extinction of wild species. However, owing to the limited availability of oocytes from wild animals, the cloning of endangered species requires the use of donor oocytes from a related domestic species. Interspecies SCNT consists of the reconstruction of a cloned embryo by use of a donor somatic cell and a recipient oocyte from a different species, but from the same genus; whereas inter-generic SCNT consists of the reconstruction of a cloned embryo in which the donor nucleus and recipient cytoplasm differ both in species and in genus, with oocyte-specific mitochondria in both cases. Several studies have demonstrated that it is possible to produce embryos from endangered species by interspecies or inter-generic SCNT (Gomez et al. 2008) (Fig. 5.1); however, few live cloned wild mammals have resulted and these animals were derived from embryos reconstructed with donor oocytes of the same genus. Viable offspring from inter-generic SCNT have not been produced in any mammalian species, although pregnancies have been established with inter-generic cloned embryos after transfer into sheep or domestic cat recipient oocytes (Gomez et al. 2000). The successful development of interspecies and inter-generic cloned embryos is dependent on a variety of factors, which are similar to those reported for intra-species SCNT including source of oocyte cytoplasm, cell cycle synchronization, mitochondrial compatibility and genotype of the donor cells. Moreover, increasing evidence shows that aberrant epigenetic alterations that arise during SCNT may be associated with perinatal and neonatal losses and the production of abnormal offspring (Wu et al. 2010).

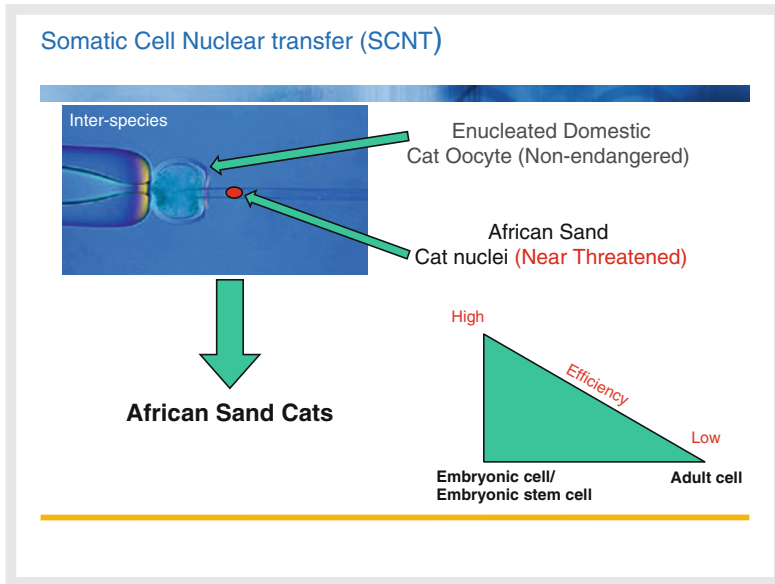


Fig. 5.1 Inter-species cloning and efficiency parameter

3 Pluripotent Stem Cells

Pluripotent stem cells differentiate into all the cell types in the body including gametes *in vivo*, while retaining the capacity for indefinite self-renewal *in vitro*. The best known example of pluripotent cells are embryonic stem cells (ESC) and the potential application of these cells are that they (1) can contribute to embryos for, e.g. chimeras, (2) can be differentiated to gametes *in vitro* and (3) can be used as a donor cell for nuclear transfer (Fig. 5.2). ESC are typically derived from inner cell mass (ICM) of blastocysts, which are destroyed in the process, raising ethical and logistical concerns for the derivation of stem cell lines from endangered species. Therefore for species in which embryos are particularly difficult to obtain or those, which are endangered, this approach was not particularly useful and feasible. In addition, to date no true ESC have been reported for any species for other than rodents (Malaver-Ortega et al. 2012; Verma et al. 2012).

4 Induced Pluripotent Stem Cells

Despite this reality check on what is possible today, it is essential to consider what might be promising for future intensive management of endangered species, especially as embryo and molecular technologies advance. So it is imperative to

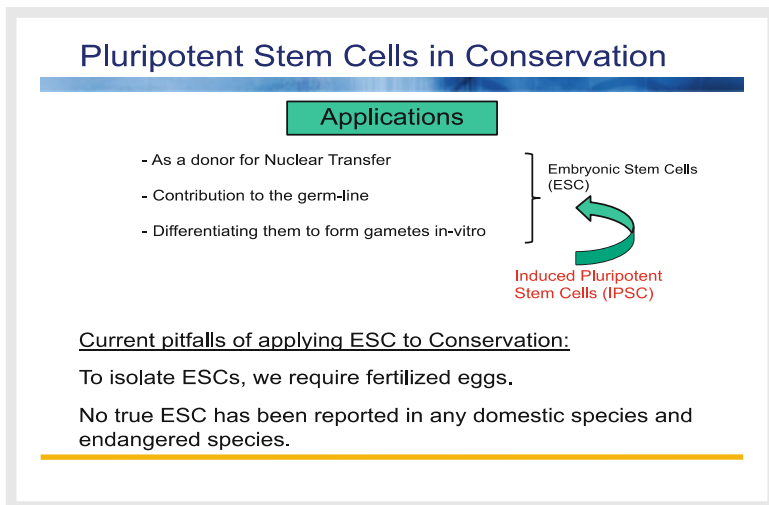


Fig. 5.2 Pluripotent stem cells and its applications in conservation

consider the potential benefits offered by induced pluripotent stem cells (iPSC) generated by reprogramming adult or differentiated somatic cells to a pluripotent stem cell-like state using defined transcription factors.

In a seminal study, Japanese scientists (Takahashi and Yamanaka 2006) used viral transduction of mouse fibroblasts to screen a combination of 24 candidate genes with putative roles in pluripotency and remarkably found that four previously known transcription factors (Oct3/4, Sox2, Klf4 and cMyc) could reprogram mouse embryonic fibroblasts (MEFs) and tail tip fibroblasts into ES-like cells, which were almost indistinguishable from mouse ESC in terms of pluripotency and coined the term iPSC.

iPSC have now been isolated from rodents (mouse and rats) (Takahashi et al. 2007; Honda et al. 2010), primates (human and monkeys) (Liu et al. 2008; Park et al. 2008), livestock (pigs, sheep, horse and cattle) (Ezashi et al. 2009; Nagy et al. 2011; Sumer et al. 2011; Khodadadi et al. 2012; Liu et al. 2012) and endangered species (Ben-Nun et al. 2011; Verma et al. 2012). The use of iPSC technology to provide a source of pluripotent cells for use in felid conservation was considered to be likely a more successful approach than isolating ESC from endangered felids embryos because it is a non-invasive technique, which only requires somatic cells. In mice, iPSC are similar to ESC and can form chimeric embryos when injected into blastocysts. This opened a new chapter, offering the possibility to convert cells from skin to ESC regardless of age and gender of donor and to use them for ART in various forms.

5 Snow Leopard

The snow leopard (*Uncia uncia*) is a moderately large cat native to the mountain ranges of Central Asia. They live between 3,000 and 5,500 m (9,800 and 18,000 ft) above sea level in the rocky mountain ranges of Central Asia. However, their secretive nature means that their exact numbers are unknown, although it has been estimated that between 3,500 and 7,000 snow leopards exist in the wild and between 600 and 700 in zoos worldwide (Kleihman and Garman 1978).

6 First Snow Leopard iPSC

Before attempting to produce iPSC from snow leopard, we came across a number of unknowns as listed below (Fig. 5.3).

Later, we reported the successful derivation of iPSC from ear fibroblasts of the snow leopard transfected with five human factors *OCT4*, *SOX2*, *KLF4*, *cMYC* and *NANOG*. We believe the high transduction efficiency (96 %) for this study, measured using a pMx-GFP construct, was important in achieving a successful outcome (Fig. 5.4).

Of particular importance was the observation that the three key exogenous pluripotency transgenes (*OCT4*, *SOX2*, *NANOG*) were silenced at later passages. Interestingly, we observed a similar requirement of transcription factors for induction of pluripotency and an identical pattern of subsequent silencing of transgenes for three globally diverse endangered felids in a subsequent study (Verma et al. 2013). During the course of our study, we observed that snow leopard iPSC at P14, when tagged with mCherry reporter and injected into the peri-vitelline space of mouse morulae, did not compromise development of the mouse embryos. We next examined whether these iPSC were able to contribute to developing mouse embryos by incorporation into the ICM (Fig. 5.5). We propose this as a novel *in vivo* assay to assess the embryo contribution ability of iPSC and pluripotent cells from exotic

LIMITATION 1: Choice of Factors; Mouse /human,
NOTE: Wild Cat Sequence not known

LIMITATION 2: Cell Type, no access to any other
cell type except ear cells

LIMITATION 3: Derivation Conditions; medium
and growth factors

LIMITATION 4: No specific stem cell markers of wild cats
for in-vitro analysis. No knowledge on differentiation

Fig. 5.3 Limitations for producing snow leopard iPSC

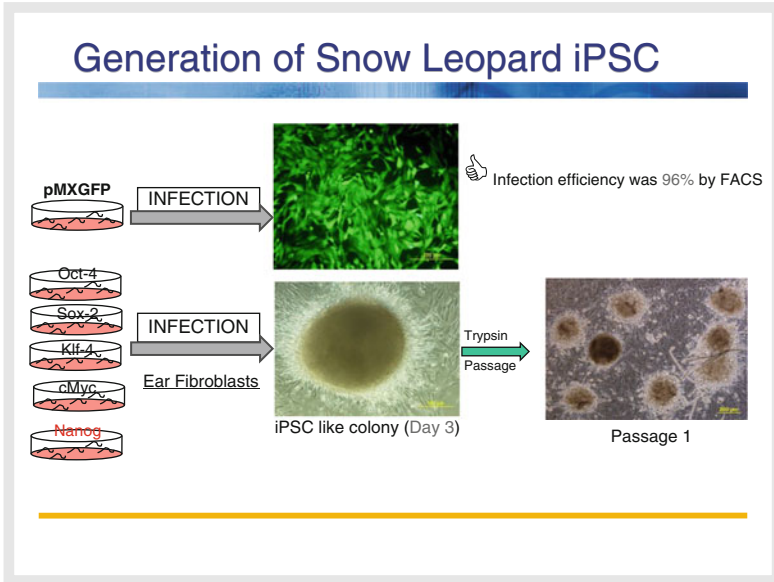


Fig. 5.4 Experimental diagram to show the procedure for producing snow leopard iPSC

Snow leopard iPSC aggregation in mouse embryos at 4-cell stage

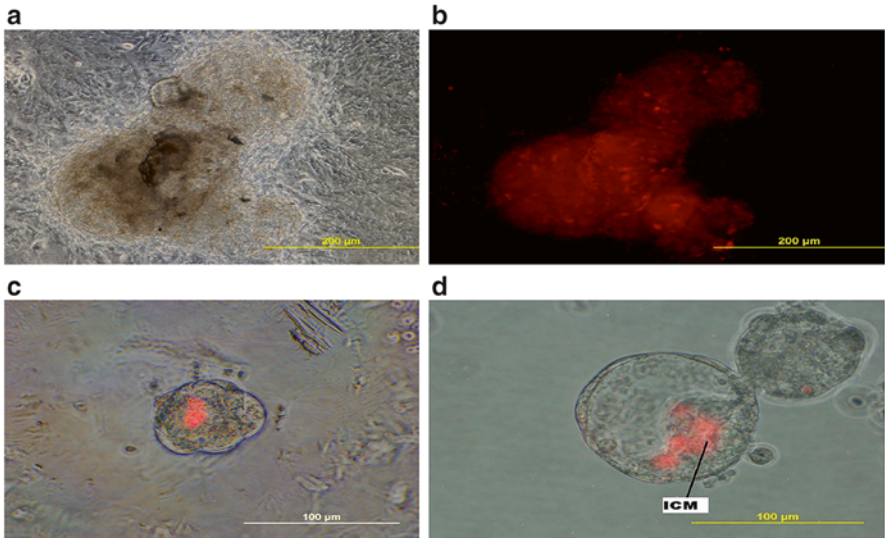


Fig. 5.5 Potential in vivo assay for assessing snow leopard iPSC. Snow leopard iPSC tagged with mCherry reporter construct and aggregated with mouse embryos at the 4-cell stage (a) Snow leopard iPSC colony (Bright field), (b) Snow leopard iPSC colony expressing mCherry reporter, (c) Snow leopard iPSC localized into a morula and (d) Snow leopard iPSC seen to appear or localize in the ICM of hatching mouse blastocyst

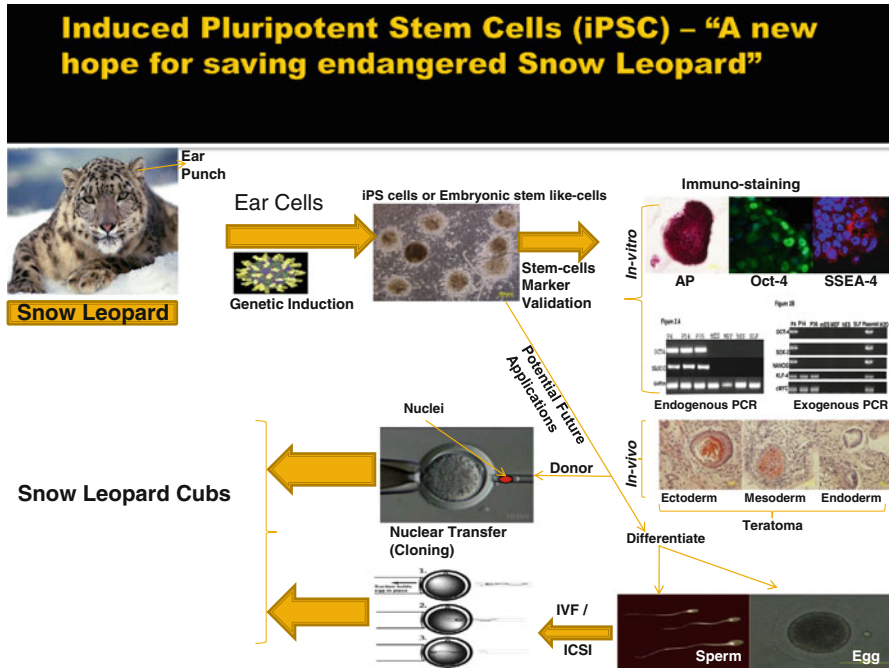


Fig. 5.6 Experimental layout of generating snow leopard iPSC and future applications

species where species-specific testing is impossible because of the often extremely limited access to gametes, especially oocytes and embryos.

In conclusion, we believe this is the first report on the derivation of iPSC from both a felid and an endangered species. This is also the first report on the induction of pluripotency in a large animal with concomitant silencing of the pluripotency associated transgenes. The iPSC technology has the potential to impact on conservation of endangered species at a number of levels. It can provide insights into pluripotency and development in species where embryos are difficult to access. Furthermore, iPSC generated from the endangered species can be easily expanded for banking of genetic material or used as a reprogrammed donor cell to improve NT outcomes. They may also create opportunities to prevent extinction in a wide range of threatened animals in the future. For example, it may eventually be possible to differentiate cell lines with proven pluripotency in vitro to produce gametes or use these cell lines in vivo in conjunction with tetraploid complementation to produce whole animals (Fig. 5.6). This report has relevance to understanding pluripotency in big cats and also has application in domestic cats, which are companion animals and are unique biomedical models to study genetic diseases (e.g. HIV, arthritis and diabetes; (Verma et al. 2013)).

7 Future of iPSC in Endangered Species

If the many challenges associated with advanced embryo culture, finding the appropriate surrogate mother and synchronizing the embryo to the uterus can be overcome, then pluripotent cells may well improve application of SCNT for producing viable offspring (Holt et al. 2004).

Generating sperm from iPSC derived from frozen somatic cell samples from long-dead animals would provide a way to infuse much needed genetic diversity using already proven AI methods. An analogous approach using pluripotent cell-derived oocytes could provide an endless resource for fundamental investigations into IVF, ICSI and SCNT (Holt et al. 2004).

There is also potential for these cells as a scalable resource of rare genetic material. Making iPSC for diverse species and populations available to every interested institution would accelerate research progress on analysing phylo-geographic structure, delineating subspecies, tracing paternities, evaluating gene flow and assessing genetic variation—information critical for decision-making in managing both ex situ and in situ wildlife populations (Pope 2000).

Therefore, wildlife-related studies will need to focus on fidelity measures for the reprogramming process to ensure the production of legitimate and ‘fit’ iPSC. As these are rare species and some methods for generating the cells rely on viral vectors, the process also has to be proven indisputably safe for offspring (Verma et al. 2013).

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Chapter 6

Pluripotent and Multipotent Domestic Cat Stem Cells: Current Knowledge and Future Prospects

Martha C. Gómez and C. Earle Pope

1 Introduction

The domestic cat (*Felis catus*) is a mammalian species of particular importance due to their evolutionary history, role as companion animals and as a research model. According to The Humane Society, in the United States 86.4 million cats are kept as companion animals. Veterinary care can be a major expense for cat owners and development of new health-related technologies has become a priority for veterinarians. Recently, stem-cell-based therapies have been developed to improve health and well-being in domestic animals (Ribitsch et al. 2010; Fortier and Travis 2011). Hundreds of cats, including a Bengal tiger and a Florida panther, have received “adult stem cells” for tissue replacement therapy. Although most of the stem-cell-based therapies have been applied by veterinarians without the support of research studies demonstrating their efficacy or safety (Cyranoski 2013), a recent pilot study in which adult stem cells were injected into the kidney, as a treatment for chronic kidney disease (CKD), induced a mild improvement in renal function of domestic cats (Quimby et al. 2011). Thus, transplantation of adult stem cells is a promising tool for cell replacement therapy and regenerative medicine.

Also, stem-cell-based technologies have been proposed as an alternative approach for the conservation of genetically valuable animals and preservation of endangered animals (Travis et al. 2009). The domestic cat has been demonstrated to be a valuable model for developing assisted reproductive technologies that can be applied to the conservation of endangered felids (Pope et al. 1993, 2006a, b, 2012). Although the feasibility of producing domestic cats and endangered felids by in vitro fertilization (Pope et al. 1993, 2006a, b, 2012) and somatic cell nuclear transfer (SCNT) (Gómez et al. 2004, 2008) has been demonstrated, practical applications depend on

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improving efficiency, particularly that of interspecies SCNT. Potential applications of using stem cells for enhancing reproduction in endangered felids range from the generation of stable characterized stem cells for use as donor nuclei to improve efficiency of interspecies SCNT (Jaenish et al. 2002), *in vitro* differentiation into germ cells (oocytes and spermatozoa; Nayernia et al. 2006; Kee et al. 2009; Yu et al. 2009b), and restoration of spermatogenesis with the haplotype of the endangered felid donor by spermatogonial stem cell transplantation (Silva et al. 2012).

Sequencing of the domestic cat genome revealed that 90 % of the 20,285 putative genes are homologous to human genes (Pontius et al. 2007). The cat provides the only naturally occurring model for human AIDS pathogenesis in its endemic fatal transmissible feline immunodeficiency virus (Siebelink et al. 1990; Willet et al. 1997). Moreover, feline Alzheimer's disease in aged animals was recognized as a feline disorder with pronounced amyloid and phosphorylated tau deposition (Gunn-Moore et al. 2006, 2007), indicative of an identical disease mechanism as seen in Alzheimer's disease in humans. Thus, the domestic cat as a model for certain human diseases may more closely resemble the human conditions than does the current mouse model for that disease. Interest in the generation of genetically modified domestic cat stem cells to produce genetically identical cats that carry genes for specific human disorders has gradually increased after recent reports on production of transgenic cats (Yin et al. 2008; Gómez et al. 2009; Wongsrikeao et al. 2011). Cat somatic cells exhibit limited proliferation, become aneuploid during *in vitro* culture (Gómez et al. 2006), and have an extremely low frequency of homologous recombination (Gómez et al. unpublished data). In contrast, pluripotent stem cells have shown the capability of long-term proliferation, which allows longer *in vitro* genetic manipulations and possibly increases the homologous recombination efficiency. The latter could represent an efficient method to produce genetically engineered cats as a model for human diseases.

Successful application of stem-cell-based therapies in cats is dependent on development of robust methods to efficiently isolate fully pluripotent stem cells and a better understanding of the mechanisms that control stem cell fate to differentiate these cells into specific phenotypes. In this chapter, we will describe recent progress on the isolation, culture, and characterization of pluripotent and multipotent stem cells and preliminary trials in regenerative medicine in domestic cats and prospects for applications to conservation and veterinary care of endangered felids.

2 Stem Cell Definition

Stem cells are characterized by their functional capacity to: (1) self-renew, either by symmetric cell division generating two stem cells, or by asymmetric cell division resulting in one stem cell and one other cell with restricted differentiation capacity, and (2) differentiate into other cell types (Weissman et al. 2001). The latter ability is known as totipotency; and can be further classified according to differentiation pattern. Stem cells are classified as either pluripotent or multipotent.

Pluripotent stem cells have the ability to proliferate extensively *in vitro* in a morphologically undifferentiated state while retaining a normal diploid karyotype during extended culture. Also, the cells are able to differentiate into all derivatives of the three embryonic layers (Murry and Keller 2008). In contrast, multipotent stem cells, isolated from tissues or organs (adult stem cells), are characterized by having limited proliferation and can only differentiate into specific cell types within the particular lineage from which they were originated.

Both types of stem cells have different potential applications for stem-cell-based therapies. Although pluripotent stem cells can differentiate into all cell types, direct injection into a host results in formation of tumors, limiting clinical use in their undifferentiated state. Thus, cell differentiation must be induced before transplantation. In contrast, multipotent stem cells are not tumorigenic after transplantation into the recipient; however, their limited ability to undergo differentiation reduces their potential applications. Regardless of the stem cell potency, both types of cells offer several advantages for their use in regenerative medicine in domestic cats and endangered felids.

3 Domestic Cat Pluripotent Embryonic Stem Cells

In the domestic cat, putative embryonic stem cells (cESC) isolated from the ICM of *in vivo*-derived and *in vitro*-produced blastocysts are the only pluripotent cells that have been generated (Yu et al. 2008; Gómez et al. 2010). In these studies, 70 % of plated embryos formed outgrowth colonies, while 50 % of the outgrowths developed into primary colonies, depending on the culture conditions (Table 6.1). Cat ESC colonies proliferated for several passages (6–12), but progressively lost their self-renewal capacity and spontaneously differentiated during *in vitro* culture. Continuous proliferation of ESC requires optimal *in vitro* culture conditions to maintain self-renewal and pluripotent characteristics. In this section, we consider the culture conditions that significantly influenced the derivation of cat ESC, the methods used for stem cell characterization and present data, acquired in the author's laboratory, which will help to reveal the intracellular signaling pathways that contribute to self-renewal in cat ESC.

3.1 Culture Conditions for Cat ESC

3.1.1 Feeder Cell Layers

Previous reports on culturing mouse and human ESC have shown that both the derivation and maintenance of ESC require the support of a feeder cell layer (Evans and Kaufman 1981; Thomson et al. 1995, 1998). Mouse feeder cell layers inhibit differentiation of human ESC by supplying growth factors and extracellular matrix

Table 6.1 Derivation of cat ESC from in vitro- and in vivo-derived blastocysts and cultured on cat (CEF) or mouse (MEF) feeder cells and medium supplemented with FBS or KOSR

Type of embryos	Type of feeder	Protein supplementation	ICM plated, <i>n</i>	ICM outgrowth, <i>n</i> (%)	Primary colony/total ICM outgrowth, <i>n</i> (%)	ESC/total ICM outgrowth (P1–P3), <i>n</i> (%)	ESC/total ICM outgrowth (P4–P12), <i>n</i> (%)
In vitro derived (Gómez et al. 2010)	CEF	FBS (15 %) + KOSR (5 %)	147	107 (73)	54 (50)	35 (65)	17 (31)
In vivo derived (Yu et al. 2008)	MEF	KOSR (20 %)	189	58 (31)	10 (17)	3 (30)	0 (0)
	CEF	FBS (20 %)	52	46 (88)	32 (61)	–	1 (3)
			49	32 (65)	18 (37)	–	2 (11)

proteins necessary to sustain self-renewal (Villa-Diaz et al. 2009). Although feeder-free systems have been developed for culturing human ESC (Xu et al. 2001; Amit et al. 2004), these systems are not completely optimized. Feeder-free systems are not a viable method for culturing pig and bovine stem cells; the cells failed to replicate and spontaneously differentiated (Talbot et al. 1995; Brevini et al. 2007, 2010). In the domestic cat, derivation or culture of ESC has not been tested in a feeder-free system. However, cat ESC have been successfully derived and cultured on homologous feeder layers of cat embryonic fibroblasts (CEF; Yu et al. 2008; Gómez et al. 2010), and a heterologous system of mouse embryonic fibroblasts (MEF; Gómez et al. 2010). We compared the effect of homologous vs. heterologous feeder cell layer on derivation of cat ESC. Derivation was influenced by the feeder cell type, where homologous feeder cells provided better cell support for the initial attachment of plated ICMs, generated higher numbers of cat ESC primary colonies and maintained more prolonged self-renewal in an undifferentiated state than did heterologous feeder cells (Gómez et al. 2010). These results demonstrated clearly the existence of a species-specific relationship between the feeder layer and the derivation of cat ESC. However, a recent study showed that MEF can efficiently maintain induced pluripotent stem cells (iPSC) generated from an endangered felid, the snow leopard, in an undifferentiated state that replicate for >36 passages (Verma et al. 2012). Despite the differences in species and type of pluripotent stem cells, these combined results confirm that pluripotent domestic cat and endangered felid cells can be cultured and maintained undifferentiated on homologous and heterologous feeder cells, but derivation of cat ESC is improved in homologous feeder cells.

There is evidence that the ability of different types of human feeder cells to support the undifferentiated growth of human ESC varies (Richards et al. 2003). Therefore, it is possible that the limited capacity of MEF to derive robust cat ESC may be due in part to the inability of that specific primary cell culture to provide the appropriate cell-surface and soluble factors required to maintain self-renewal and proliferation (Villa-Diaz et al. 2009). Currently, we use a CEF cell culture as a feeder cell layer which has been tested to consistently support the derivation of new cat ESC. Nonetheless, this cell culture does not maintain cat ESC undifferentiated for prolonged periods during *in vitro* culture. The detection of cat feeder cells that express some of the signal pathway genes required to support cat ESC self-renewal in an undifferentiated state will facilitate the maintenance of robust cat pluripotent cells *in vitro*.

3.1.2 Protein Supplementation

In addition to feeder cells, the presence of serum in the culture medium is an important factor for maintaining cat ESC replication. Higher numbers of cells per colony (972 ± 89) at day 5 after plating were observed when ICMs were cultured in medium supplemented with 20 % FBS in comparison to that of ICMs cultured in medium supplemented with 20 % knockout serum replacement (KOSR; 476 ± 65). Moreover, only cat ESC cultured in 20 % FBS continued replication after passage four

(Yu et al. 2008). Similarly, we have observed that cell growth rate is significantly reduced when cat fibroblast cells are cultured in a defined commercial medium containing human albumin, instead of FBS (Gómez et al. unpublished data). Although it is not possible to make a direct comparison between fibroblasts and ESC, due to differences in cell type and cell cycle pathway, these results corroborate that factors in FBS positively influence cell growth in cat cells. It is not clear why supplementing the culture medium of cat ESC with KOSR did not maintain prolonged self-renewal in an undifferentiated state as did FBS, but it appears that differences in lipid content between the two supplements may be an important influence. In one study with human ESC, it was found that lipid-rich bovine serum albumin was the active ingredient in KOSR and that lipids, not albumin apoprotein, were responsible for stimulating the self-renewal effect (Garcia-Gonzalo and Izpisua 2008). In a later study, it was found that retinol, conjugated linoleic acid, and eicosapentaenoic acid were some of the lipids present in lipid-rich bovine serum albumin that were actively involved in self-renewal of human ESC; however, the authors clearly specified that the optimal response of cells to lipid-rich serum albumin may be due to synergism between the different lipids (Rajala et al. 2011). In contrast, relatively little is known about the complete lipid content of FBS and about the impact of lipids on cell growth, but it is generally accepted that the composition of FBS may directly influence the outcome of cell culture. FBS supported spontaneous differentiation of human ESC into beating cardiomyocytes during in vitro culture (Bettiol et al. 2007). Similar effects of prolonged supplementation of human ESC with human serum have been reported. In fact, differentiation rates of human ESC were significantly increased when cells were cultured with human serum for more than ten passages, possibly due to the presence of molecules that may accelerate cell differentiation (Richards et al. 2003). Similarly, we observed that cat ESC cultured in medium supplemented with 15 % FBS and 5 % KOSR can spontaneously differentiate into beating cardiomyocytes and differentiation is significantly increased after passage six (Gómez et al. 2010). Additional studies are required to determine which active lipids and additional factors have a positive effect on self-renewal of cat ESC to develop a supplement that could be used as a replacement for FBS.

3.1.3 Cell Dissociation

The method of dissociating cat ESC colonies for further passages is another factor that affects their stability. Enzymes such as trypsin are used to dissociate mouse ESC into single-cell suspension during routine passage without evident negative effects on cell survival and their pluripotency. In contrast, monkey, pig, and human ESC died after enzymatic single-cell dissociation (Thomson and Marshall 1998; Brevini et al. 2007; Xu et al. 2010). Cat ESC differentiate within one to two passages after dissociation with trypsin-EDTA (Yu et al. 2008). In a recent human ESC study, it was demonstrated that cell death and low plating efficiency after enzymatic dissociation was caused by downregulation of the E-cadherin signaling pathway, as

a consequence of the disruption of cell–cell interaction (Xu et al. 2010). E-cadherin is a molecule, highly expressed in human ESCs, that is involved in maintaining cell–cell adhesion and cortical actin cytoskeletal arrangement (Eastham et al. 2007); thus, loss of its activity is associated with cell death. Xu et al. (2010) also demonstrated, that mouse ESC are more tolerant to single-cell dissociation due to their capacity to synthesize new E-cadherin after enzymatic dissociation. The authors suggest that distinct mechanisms are used by cells of each species to stabilize E-cadherin and this may be influenced by their specific culture conditions. Although little is known about the mechanism of self-renewal in cat ESC, it is possible that they are using a similar E-cadherin signaling pathway as human ESC; thus, susceptibility to enzymatic dissociation may be due to the loss of cell–cell contact.

Rho-associated coiled-coil forming protein, serine/threonine kinase (ROCK), is a downstream effector of the Rho signaling involved in cytoskeleton remodeling of actin stress fibers and focal adhesion (Leung et al. 1996) and Rho-ROCK signaling pathway is stabilized by E-cadherin (Xu et al. 2010). When human ESCs were dissociated with trypsin, the activity of Rho-ROCK signaling was up-regulated, and this up-regulation induced cell hypercontraction and consequently, lower plating efficiency. When ESC were treated with a ROCK inhibitor, the stress on the cell fibers disappeared and focal adhesion was observed with an increase in cell plating, and the levels of E-cadherin were significantly increased. These results not only demonstrated that Rho-ROCK signaling is involved in stabilization of E-cadherin, but also confirmed that inhibition of the signaling pathway, when levels of E-cadherin are low, is beneficial to increase cell–cell adhesion and survival rates. ROCK inhibition also attenuates induced apoptosis in human ESC and significantly reduces cell death after single-cell dissociation without affecting their pluripotent ability (Watanabe et al. 2007). Accordingly, based on these results, and the fact that cat ESC must be passaged as aggregates due to their sensitivity to clonal expansion, for further passages the author's currently pretreat cat ESC with ROCK inhibitor and mechanically disaggregate colonies by cutting them with an ultra-sharp splitting blade.

3.2 Characterization of Cat ESC

Several methods have been used to characterize the “stemness” and potency of pluripotent stem cells. Traditionally, the pluripotent characteristic of a stem cell is measured by their cell morphology, expression of pluripotent markers, self-renewal capacity, and in vitro differentiation potential into several cell types. However, pluripotent cells must meet additional criteria, with more rigorous assays, to establish true pluripotency. These additional criteria include the generation of teratomas that express cells from the three germinal cell layers, production of chimeras by injection of cells into a blastocyst and integration of the cells into the host embryo, and germline transmission. The majority of domestic mammalian species ESC are defined as putative or-like ESC because they do not fulfill all criteria to be defined as true pluripotent cells. Similarly, true pluripotency in cat ESC has not been accomplished to date.



Fig. 6.1 Morphology of cat ESC at 6 days after plating. Primary colonies can be clearly identified by marked borders, as uniformly dense cluster of cells that have a large nucleus: cytoplasm ratio, prominent nucleoli, and a distinct dome shape. A flat monolayer of TPD cells can be also visualized around the ESC colony. The TPD cells contain lipid droplets, and grow usually as a flat monolayer

3.2.1 Morphological Characteristics of Cat ESC

The cell morphology of cat ESC can be described as a uniformly dense cluster of cells that have a large nucleus:cytoplasm ratio, prominent nucleoli, and a distinct dome shape (Fig. 6.1). When a whole embryo is plated, a flat monolayer of trophectoderm (TPD) cells can be also visualized around the ESC colony. The TPD cells contain lipid droplets, and grow usually as a flat monolayer (Fig. 6.1). Morphology of cat ESC colonies can be heterogeneous. Most colonies, whether cultured with leukemia inhibitory factor (LIF), or a combination of LIF and basic fibroblast growth factor (bFGF), displayed a compact dome shape similar to that of mouse ESC (Yin et al. 2008; Gómez et al. 2010). However, large flattened colonies similar to those of human ESC were also seen in cat ESC colonies cultured in stem cell medium supplemented with 20 % FBS (Yu et al. 2008).

3.2.2 Expression of Pluripotent Markers

The pluripotent state of ESC is mainly regulated by a “core” of transcription factors, octamer-binding transcription factor Oct-4 (*OCT-4*), SRY-box 2 (*SOX-2*), and *NANOG*, which act in conjunction with other transcription factors to establish and maintain a robust pluripotent state (see review; Young 2011). Cat ESC have been further characterized by the expression of the core *OCT-4*, *SOX-2* and *NANOG* transcription factors and other markers involved in pluripotency. Alkaline phosphatase activity was observed with different degrees of intensity, depending on the undifferentiating state of the colony. Expression of cell-surface stage-specific embryonic antigens *SSEA-1*, *SSEA-3*, and *SSEA-4*, as well as the expression of *OCT-4* and *NANOG*, was also confirmed by immunocytochemical staining (Yu et al. 2008; Gómez et al. 2010). On the other hand, the expression of pluripotent associated markers *OCT-4*, *NANOG* (Yu et al. 2009a; Gómez et al. 2010), and *SOX-2* and the proto-oncogene *C-MYC* (Gómez et al. 2010) were detected at the mRNA level,

suggesting that cat ESC colonies express the “core” of transcription factors reported in other mammalian ESC. Interestingly, we found that up-regulation of *NANOG* without the expression of *OCT-4* is capable of maintaining cat ESC colonies in an undifferentiated state. Instead, at the onset of differentiation, *NANOG* is downregulated (Gómez et al. 2010). These results clearly demonstrated the significance of *NANOG* for maintaining pluripotency in cat ESC. Our observation was recently confirmed when *NANOG* was shown to be an essential factor for reprogramming and maintaining prolonged self-renewal of iPSC from endangered felids (Verma et al. 2013).

3.2.3 In Vitro Differentiation of Cat ESC

The ability of cat ESC to differentiate into derivatives of the three embryonic layers has been tested with the embryoid body (EB) formation assay. In vitro culture of EBs showed that cat ESC were able to differentiate, when cultured without LIF and without a feeder cell layer, into cells morphologically similar to neurons and epithelial cells (Yu et al. 2008). However, proper characterization of the three germ cell layers by expression of specific protein markers for each cell type was not reported. Also, cat ESC exhibited spontaneous differentiation into well-developed contractile functional cardiomyocytes when grown in a mass and in the presence of a feeder cell layer and growth factors which expressed alpha actinin (Yu et al. 2008) and desmin (Gómez et al, unpublished data) confirming mesoderm differentiation.

3.3 Signaling for Self-Renewal in Cat ESC

Developmental signaling pathways control the self-renewal and differentiation of stem cells. As mentioned in the previous section, feeder cell layers play an important role in derivation and proliferation of cat ESC. However, the undefined nature of feeder cell layers posed a significant challenge in determining the signaling pathways involved in self-renewal in cat ESC. Nonetheless, there are additional factors other than feeder cell layers that selectively activate self-renewal signaling pathways. Several studies have clearly suggested that stem cells from different species use diverse self-renewal mechanisms. Mouse ESC self-renewal is regulated by the gp130/JAK/STAT pathway as a result of LIF-dependent activation (Niwa et al. 1998), as well as Bone morphogenic protein 4 signaling pathway (BMP4; Ying et al. 2003). In contrast, human ESC self-renewal is regulated through fibroblast growth factor (FGF), and transforming growth factor- β (TGF- β)/Activin/nodal pathways (Thomson et al. 1998; Laping et al. 2002). Cat ESC have been cultured on MEF feeder cell layers with cytokines and growth factors that were shown to be beneficial for mouse and human ESC. However, none of the factors were able to promote prolonged self-renewal (Yu et al. 2008; Gómez et al. 2010). The signaling pathway(s) for self-renewal of cat ESC have not been elucidated.

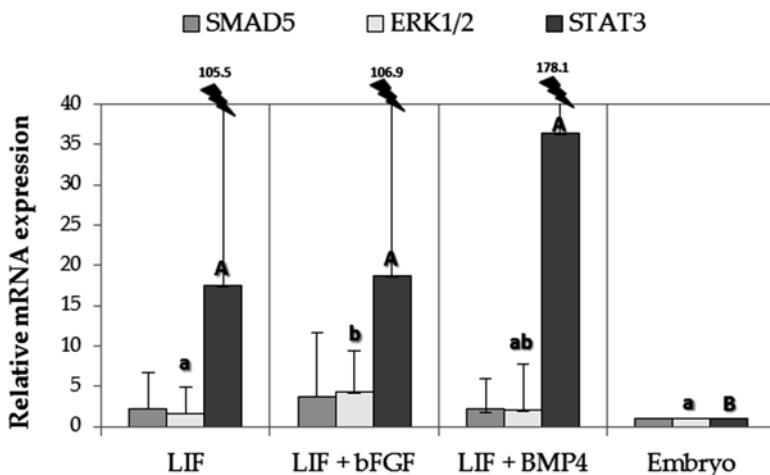


Fig. 6.2 Relative transcript abundance of SMAD5, ERK1/2, and STAT3 genes in in vitro produced cat blastocysts at day 8 and cat ESC derived on cat feeder cell layers and LIF, and cultured in: (1) LIF only, (2) a combination of LIF and bFGF, and (3) a combination of LIF and BMP4. mRNA transcript levels for each gene were determined by qRT-PCR and signals were normalized with their corresponding 18SrRNA signal. Relative mRNA expression was expressed as *n*-fold differences relative to embryos which were set at 1. Different lower and capital letters denoted samples that differ significantly in the relative expression of ERK 1/2 and STAT3 within treatments and embryos ($P < 0.05$). Jagged arrow values are greater than the ones shown in the graph

In the author's laboratory, preliminary experiments were performed to identify if downstream transcription factors of the intracellular signaling pathway that are involved in self-renewal of pluripotent mouse and human ESC are also involved in self-renewal of cat ESC. We first designed primers for transcription detection of signal transducer and activator of transcription 3 (STAT3), the extracellular regulated kinase 1/2 (ERK1/2), and the receptor-regulated SMAD 5 (SMAD5), molecules which are phosphorylated and activated by different signaling pathways. Primer sets were tested for efficiency and amplification by standard curve analysis and sequencing of PCR products in in vitro-produced cat blastocysts. qRT-PCR analysis showed the presence of the three transcripts (Fig. 6.2), suggesting that downstream transcription factors involved in self-renewal of mouse and human ESC are expressed in cat embryos.

Mouse ESC require the presence of LIF to maintain the cells in an undifferentiated state (Niwa et al. 1998), while human ESC do not (Thomson et al. 1998). In the mouse, the mechanism of self-renewal is dependent on LIF activation of STAT3 through the heterodimerization of gp130 and the LIF receptor (LIFR), and activation of the Janus-associated tyrosine kinase (JAK) pathway (Niwa et al. 1998). However, LIF also up-regulates ERK1/2 activity, which leads to the loss of pluripotency and the onset of differentiation (Burdon et al. 1999). Similarly, our study showed that LIF signaling resulted in significant up-regulation of STAT3 transcripts in cat ESC cultured in LIF or in a combination of LIF and bFGF (Fig. 6.2).

However, the transcript levels of ERK1/2 were not increased in cat ESC cultured with LIF only, but expression levels of ERK1/2 were greater when cat ESC were cultured in the combination of LIF and bFGF (Fig. 6.2). It is not clear why combined treatment of LIF and bFGF induced higher levels of ERK1/2 transcripts, but since both factors induced ERK1/2 activation through different pathways, it is possible that accumulation of ERK1/2 occurs and consequently higher transcript levels were present, as recently reported in rabbit ESC (Hsieh et al. 2011).

FGF signaling activates the mitogen-activated protein kinase (MEK)/ ERK1/2 and the PI3L/AKT pathways. Activation of these pathways plays a dissimilar role in ESC self-renewal of different mammalian species. For example, inhibition of the PI3L/AKT pathway in mouse ESC cultured in LIF only results in an increased expression of ERK1/2 and cell differentiation. However, inhibition of both the PI3L/AKT and MEK/ERK1/2 pathways results in self-renewal (Paling et al. 2004). In contrast, activation of both PI3L/AKT and MEK/ERK1/2 pathways in rabbit ESC cultured with a combination of LIF and bFGF results in robust self-renewal (Hsieh et al. 2011). In our study, although we did not measure the expression of AKT, we similarly observed that activation of ERK1/2 with a combination of LIF and bFGF results in prolonged self-renewal and enhanced cell proliferation (Gómez et al. 2010). The biological significance of this effect in rabbit ESC was reported to be improved colony morphology and stronger expression of some pluripotent markers. Nonetheless, similar effects in colony morphology were not observed in cat ESC and expression of pluripotent markers was not tested, although supplementation with higher doses of bFGF (20 ng/mL) promoted cell proliferation and prolonged self-renewal (Gómez et al. 2010). These combined results suggest that the JAK/STAT signaling pathway is activated by LIF, and that cat ESC use this pathway for self-renewal. The beneficial self-renewal effect observed when bFGF was added to the culture medium suggests that the FGF signaling pathway is also involved in self-renewal of cat ESC. However, the two pathways were not sufficient to maintain cat ESC in their pluripotent state.

Inhibition of the MEK/ERK pathway with BMP4 enhanced self-renewal of mouse ESC (Ying et al. 2003; Qi et al. 2004). Thus, we did an experiment to see if culturing cat ESC with a combination of BMP4 and LIF enhanced self-renewal. Our results showed that BMP4 did not have an inhibitory effect on ERK1/2. In fact, transcript levels of ERK1/2 in cat ESC cultured with both BMP4 and LIF were not downregulated, and the levels of expression were similar to that of cat ESC cultured in LIF only or a combination of LIF and bFGF (Fig. 6.2). Moreover, BMP4 induced cell differentiation of cat ESC, as previously reported in humans (Schuldiner et al. 2000). In human ESC, the BMP-signaling pathway activates SMAD1/5/8, which promotes trophectodermal differentiation (Lyssiotis et al. 2011). To determine if BMP4 induced cell differentiation in cat ESC through the BMP-signaling pathway, we measured expression levels of SMAD 5 in cat ESC cultured in both BMP4 and LIF. Interestingly, an increase in SMAD5 was not observed upon stimulation of the BMP-signaling pathway (Fig. 6.2). It is possible that paracrine molecules secreted by the CEF feeder cells were interacting indirectly with the ESC and reducing expression levels of SMAD5, but not enough to compensate for the differentiation

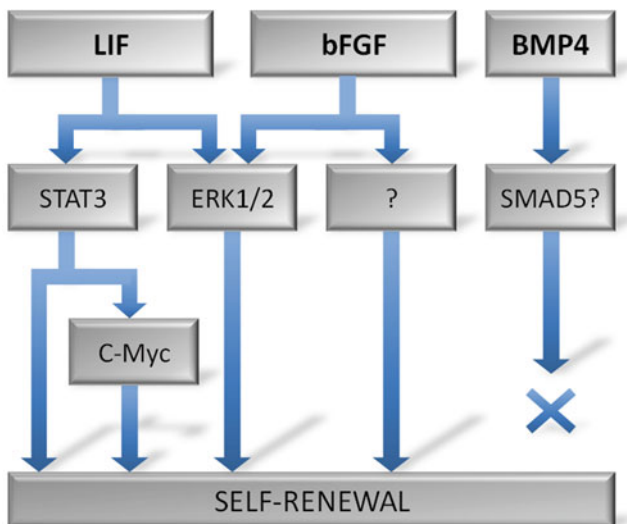


Fig. 6.3 An interpretation of the effect of LIF, bFGF, and BMP4 on activation of downstream transcription factors associated with self-renewal signaling pathways in cat ESC. LIF signaling activates STAT3 pathway and STAT-3 induces c-MYC expression, as detected by RT-PCR in cat ESC colonies. The STAT3 pathway acts as one of the self-renewal pathways in cat ESC. bFGF signaling in combination with LIF activates the downstream protein ERK 1/2 which is involved in self-renewal in cat ESC. bFGF may also activate other signaling pathways that are involved in self-renewal and maintaining cat ESC in an undifferentiated state. BMP4-signaling was responsible for inducing cell differentiation. BMP4 did not have an inhibitory effect on ERK 1/2 and did not increase SMAD 5. It is possible that paracrine molecules secreted by CEF feeder cells were interacting indirectly with ESC and reducing expression levels of SMAD5, but not enough to compensate for the differentiation effect induced by BMP4. Cat ESC do not use the BMP-signaling pathway for inhibiting ERK 1/2 activity and self-renewal

effect induced by BMP4. Thus, our results indicate that cat ESC do not use the BMP-signaling pathway for inhibiting ERK1/2 activity and self-renewal.

An interpretation of the effect of LIF, bFGF, and BMP4 on activation of downstream transcription factors associated with self-renewal signaling pathways in cat ESC is given in Fig. 6.3. In summary, the JAK/STAT pathway is activated by LIF signaling and possibly acts as one of the self-renewal pathways in cat ESC. In addition, the MERK/ERK pathway activated by bFGF signaling may be involved in self-renewal through activation of the downstream protein ERK and other signaling pathways. The BMP-signaling was responsible for inducing cell differentiation, while it is not clear if ERK activation by a combination of LIF and bFGF is also involved in cell differentiation. Additional signaling pathways are involved in self-renewal and maintaining ESC in an undifferentiated state. Ongoing studies detecting downstream transcription factors that are influenced by specific regulatory pathways will provide a means to identify modulators of ESC fate. Ensuring proper culture conditions is required for the maintenance of robust and stable cat ESC lines.

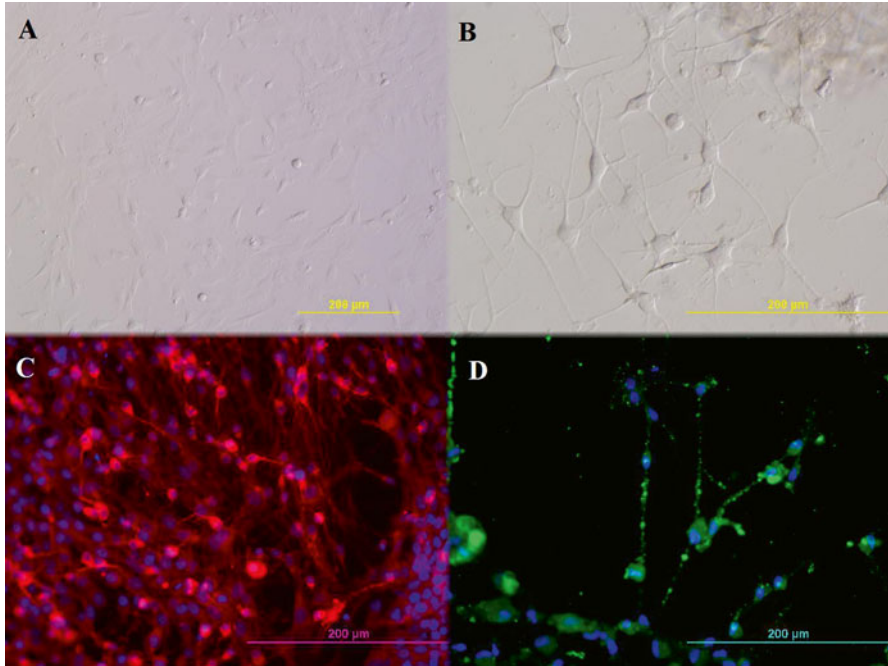


Fig. 6.4 Morphological characterization and neuronal differentiation of cat ESC. (a) Pre-induction: Single cat ESC in growth medium 48 h after colony dissociation. Single cat ESC appear with a fibroblastic morphology. (b) After pre-induction treatment with bFGF followed by addition of neuronal induction medium. Cat ESC change to a neuronal morphology with long, thin processes and round cell bodies. Neuronal phenotype cells showing positive expression for neuronal specific markers (c) MAP2 (red), and (d) NeuN (green). Nuclei are stained with DAPI (blue)

3.4 Induced Differentiation of Cat ESC

Clearly, spontaneous *in vitro* differentiation of cat ESC is not a viable method to obtain a pure culture of a desired cell type; even less so when true pluripotency has not been confirmed. Thus, efficient methods that direct the differentiation of cat ESC and produce a homogeneous population of a specific cell type, before cells spontaneously differentiate, would benefit the production of cat cells for future stem-cell-based therapies.

3.4.1 Neuronal Differentiation

In the author's laboratory, a preliminary experiment was done to determine the ability of cat ESC to differentiate into neuronal cells. Cat ESC colonies were induced with neuronal induction medium, using the same protocol effectively tested for the induction of feline bone marrow into neuron-like cells (Martin et al. 2002). We found that high numbers of cat ESC rapidly differentiated into neuronal phenotype cells that possessed a round cell body with long and thin processes (Fig. 6.4b), some

of which made contact with other cells. The majority of the neuronal induced cells expressed neuron-specific microtubule associated protein 2 (Map-2), while lower proportions expressed neuron-specific nuclear protein (NeuN; Fig. 6.4c, d) that appears to be expressed in the neuron at withdrawal from the cell cycle and/or with the initiation of terminal differentiation. These preliminary results demonstrated that cat ESC are capable of differentiating *in vitro* into neuronal cells. Further studies are required to differentiate cat ESC into mature physiologically functional neuronal cells. Ultimately, derived neuronal cells can be used to study neurological diseases in cats, serve as a model of human diseases, and to generate cells for stem-cell-based therapies. Additional applications for derived neuronal cells are given in another section of this chapter.

3.5 Current and Future Applications of Stem-Cell-Based Therapies Using Cat ESC

There are no reports indicating that cat ESC are being used currently for stem-cell-based therapies for regenerative medicine, possibly due to the limited stem cell proliferation of the cells *in vitro*. However, our recent results confirming that cat ESC can be induced to differentiate *in vitro* into neuronal cells opens the door for future studies focusing on neuronal diseases. One possible application of derived neuronal stem cells could be for the study and treatment of Alzheimer's disease (AD). Histopathological features of AD in humans consist of extracellular deposition of amyloid- β peptide, an enzymatic product of amyloid precursor protein (App), and intracellular neurofibrillary tangles (NFT). Aged cats have a similar AD that is recognized as a feline disorder with pronounced amyloid and phosphorylated tau deposition (Gunn-Moore et al. 2006, 2007) that is indicative of an identical disease mechanism to that of AD in humans. Moreover, the human brain and the cat brain both have cerebral cortex with similar lobes, sharing some neurological aptitudes (Mann 1979). These properties and conditions are indicative that cats could be a potential model for human AD.

4 Domestic Cat Multipotent Stem Cells

In the domestic cat, two types of multipotent stem cells, mesenchymal and spermatogonial, have been isolated, partially characterized, and initial transplantation trials have been done. One drawback to developing stem-cell-based technologies is that most isolated multipotent adult stem cells have more limited life-spans in culture than do ESC. Understanding self-renewal and how these cells commit to differentiation has progressed slowly, in part due to the absence of molecular markers that reliably define the target cell and the lack of optimal *in vitro* culture conditions. In this section, we will give an overview of current knowledge for the molecular

characterization of mesenchymal and spermatogonial stem cells and describe some preliminary transplantation trials performed in the domestic cat using these adult stem cells.

4.1 Cat Mesenchymal Stem Cells

Mesenchymal stem cells (MSC) are a heterogeneous population of multipotent cells that can be isolated from any adult and fetal tissues and exhibit a capacity to give rise to cells of multiple cell lineages. Adipose-derived MSC (AD-MSC) are a good cell source alternative that offers promising potential in organ-specific tissue engineering and tissue reconstruction. AD-MSC have the ability to differentiate into mesodermal cells, such as adipocytes, fibroblasts, myocytes, osteocytes, and cartilagocytes, a process known as lineage-specific differentiation (Lin et al. 2006). Inducing specific cell differentiation of AD-MSC into chondrogenic or osteogenic cells provides a possible clinical application for the treatment of several diseases in humans, including inherited, traumatic, or degenerative bone, joint, and soft tissue defects.

In cats, the functional capacity of bone-marrow-derived MSC (BM-MSC) was measured by their capacity to give rise to cells of multiple cell lineages, including adipocyte, osteocyte, and neural-like cells (Martin et al 2002; Zhang et al 2011). Similarly, cat AD-MSC isolated from subcutaneous adipose tissue differentiated into mesodermal cells when stimulated with inductive factors (Quimby et al. 2011). AD-MSC offer several advantages for use in endangered species compared to BM-MSC, because adipose tissue is easier to access, simpler enzyme-based protocols for cell isolation have been described, and seemingly their potential to differentiate into osteoblasts, chondrocytes, and adipocytes is similar to that of BM-MSC (Schäffler and Büchler 2007).

4.1.1 Characteristics of Cat MSC

In the domestic cat, MSC isolated from BM had morphological characteristics that were similar to those reported in human and mice. Cat BM-MSC grew as isolated colonies between 7 and 10 days after initial plating, but after further expansion, cells were fibroblast-like, appearing polygonal or spindle-shaped with long processes (Martin et al. 2002). Cat BM-derived MSC do not grow well when cultured at low cell densities; however, when they were cultured with an initial concentration of 1.5×10^7 BM mononuclear cells, frequency of colony formation was increased (Martin et al. 2002). Cat AD-MSC were plastic adherent, spindle-shaped cells that formed within 24–72 h of culture with a replication rate significantly greater than that of BM-MSC (Webb et al. 2012). These studies suggest that cat BM and AD-MSC shared similar morphological characteristics, but AD-MSC can be generated more quickly due to its higher proliferative rates (Webb et al. 2012).

4.1.2 Molecular Signature of Cat MSC

MSC isolated from cat BM have shown similar patterns of cell-surface antigens as those reported for other mammalian species, where cells are strongly positive for CD9, CD44, and MHC-1, and negative for CD45 (Martin et al. 2002). Moreover, cat AD-MSC express high levels of CD44 and CD105, and expression of the CD90 surface antigen was higher in AD-MSC than that of BM-MSC (Webb et al. 2012). Studies on MSC gene expression have led to identification of some genes associated with multipotency (Song et al. 2006; Mitchell et al. 2006). However, molecular mechanisms regulating proliferation and multipotency of MSC are yet to be identified. Expression of transcription factors involved in the maintenance of pluripotency in ESC (*OCT-4*, *SOX-2* and *NANOG*) has been proposed to play a similar role in adult multipotent cells, including MSC. In humans, expression of *OCT-4* and *SOX-2* transcripts has been reported in MSC isolated from different tissues (Kolf et al. 2007; Riekstina et al. 2009; Greco et al. 2007). Similarly, expression of the three transcription factors *OCT-4*, *SOX-2*, and *NANOG* has been reported in cat BM-MSC (Zhang et al. 2011).

4.1.3 Induced Differentiation of Cat MSCs

Cat AD-MSC isolated from subcutaneous adipose tissue differentiated into mesodermal cells (osteoblasts, chondrocytes, and adipocytes) when stimulated with inductive factors (Quimby et al. 2011). The original anatomical location of the adipose tissue may influence both the proliferation rate and the differentiation capacity of the cells. For instance, in rabbits, the osteogenic potential of AD-MSC isolated from abdominal adipose tissue was greater than that of AD-MSC isolated from subcutaneous adipose tissue (Peptan et al. 2006). Conversely, in humans, osteogenesis was more robust in adipose-derived stromal cells from the flank and thigh, as compared with cells from the abdomen (Levi et al. 2010). It is not clear why MSC isolated from different adipose tissues are committing to differentiate into a specific cell lineage, but it is known that certain transcriptional and molecular events activate cell differentiation (Hong et al. 2005; Hong and Yaffe 2006). Although, cat AD-MSC differentiate into mesodermal cells, it is important for future clinical applications to determine if expression of transcription factors between AD-MSC isolated from different anatomical locations may favor one cell lineage, rather than another.

4.1.4 Current and Future Applications of Stem-Cell-Based Therapies Using Cat Adipose-Derived MSC

CKD is a major cause of morbidity and mortality in cats. At present, the only definitive treatment option for cats with CKD is renal transplantation (Adin et al. 2001; Schmiedt et al. 2008). Similarly, the black-footed cat (*Felis nigripes*), one of the smallest wild cats and listed as threatened, suffers a high mortality rate due to

kidney failure resulting from a condition known as amyloidosis (Terio et al. 2008). At our institute, we have black-footed cats, which are part of our assisted reproduction research program, that have been dying at 3–5 years of age due to amyloidosis. Similarly, San Diego and other zoos also reported the deaths of black-footed cats at early ages due to the same condition. Amyloidosis is a progressive, incurable, metabolic disease characterized by abnormal deposits of protein in one or more organs, in this instance, the kidneys. Even though, supportive care and treatment were provided to black-footed cats to stabilize renal function and reverse metabolic complications, the disease is progressive and animals die prematurely. Recently, BM and AD-MSK were transplanted into domestic cats for treatment of CKD (Quimby et al. 2011). Although the pilot study did not report a cure for the disease, the intrarenal injection of autologous MSC was well tolerated and induced a mild improvement in renal function. Thus, MSC transplantation is a promising tool for cell replacement therapy and regenerative medicine in endangered species and possibly an effective treatment for amyloidosis in black-footed cats.

Many rare and endangered animals held in zoological institutions are predisposed to chronic joint and ligament problems. This may be due to a combination of their captive environment and the longer life-span they usually achieve therein. Some treatments are not curative and, instead, are palliative for treating pain and offer a certain level of well-being to the animals. MSC have promising application for tissue repair, treatment of autoimmune diseases, and regenerative medicine (Huang et al. 2011). MSC transplantation for regenerative medicine has become an important area of research for treating human diseases and recently adopted in veterinary medicine. Currently, veterinarians are using MSC for treatment of tendon, ligament, or joint/cartilage injuries, mainly in dogs and horses, but also in domestic cats. Recently (2011), a Florida panther at the Tallahassee Museum of Natural History, was treated with AD-MSK to treat chronic arthritis in his elbow. Over a period of 7 weeks, the panther recovered the complete use of his elbow without any apparent pain. Thus, it is possible that the cells transdifferentiated into several types of lineages that may be beneficial when transplanted into endangered felids as a treatment to regenerate skeletal elements and repair cartilage.

4.2 *Spermatogonial Stem Cells*

In domestic cats, SSC are single cells localized in the basal membrane of the seminiferous tubules (Powell et al. 2013) that are capable of differentiating into mature spermatozoa after testicular transplantation (Silva et al. 2012). Domestic cat SSC have been isolated from prepubertal and adult testis (Kim et al. 2006; Powell et al. 2012, 2013; Silva et al. 2012; Vansandt et al. 2012; Tiptanavattana et al. 2013). SSC constitute only a small fraction of the adult testicular germ cells. Identification of germ-cell-specific markers that are expressed uniquely on SSC, but not on other somatic cells or differentiated spermatogenic cells, facilitates enrichment of a pure population of SSC for further studies or spermatogonial cell transplantation (SCT).

4.2.1 Molecular Signature SSC

Cat SSC have been characterized phenotypically by the expression of spermatogonial markers previously identified in rodents. Expression of cell-surface markers *GFR α 1* (glial cell-derived neurotrophic factor (GDNF) receptor) which is a signaling molecule involved in SSC self-renewal, *GPR125* (G protein-coupled receptor 125) which is expressed in SSC, and *C-Kit* (which is a receptor for stem cell factor and an early marker for germ cell differentiation) was detected by immunohistochemistry of adult and prepubertal cat testes (Powell et al. 2012). Moreover, it also was demonstrated that *GFR α 1* and *GPR125* cell-surface markers were localized both at the basement membrane of the seminiferous tubules and also across the seminiferous tubules section (Powell et al. 2013); a clear indication that these two cell-surface markers are not only expressed by SSC but also by more differentiated spermatogonial cells, and may not be used for isolation of a purified population of cat SSC.

Expression of the major pluripotent ESC surface markers *SSEA-4*, *SSEA-1*, *TRA1-81*, *TRA-1-60*, and transcription factor *OCT-4* also were detected in cat SSC, but all of them were located only along the basement membrane of the seminiferous tubules (Powell et al. 2013). On the other hand, expression of cell-surface markers (*GFR α 1*, *GPR125*), and thymus cell antigen-1 (*THY1*), and intracellular markers (ubiquitin carboxi-terminal hydrolase I—*UCHL*1; promyelocytic leukemia zinc finger—*PLZF*, and *OCT-4*), previously identified in rodents, were detected at the mRNA level in a pool of spermatogonial cells isolated from prepubertal and adult cat testes (Vansandt et al. 2012). Overall, these studies confirm that SSC of prepubertal and adult cats express cell-surface and intracellular germ-cell-specific markers in common with SSC of other mammalian species, as well as pluripotent ESC markers. The location of cat SSC along the basement membranes of the seminiferous tubules, and the positive expression of pluripotent ESC markers provide further support for classifying the cells as SSC.

Further characterization by flow cytometry revealed that cat testes contain distinct cell populations expressing *GFR α 1* (47 %), *GPR125* (52 %), *TRA-1* (18 %), and *TRA-1-81* (16 %), and smaller populations expressing *SSEA-1* (7 %) and *SSEA-4* (3 %). Dual staining of germ cells also revealed the presence of three distinct cell populations that either express *GFR α 1* only (23 %), are positive for both *GFR α 1* and *SSEA-4* (6 %) or are positive only for *SSEA-4* (1 %; Powell et al. 2013). Recently, immunohistochemistry in sections of adult cat testis showed a distinct cell population of SSC, located at the basement membrane of the seminiferous tubules, showing positive expression for *GFR α 1* and a germ cell DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (DDX-4) marker (Tiptanavattana et al. 2013). These results demonstrated that cat SSC can be purified into different subpopulations by determining the expression of specific markers and can be isolated from more differentiated spermatogonial cells.

4.2.2 Transplantation of Spermatogonial Cells in Felids

SCT has been carried out in the domestic cat. Kim et al. (2006) investigated the ability of domestic cat germ cells to colonize the seminiferous tubules of mice by

xenogenic transplantation. Successful colonization was observed, but spermatogenesis was not supported by the mouse testes. The taxonomic distance between the donor cat and recipient mice may have influenced the results. In contrast, in a recent study, it was demonstrated that xenogenic transplantation of ocelot spermatogonial cells, selected by the expression of the *GFRa1* marker, into domestic cat testes, a taxonomically similar species, resulted in both colonization and spermatogenesis (Silva et al. 2012). Nonetheless the production of live offspring from spermatozoa generated from SCT has not been accomplished.

Several steps during the process of SCT may affect the success of the technique. One critical step is preparation of recipient testes. Endogenous differentiated germ cells in the recipient male must be depleted to provide additional space at the basal compartment of seminiferous tubules for colonization and expansion of the transplanted cells (Brinster et al. 2003). The approaches applied to deplete germ cells in the domestic cat were similar to those reported in mice. The first approach involved focal irradiation with a total of 9–10 Gy (Kim et al. 2006; Silva et al. 2012), and in the second method the cat was treated with busulfan (Silva et al. 2012). The ability of both methods to deplete male germ cells was demonstrated, but treatment with busulfan at doses that depleted germ cells was toxic and resulted in animal mortality (Silva et al. 2012). Thus, in the domestic cat, focal irradiation is the recommended method to induce germ cell depletion.

Another critical step that may affect the success of SCT is the age of the recipient. Transplantation of SC into the testis of prepubertal mice and goats resulted in higher rates of colonization and spermatogenesis than was seen in older animals (Shinohara et al. 2001; McLean et al. 2003; Honaramooz et al. 2003). However, spermatogenesis of ocelot germ cells was supported after transplantation into testis of adult domestic cats. With the limited number of animals in which spermatogenesis was restored after SCT into domestic cat testes, it is not possible to conclude that adult cats were better recipients than younger cats. Moreover, depletion of germ cells in domestic cats before SCT may not be needed. Most of the original studies in SCT were performed in the mouse. Possible species-specific differences, including the dissimilarity between the mouse and the cat in size of the testes, may be suggestive evidence that depletion of germ cells before SCT may not be necessary. Further studies are required to better understand the factors that affect SCT success in domestic cats.

5 Conclusions

Understanding the transcriptional network of the signaling pathway(s) for self-renewal in pluripotent and multipotent cat stem cells is essential for optimization of the *in vitro* culture conditions required to produce robust cat stem cells. In these studies, it was shown that “core” transcription factors *OCT-4*, *SOX-2*, and *NANOG* are expressed by pluripotent and multipotent cat stem cells. At the transcriptional level, in cat ESC, LIF and bFGF signaling is able to induce activation of the

downstream transcription factor STAT3 and protein kinase ERK1/2, and possibly by activation of other signaling pathways, up-regulate expression of the core of transcription factors *OCT-4*, *SOX-2*, and *NANOG*. Even though, cat ESC use the JAK/STAT and MEK/ERK signaling pathways for self-renewal ESC were not maintained in an undifferentiated state for prolonged periods of time. Additional studies are required to define if other signaling pathways, such as Wnt or Activin/nodal signaling, which sustain pluripotency in mouse and human ESC, may also regulate self-renewal in cat ESC. Similar studies are needed to determine if self-renewal pathways are conserved in multipotent cat stem cells.

Lastly, for application in regenerative medicine, it is relevant to elucidate the mechanisms that regulate lineage commitment in pluripotent and multipotent cat stem cells at the single-cell level to improve the efficacy of differentiation into specific cell types.

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Chapter 7

Brief Introduction to Coral Cryopreservation: An Attempt to Prevent Underwater Life Extinction

Tiziana A.L. Brevini, Sara Maffei, and Fulvio Gandolfi

In recent decades, the coupling of climate change and anthropogenic stressors has caused a widespread and well-recognized reef crisis (Hughes et al. 2003; Veron et al. 2009) (Table 7.1). Throughout the world coral reefs are being degraded at unprecedented rates. The increased greenhouse gases make corals more susceptible to stress, bleaching, and newly emerging diseases. Locally, reefs are damaged by pollution, nutrients, and sedimentation from outdated land use, fishing, and mining practices (Hughes et al. 2003).

Although in situ conservation practices, such as marine protected areas, reduce these stressors and may help slow the loss of genetic diversity on reefs, the global effects of climate change will continue to cause population declines.

Gamete cryopreservation has already acted as an effective insurance policy to maintain the genetic diversity of many wildlife species, but has only just begun to be explored for coral.

So far Mary Hagedorn reported having a great deal of success with cryopreserving sperm and larval cells from a variety of coral species. Building on this success, she has now begun to establish genetic banks using frozen samples (Hagedorn et al. 2012a, b). She demonstrated that cells that are cryopreserved and banked properly can retain viability for years, or even centuries, without DNA damage (Hagedorn et al. 2012b).

Of all the reefs, Caribbean reefs are suffering the most severe declines, and their fate may predict the future of corals throughout the world. To build new tools for the continued protection and propagation of coral from the Great Barrier Reef, an international group of coral and cryopreservation scientists known as the Reef Recovery Initiative joined forces during the November 2011 mass-spawning event.

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Table 7.1 Extinction risk of different coral species

Species	Extinction risk
<i>Acropora austera</i>	++
<i>Acropora cervicornis</i>	+++
<i>Acropora Florida</i>	++
<i>Acropora formosa</i>	++
<i>Acropora humilis</i>	++
<i>Acropora hyacinthus</i>	++
<i>Acropora millepora</i>	++
<i>Acropora nasuta</i>	++
<i>Acropora palmata</i>	+++
<i>Acropora paniculata</i>	+
<i>Acropora tenuis</i>	++
<i>Acropora verweyi</i>	+

+ Low risk, ++ moderate risk, +++ high risk

Storage of important coral and related cells through cryopreservation will profoundly advance basic research in embryology, genetics, systematics and molecular biology, as well as enhance management strategies for reef restoration.

Current protocols for coral cryopreservation and associated organisms are not fully developed, and so the associated programs that could employ these important genetic resources have not reached their full potential. In the past 9 years some of the fundamental cryobiology for coral sperm, larvae, and associated symbionts have been characterized (Hagedorn et al. 2010; Hagedorn et al. 2006a, b). Additionally, sperm have been successfully cryopreserved and used to create new coral in vitro, and have been added, along with dissociated larval cells, to frozen repositories in the US and Australia (Hagedorn et al. 2006a, b).

Eight individual *Acropora tenuis* (Fig. 7.1) and eight *Acropora millepora* (Fig. 7.2) colonies were collected from western Pelorus Island (146_29.3040 E, 18_33.0010 S) and were transported in seawater to Australian Institute of Marine Science (AIMS) within 24 h. Each colony was labeled and maintained in 1-lm filtered seawater in 1,000 L tanks for the duration of the spawning event. Each night, colonies were examined for evidence of pre-spawning behavior or “setting” (Hagedorn et al. 2006a) and then isolated into individual bins.

A. tenuis and *A. millepora* are hermaphrodites, the sperm and buoyant eggs from individual colonies were allowed to separate and were then isolated using pipettes. The coral sperm cells were successfully cryopreserved, and after thawing, samples were used to fertilize eggs, resulting in functioning larvae.

In addition, developing larvae were also dissociated, and these samples with pluripotent cells (Heyward and Negri 2012) were cryopreserved and alive after thawing.

With advances in stem cell technologies, these frozen embryonic cells are likely to respond to specific media be coaxed to form developing larvae.

The outcome of these studies was the creation of the first frozen bank for Australian coral from two important reef-building species, *A. tenuis* and *A. millepora*.



Fig. 7.1 *Acropora tenuis*



Fig. 7.2 *Acropora millepora*

Moreover, the successful results from these studies indicated that cryomethods previously devised for coral specific (Hagedorn et al. 2006a, b) are widely applicable to other coral species.

The data described in these experiments also demonstrated that using frozen sperm, creating new coral and increasing genetic diversity in small populations is now possible.

However, the possibility to collect germplasm from coral is extremely limited by the short reproductive season (3 consecutive days each year) of most coral.

Besides reproducing sexually, a second way that coral can reproduce is through asexual fragmentation. These fragments consist of a host-symbiotic partnership of coral tissue and intracellular symbiotic *Symbiodinium* sp., commonly called zooxanthellae.

If it could cryopreserve these asexual fragments the speed of conservation efforts would be increased.

Since the fragment is so complex, the studies of Mary Hagedorn et al. had to take into account both the cryosensitivities of the coral tissue and those of their endosymbionts.

These studies used small fragments of *Pocillopora damicornis* of a size that would make them suitable for long-term cryopreservation storage in cryovials, yet still large enough to outplant and form a new colony. *P. damicornis* colonies were collected from various shallow reef flats around Coconut Island in Kaneohe Bay, Hawaii, and then maintained at the Hawaii Institute for Marine Biology, University of Hawaii. All fragments were labeled and followed individually throughout the treatments.

During the post-treatment recovery identified fragments were returned to water tables for visual monitoring over a 3-week period. The number of intact and missing polyps was determined by visually examining each fragment under a microscope at the end of the 3-week period. The presence of intracellular zooxanthellae was critical to the health of the coral polyp. Therefore, zooxanthellae number and the robustness of their photosystem were monitored over time to examine relative levels of photosynthesis in photosystem I and II between the treatments and controls. If a fragment had been affected by chilling or the toxicity of a cryoprotectant, it may lose tissue, zooxanthellae or both.

To understand the fragments' sensitivity to cryoprotectants, *P. damicornis* fragments were exposed to dimethyl sulfoxide solutions at different concentrations (1.0, 1.5, and 2.0 M) for different durations (1.0, 1.5 or 2.0 h) and monitored.

Coral embryos are extremely sensitive to chilling (Hagedorn et al. 2006a, b), therefore assessing coral fragments' sensitivity to chilling injury was a very important point.

Coral fragments chilled in the winter demonstrated a robust coral tissue with little loss to chilling, whereas fragments chilled in late spring were far more sensitive. In contrast regardless of the season, the zooxanthellae were consistently chill sensitive, demonstrating significant loss of zooxanthellae numbers even after a 5 min chilling event (the fragments appeared pale after 24 h).

These seasonal sensitivities have been demonstrated in other corals as well.

Successful cryopreservation of cells, germplasm, and tissues must address intrinsic biophysical properties (e.g., water and cryoprotectant permeability, osmotic tolerance limits, and intracellular ice nucleation) to maximize survival.

A similar systematic approach is vital to improving post-thaw survival of coral and its associated organisms.

Coral fragment cryopreservation is an entirely new field of research with great potential opportunities because the process would store the valuable whole, diploid fragments of the coral. Coral fragment storage is important because a whole fragment can grow much more quickly than coral re-derived from germplasm.

Being able to cryopreserve coral fragments would rapidly move cryoconservation efforts forward avoiding the limits deriving from the present dependence yearly spawning events. Nevertheless, all types of tissues, including sexually derived germplasm and asexual fragments, should be part of a comprehensive national plan for maintaining reef diversity.

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Part III
Stem Cell Banking for the Future

Chapter 8

Fundamental Principles of a Stem Cell Biobank

Ida Biunno and Pasquale DeBlasio

1 Introduction

Alternatives to transplantation medical practice are of utmost importance since, dramatically successful in some cases, remains an option for a limited number of patients due to the shortage of donated organs, to the epidemic of chronic diseases and to the high costs associated with patient transplantation and follow-up (Nelson et al. 2008; Dalay and Scadden 2008). Recent scientific advances in stem cell-based regeneration provides enormous potential to the replacement of damaged tissues with “new” ones, indeed pluripotent (PSCs) stem cells are becoming promising tools for replacing, repairing, regenerating, and rejuvenating dead, degenerating or trauma injured affected tissues. PSCs essentially are of two types: embryonic (ESCs), derived from embryos, and induced pluripotent (PSCs) generated by molecular reprogramming.

2 Stem Cells Are Pillar to the “Bioeconomy” in Many Countries

The bioindustry requests for stem cell products of human PSCs has increased over the last few years, the global market was \$3.8 billion in 2011 and expected to reach \$6.6 billion by 2016, increasing at a compound annual growth rate (CAGR) of

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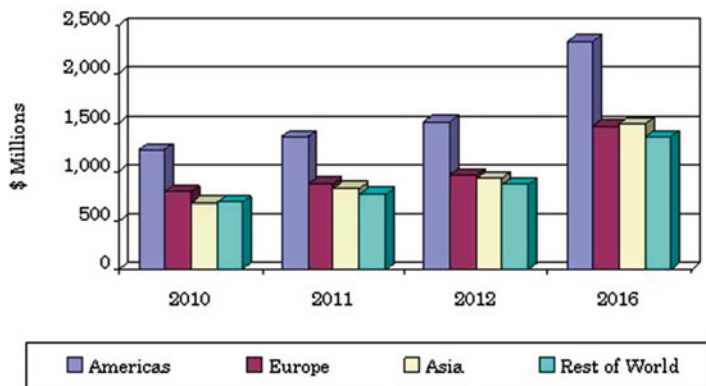


Fig. 8.1 Global stem cell market and forecasts, 2010–2016 (Source: BCC Research)

11.7 % from 2011 to 2016. The American market for stem cell products was \$1.3 billion in 2011. This sector is expected to rise at a CAGR of 11.5 % and reach nearly \$2.3 billion by 2016. The European market for stem cell products was \$872 million in 2011 and is expected to reach nearly \$1.5 billion by 2016, a CAGR of 10.9 % (bcc/research Market forecasting. Global Market for Stem Cells, Report code BI0035D, July 2012) (Fig. 8.1).

Neurological applications is forecasted to exert the highest supporters of this growth with a CAGR (Compound annual growth rate) of 24.4 % during 2010–2015, hematology and diabetes will closely follow the expected rate during the same time frame (Research and Markets May 11, 2011). North America and Europe are expected to have a lead position in stem cell market growth although Asian economies probably will maintain a large CAGR.

3 Stem Cells Types

Totipotent stem cells are found only in embryos. Each cell can form a complete organism (e.g., identical twins). Pluripotent stem cells exist in the undifferentiated inner cell mass of the blastocyst and can form any of the over 200 different cell types making up a living organism. Multipotent stem cells are derived from fetal tissue, cord blood, and adult stem cells. Although their ability to differentiate is more limited than pluripotent stem cells, they already have a track record of success in cell-based therapies (Bongo and Lee 2005) (Fig. 8.2).

Here is a current list of stem cell sources and basic characteristics:

1. *Embryonic stem cells*—are harvested from the inner cell mass of the blastocyst (Riazi et al. 2009) 7–10 days after fertilization. These cells have no natural environment as they exist only in vitro. When implanted, interact minimally with the surrounding tissues, form teratomas or structures reminiscent of in vitro embryoid bodies.

Stem Cells Types Type	Description	Examples
Totipotent (days)	Each cell can develop into new individual	Early embryos cells (1-3)
Pluripotent	able to form any of the 200 cell types that make the organism	Blastocyst cells
Multipotent	differentiated cells able to form several tissues	Fetal tissues, cord blood and adult stem cells

Fig. 8.2 Stem cells can be classified into three broad categories, based on their ability to differentiate

2. *Fetal stem cells*—are taken from the germline tissues that make up the gonads of aborted fetuses. These cells overall are the most plastic since they must adapt constantly to their evolving environment hence their potency must vary over time as the organs develop.
3. *Umbilical cord stem cells*—Umbilical cord blood contains stem cells similar to those found in bone marrow.
4. *Placenta derived stem cells*—up to ten times as many stem cells can be harvested from a placenta as from cord blood.
5. *Adult stem cells*—Many adult tissues contain stem cells that can be isolated. These cells are multipotent capable of persistent self-renew, live in a quiescent state and stable environment, in the body throughout life.
6. *Reprogrammed stem cells*, iPS, or retro-differentiation of tissue-restricted stem cells are obtained by the conversion of somatic donor cells (harvested from organs) into pluripotent progenitors to derive patient-specific progenitors.

Particular interest is being placed on induced Pluripotent Stem (iPS) cells by virtue of their enormous potential to model a variety of human cell types and human conditions, in particular can model: (1) in vitro diseases, particularly for those disorders for which no animal model system is available; (2) to implement drug discovery, assess its efficacy and safety; (3) in preclinical and clinical toxicity studies; (4) in vitro stratification of patients based upon the phenotype; (5) can become in vitro reference control stem cell lines particularly useful in those genetic diseases where the pathophysiology is still controversial; (6) in iPS-derived organotypic cell lines as model of organ response to drug metabolism based upon individual genotype and phenotype. The successful use of these cells in drug discovery, toxicology (Pistollato 2012) studies, and product safety assessments will also lead to a reduction in the use of animals for laboratory research and commercial testing (Baker 2007; Takahashi and Yamanaka 2006; Nobel Foundation Retrieve 2012).

4 Stem Cells in Translational Medicine

The clinical application of stem cells depends on the fundamental principle that the final cell phenotype rely on a combination of two factors: the starting cell population and the environment in which the cells are eventually implanted (Hipp and Atala 2008). This combination is fundamental and can be obtained by implanting: (i) raw tissue-restricted stem cells, (ii) progenitors capable to differentiate only after transplantation or (iii) pre-differentiating stem cells. The choice of the strategy depends on the tissue “niche” where the cells will ultimately be placed and essentially whether the “niche” has the appropriate differentiation program. Traumatic or ischemic disorders probably would benefit from implantation of raw tissue restricted progenitor cells, on the other hand, degenerative type of disorders would probably benefit from in vitro differentiated cells (Simara et al. 2013; Baker 2007; Takahashi et al. 2007).

Stem cell research and clinical translation constitute fundamental and indivisible modules catalyzed through the biobanking activity and eventually will generate a return of investment since banking through long-term storage represents a fundamental source to preserve the cells’ original features (Bardelli 2010).

5 Stem Cell Specialized “Repository”

Clinics around the world offer stem-cell-based therapies to desperate patients for an array of intractable medical conditions and, in most cases, the benefits are overpriced and the risks are ignored (Gorski 2013). While the derivation and use of ES and iPS cells are increasing rapidly for biomedical research purposes, information on the actual number, provenience, characteristics, and accessibility of these cells remain “SCARSE”. The use of poorly defined stem cells hampers: disease modeling, toxicology studies, drug discoveries, and most of all affects research results credibility. To decrease the use of poorly characterized cells in “stem-cell-based therapies” it is auspicious to encourage the establishment of STEM CELL BANK(S) with a CENTRALIZED DATABASE and discourage “colleague-supply-routes.”

There are a number of key elements required to support the development of high quality PSC-based research and human applications of stem cells of which the most fundamental are (Day and Stacey 2008; Diaferia et al. 2012; Inamdar et al. 2012):

- The establishment of pristine early passage stocks of cell lines to enable accurate and independent verification of research data and to sustain these tools for use in future research developments.
- Availability of well characterized and quality controlled sources of PSC lines established through implementation of up-to-date scientific norms, to ensure that research efforts are not wasted on cells that are unauthenticated, contaminated, or inadequately reprogrammed.
- Direct linkage of PSC stocks with a well documented and accessible database of donor and PSC characteristics.

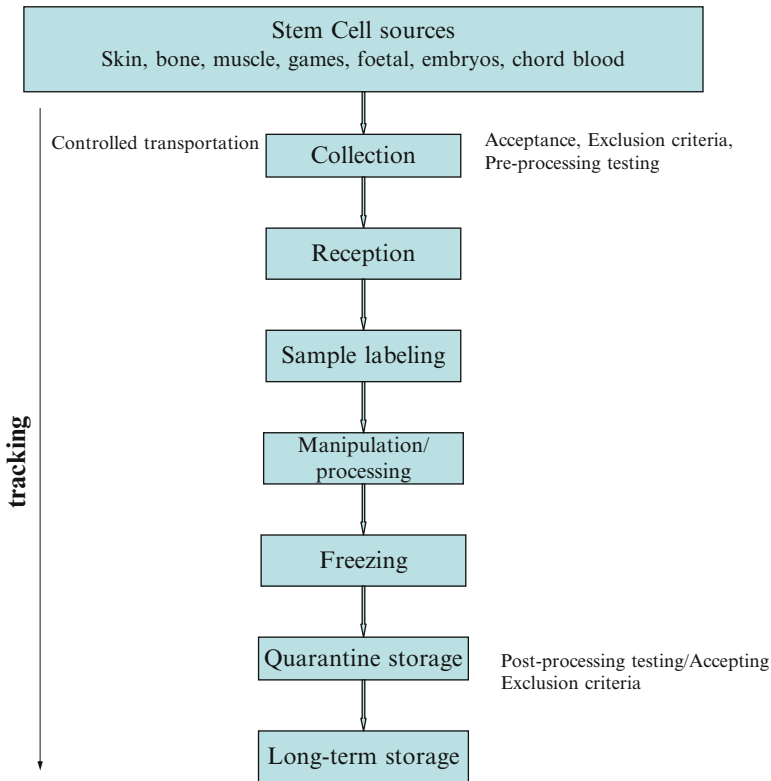


Fig. 8.3 Database and tracking system

- Assurance that cells intended for human application have appropriate traceability (Knoppers and Isasi 2010), and processing/storage history to meet regulatory demands and that they can be made available at the right time and in the right condition for therapeutic use.
- Provision of specific cell types, that can be produced at a sufficient scale with minimized batch-to-batch variability.

In general, to be operative the stem bank must implement a quality management system to make the banking facility function properly in every step of its activity; the standardization of procedures and protocols for sample collection (Murdock 2012), transport, processing, testing and storage, and release (Fig. 8.3). Standardization will guarantee that all the samples will share the same conditions and same quality controls. There must be sample traceability, meaning proper sample coding and identification; computer management and paper copies of all applications and processes to which samples and associated data have been submitted; employment of qualified personnel who will exclusively be dedicated to the different operations of the biobank.

Stem cell samples, collected upon informed consent should be sent to the biobank in a special transportation kit in order to preserve their viability and quality. According

to the proper quality management system, samples are then accepted or rejected and if accepted, after being labeled by a barcode, they can proceed to manipulation. Sample freezing is normally operated through computerized controlled systems in order to retain a traceable graph of the freezing process. Samples are then stored in quarantine containers until all the post-processing tests are performed and samples undergo thorough acceptance/exclusion criteria. Sample tracking is maintained all throughout the process, starting from collection to long-term storage (Healy et al. 2011).

Stem-Cell-specific banks should not be regarded as separate academic entities with limited activities but rather, as integrated infrastructural modules with experienced workforce. An essential prerequisite for biobanking is a robust sample tracking system, as described in the Fig. 8.3, which describes how to handle the sample, its codification or identification. All the processes performed on the samples must be managed by computer assisted programs as well as archives of paper copies. This requires the employment of qualified personnel who is exclusively dedicated to the overall management of the biobank. Sample tracking must be maintained throughout the entire process, starting from the collection to the long-term storage and distribution and must be efficient in order to follow the many phases of the biobanking process starting from: recruitment and informed consent of cell donation; sample collection and transportation; registration, processing, freezing, and testing; and the potential release of the stored units for therapeutical purposes. This necessitates the application of standard operative procedures for sample collection, transport, identification, processing, storage at the proper temperature, and informatic data collection and processing. In general, to be operative the stem bank must implement a quality management system to make the banking facility function properly in every step of its activity by standardizing all the procedures including lines release.

6 Stem Cells Cryopreservation

Whilst effective methods exist for the cryopreservation of a wide variety of lines, many cell types (including few stem cells) still prove to be refractory to freezing, or yield unacceptably low rates of recovery. Effective cryopreservation requires an understanding of the basic physical principles that underlie in the freeze/thaw process and the cells' response to the imposition of such a process (Pegg 2007; Maryman 2007). In order to guarantee that all the samples share the same conditions and the same quality controls it is auspicious to employ qualified personnel who will exclusively be dedicated to the different biobanking operations.

7 Freezing and Thawing Process of Human Neural Stem Cells

We determined the best procedure, equipment, and velocity of NS cell freezing and thawing. Freezing media, conditions, and equipment for human glioblastoma NS cells (GliNS2, G179, G144, G166) and human fetal NS cell lines (CB660, CB660SP,

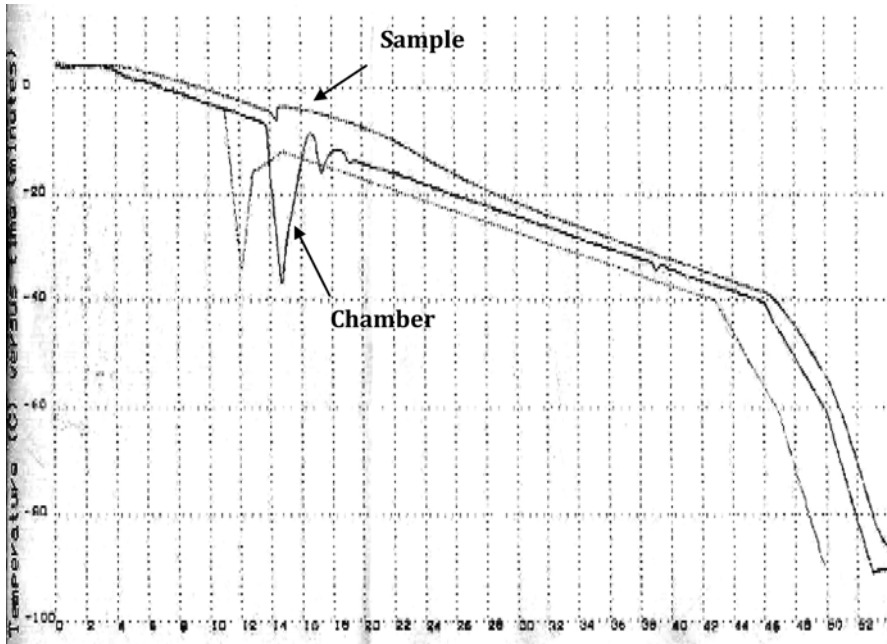


Fig. 8.4 Cooling rate profile recorded with a probe in the chamber along with the sample

CB541) were optimized. Using a rate freezer (Planer 560-16) with a programmable cooling rate, equipped with probes to measure and control the cooling chamber and the sample temperature, we were able to obtain a cooling rate of ~ 1 °C/min following these steps tested to control ice crystal formation: step 1, hold 3 min at 4.0 °C; step 2, -1.0 °C/min to -4.0 °C; step 3, -28.0 °C/min to -35.0 °C; step 4, $+25.0$ °C/min to -16.0 °C; step 5, $+2.0$ °C/min to -12.0 °C; step 6, -1.0 °C/min to -40.0 °C; step 7, -5.0 °C/min to -60.0 °C; step 8, -10.0 °C/min to -90.0 °C (Fig. 8.4).

To assure a better temperature exchange and minimum chance of contamination, cells were dispensed and sealed in glass ampoules and then stored in liquid nitrogen tanks. As cryopreservation media was used a protein-free, GMP manufacturing, freezing solution formulated with 10 % DMSO (CryoStor™, BioLife Solution) that proved to better control ionic and hydraulic balance in cells during hypothermia. After rapid thawing in water bath (for 1–2 min) cells were put back in culture and assessed for viability and apoptosis by flow cytometry. The use of a rate freezer with a programmable cooling rate and an intracellular-like freezing solution improved NS cell survival and reduced apoptosis at 24 h after thawing when compared with the manual step-down procedure using the isopropanol chamber and culture medium supplemented with 10 % DMSO as freezing medium (Fig. 8.5). Several studies showed that the freezing procedure displays its detrimental effects mainly at 24 h after thawing, due to failure to recover from the freezing damages, so we focused on this time point to evaluate survival and apoptosis.

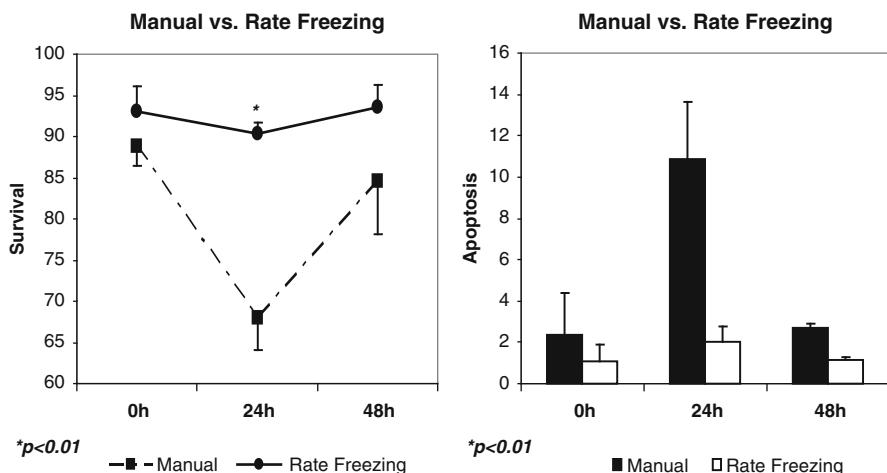


Fig. 8.5 Quantification of the survival (*left panel*) and apoptosis (*right panel*) rate of mouse neural stem cells embryo-derived (NS46C) comparing standard manual freezing procedure vs. rate freezing in CryoStor™

The same parameters used to freeze mouse neural stem cells were successfully used to cryopreserve other human cell types (human fetal neural stem cells and glioma neural stem cells) obtaining an optimal cooling rate profile of ~ 1 °C/min (Fig. 8.6).

Local import regulations with the recipient (helpful information is available from the world federation of culture collection, www.wfcc.org) and include a “Material Transfer Agreement” (MTA) that needs to be signed by the recipient scientist before distribution. In this document all the terms and conditions for the use of the biomaterial are explained to assure intellectual rights to the scientists that generated the cell lines. Furthermore a custom declaration form and a material datasheet are included in the shipment to assure proper clearance and handling of the material upon delivery.

8 Stem Cell Registry

Along the development of a high quality management system, a database is essential to capture scientific and other relevant data that are directly linked to the physical stocks of cells. This will be achieved by implementing a registry of all the cell lines including hESC and a comprehensive and fully relational database providing access to details of the cell lines and other information held in the biobanking network facilitating sourcing and shipments directly to the researchers under standardized and non contentious transfer agreements. The database should contain informations regarding the origin of the cell donor (anonymous identity),

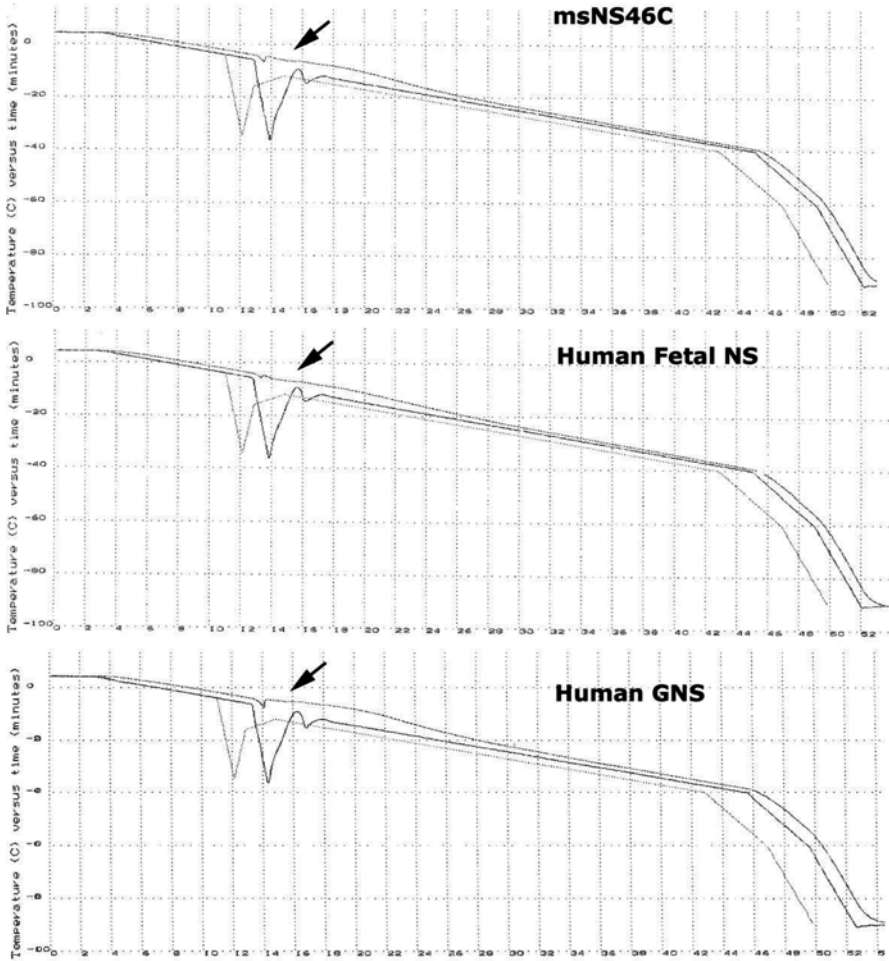


Fig. 8.6 Cooling rate profile recorded with a probe in the chamber along with the sample

phenotype (healthy controls as reference line), diseases, susceptibility to certain toxicities, HLA diversity, drug response, age, sex, gender, genotype, original tissue source, epigenetic signature, pluripotency status, gene expression profiles, epigenetic changes, and stability upon passages. The database allows the downloading of individual datasets, gives support to queries across multiple experiments and links together different types of data obtained from other ongoing projects. Added value to the database will be the addition of protocols in SOPs format, primers sequences, and experimental conditions.

(Stem Cell Information: NIH guidelines (Young et al. 2010) July 7, 2009)

9 Quality Control (QC) Stem Cell Pipeline

Whilst the quality and data management resources are essential to the work of the biobank, the delivery of sustainable supplies of lines that are scientifically and ethically sourced (based on solid scientific methodologies) is both a practical and legal prerequisite for the continued progression of stem cell research towards clinical therapies. Stem cell banks are indeed invaluable resources and will provide access to high quality controlled and characterized lines that meet the needs of current research and safety standards. The QC stem cell workflow pipeline of Fig. 8.7 describes in the workflow which was designed following *ISCB* (*International Stem*

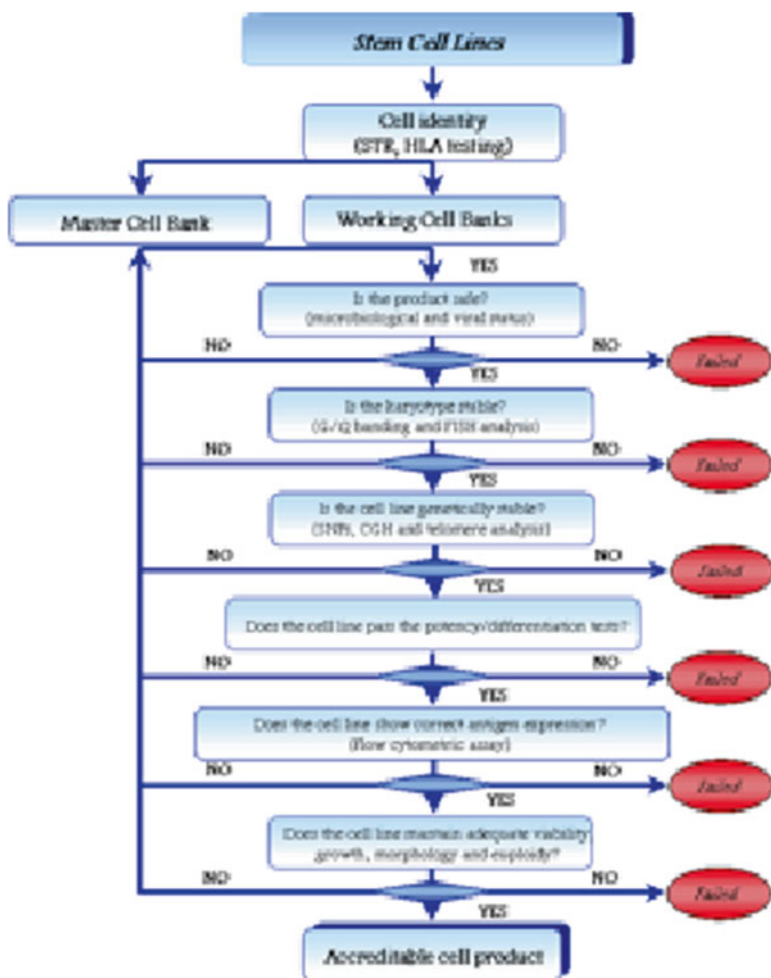


Fig. 8.7 Pluripotent stem cells bank workflow designed to reduce the use of uncharacterized lines and distribution using “colleagues supply route”

Cell Banking Initiative) (Adewumi et al. 2007; Crook et al. 2010) best practice document applicable only for research-grade stem cells supply.

The aim of the workflow is to reduce the number of stem cell lines that are being produced, reduce the use of unproven stem cells, and substitute the uncharacterized lines for those that are well characterized. The successful use of stem cells and their derivatives in drug discovery, toxicology, and safety assessments requires (1) their production at a sufficient amount, (2) reduce batch-to-batch variability, (3) use of appropriate in vitro QC assays, and (4) animal testing drastic reduction. The QC pipeline consists in performing sequentially and systematically a number of activities:

1. Identify the iPS and derived cell line products following the nomenclature committee specifications. This step is fundamental to ensure that research efforts are not wasted on cells that are unauthenticated, contaminated, or inadequately reprogrammed. Cell identity can be obtained by various means, *NSCB* (National Stem Cell Bank) profiles the NIH registry of hESC lines using PowerPlexSTR testing (16 STR alleles) using the WiCellPopwerPlex-16. STR analysis must be performed on DNA extracted upon cell arrival to the bank (master bank) and the working bank line. The same tests need to be re-performed after cell lines cryostorage and reseeded to confirm cell identity. The following STR are usually evaluated: Fluorescent D8S1179; D21S11; D7S820; CSF1PO; D3S1358; TH01(chr.11p15.5); D13S317; D16S539; D2S1338; D19S433; vWA(chr.12p13.31); TPOX(chr.2p25.3); D18S51; Amel (sex-typing); D5S818, and FGA (chr. 4q28). HLA typing is another form of cell authentication.
2. Establish pristine early passage stocks of cell lines defined as a master cell bank to enable accurate and independent verification of the working cell bank
3. Establish and standardise contamination quality control biological assays including second line (cross-contamination) and microorganism contamination. Two lines of quality controls can be identified: (i) first line, contamination by pathogens and cross-cell contamination and (ii) chromosomal stability.
 - (i) First line QC: (a) cross-cell contamination and (b) pathogens detection
 - (a) Cross-cell contamination may be explained by the presence of an additional cell population within a cell line that makes it “impure”, this may be caused by the overgrowth of a second (human or animal) cell line during the initial establishment or maintenance, by mislabeling or poor tissue culture practices.
 - (b) Among the microorganisms (bacteria, fungi, viruses) contaminations particular attention must be given to the presence of mycoplasma. These pathogens are the smallest self-replicating organisms (0.3–0.8 μm), difficult to detect and their contamination is frequent in laboratories. It has been reported that mycoplasma contamination occurs in 1 % of primary and 5 % of early passage cultures, increasing to 15–35 % in continuous long-term culturing cell lines. The effects of the contamination on the cells vary and depend on the type of mycoplasma specie present. Several species which are known to occur in 98 % of the laboratory infections of cell cultures: *Mycoplasma hyorhina*, *Mycoplasma arginini*, *Mycoplasma*

Table 8.1 lists three mycoplasma detection assays and detection limits generally used in cell biology labs

MycAlert®	PCR	Enrichment + DAPI staining
Most species	All TC related	Many species
10^4 – 10^5 cfu/mL	10^2 – 10^3 cfu/mL	1–10 cfu/mL

orale, *Mycoplasma fermentans*, *Mycoplasma salivarium*, *Mycoplasma hominis*, and *Acholeplasma laidawii*. The effects of mycoplasma infection are more insidious than those produced by other bacterias, fungi, and yeast. They may remain undetected by microscopic observation but can cause a reduction of growth rate, morphological changes, chromosomal aberrations, induction or suppression of cytokine expression, changes in membrane composition and alterations in the amino acid, and nucleic acid metabolism. A variety of tests for mycoplasma detection are available, and it is usually recommended to use at least two techniques for testing cell banks to ensure optimum sensitivity and specificity. The kits usually used in biobanks are: Detection of Mycoplasma using indirect DNA staining (Hoechst 33258); Mycoplasma detection using PCR and Detection of mycoplasma using the GenProbe mycoplasma tissue culture non-isotopic detection system (Cobo et al. 2005) (Table 8.1).

Contamination from adventitious agents

As part of the quality control process it is necessary to test for Adventitious Agents. Following the blood transfusion guidelines and the geographical areas of the incoming cells or tissues, blood-borne viruses such as Hepatitis B Virus (HBV), Human Immunodeficiency Virus (HIV), and others such as Hepatitis C virus (HCV) easily transmissible must be assayed. Viral contaminations may be due to the operator, cell or tissue culture reagents of animal or human origin and viral latency. Assays for a defined set of viruses must include: HCV, HBV, EBV, HCMV, HSV1-2, HPV, and HIV using the sensitive NAT technology with a detection efficiency ranging from 1×10^4 to 30×10^3 copies/mL.

- (ii) Genetic/genomic stability is a fundamental process in stem cell monitoring Fig. 8.8 (Diaferia et al. 2011). Characterize the genetic stability of the cell lines using G/Q-banded chromosome spreads able to detect deletions of greater than 10 Megabases in addition to comparative genomic hybridization (CGH) by microarray to look for micro chromosomal aberrations. This allows for an immediate evaluation of the population dynamic with regard to chromosomal stability, other “omics” technologies can be used to monitor the consistency of the results. To further validate the genomic stability of these cells, telomere length and telomerase activity can be analyzed. Telomere maintenance appears to be essential for chromosomal integrity and the prolonged persistence of stem cell function. hTERT (human telomerase reverse transcriptase) mRNA expression, relative telomerase activity by

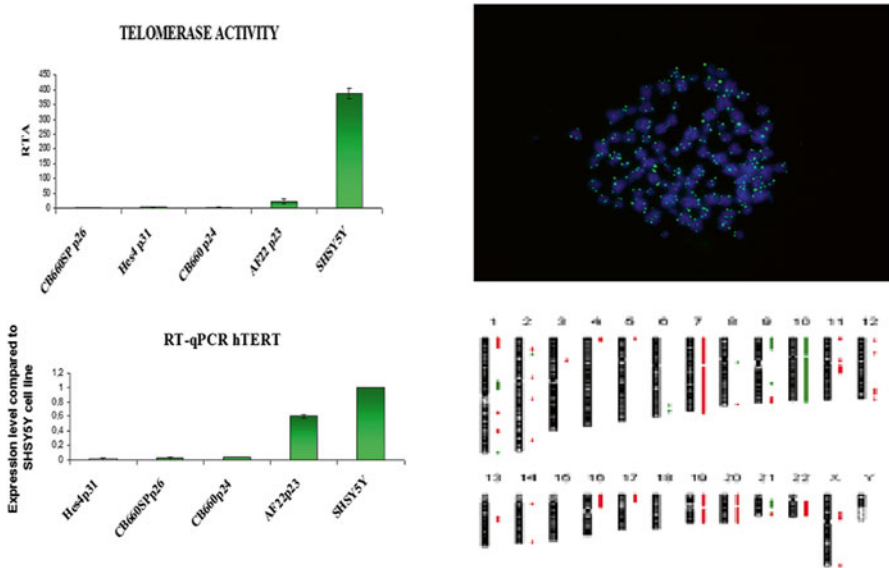


Fig. 8.8 Genetic stability

TRAP assay, average telomere length by fluorescent in situ hybridization (FISH), and telomere restriction fragment (TRF) length analysis and evaluation of γ -H2ax focus formation as a sign of DNA damage response (DDR) can all be determined as a means to follow the safety of the cell lines.

9.1 Epigenetic/Epigenomics Analysis

A process which can be performed in stem cell biobanks but not necessarily be part of the QC pipeline is to assess the epigenetic/epigenomic stability of the cells during extended passages. Emerging studies uncovered genetic and epigenetic alterations both in hES and hiPS whose origin can be attributed to culture adaptation, in vitro differentiation, and in reprogramming of iPS to preexisting parental somatic cell modifications. The genomic and epigenomic differences often involve cancer related genes and onco-microRNAs with negative effects on the function, stability, differentiation potential, and safety of the cells (Baronchelli et al. 2003).

Stem Cell lines pluripotency and differentiation characterization is to be assayed before cryopreservation and after thawing. Pluripotency markers (referring to those markers that indicate the ability to differentiate the cells into the three germ layers) following a combination of qRT-PCR assay (looking at the expression of at least 12 genes) and protein level using flow cytometry and immune-cytochemistry (Figs. 8.9 and 8.10).

Quality control 5 pre/post thawing differentiation

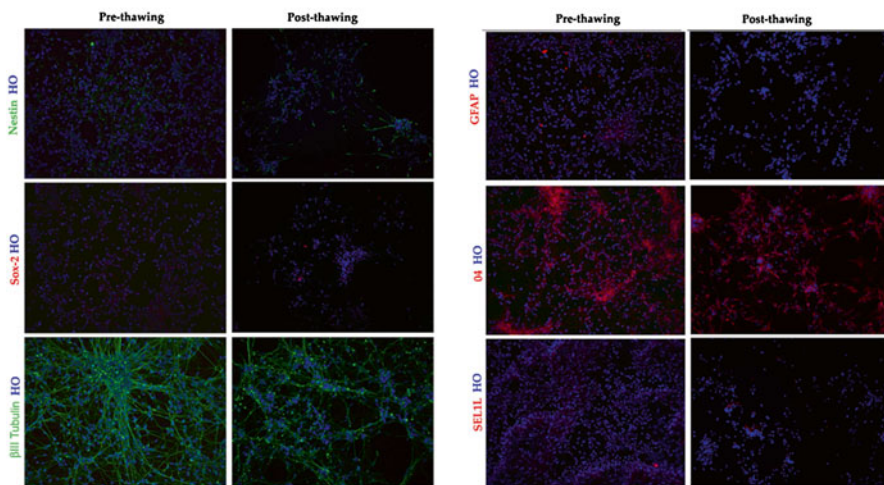


Fig. 8.9 Immunofluorescence analysis of neuronal derived human stem cells showing pluripotency and positivity to differentiation markers. The same cells were also assayed by q-PCR (not shown)

ISENET (www.isenet.it) human research stem cell bank workflow

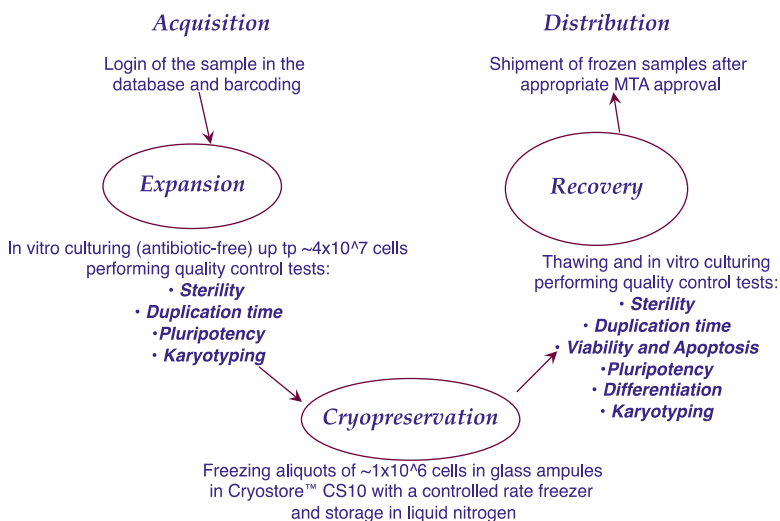


Fig. 8.10 ISENET cultures the stem cell lines in antibiotic free media and controls the sterility and stability of all the stem cells for research purposes before and after cryopreservation. Cells are cryopreserved in 10 % DMSO and/or in Cryostore CS10 (BioLife solution), a GMP cryoreagent containing 10 % DMSO but free of animal proteins

10 Important Elements to Include in the Material Transfer Agreement (MTA)

The MTA needs to be signed by the recipient scientist before product distribution. The RECIPIENT SCIENTIST must agree that the MATERIAL (Isasi et al. 2012):

- Is to be used solely for teaching and academic *research purposes* and for the Project indicated in the implementing letter annexed to the Agreement
- Will not be used in human subjects, in clinical trials, or for diagnostic purposes involving human subjects
- Is to be used only at the RECIPIENT organization and only in the RECIPIENT SCIENTIST's laboratory under the direction of the RECIPIENT SCIENTIST or others working under his/her direct supervision
- Will not be transferred to anyone else within the RECIPIENT organization without the prior written consent of the PROVIDER

Acknowledgment Ministero della salute# RF-MUL-2008-1248034.

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Chapter 9

Freezing and Freeze-Drying: The Future Perspective of Organ and Cell Preservation

Sara Maffei, Tiziana A.L. Brevini, and Fulvio Gandolfi

1 Cryobiology

1.1 Historical Background

Cryopreservation originates from the Greek word “kryos,” which means “cold or frost,” it indicates storage of cells or tissue, usually in liquid nitrogen, at temperatures below $-130\text{ }^{\circ}\text{C}$. The lowest natural temperature on earth is $-80\text{ }^{\circ}\text{C}$. Under normal pressure, the inert gas nitrogen, which is commonly used in cryopreservation, becomes a liquid at $-196\text{ }^{\circ}\text{C}$.

A small number of species like various fish, frogs, and insects can survive at low temperature using two different biological principles or a combination of these. The first process is cell dehydration, which reduces the freezing point secondary to a concentration of solutes. The other mechanism is by endogenous production of special antifreeze molecules, like sugars, which prevent formation of large ice crystal. However, recent studies provide the first demonstration that freeze tolerance can also occur amongst vertebrate species. A freeze tolerance vertebrate would make an excellent model system for studies of the medical cryopreservation of tissues and organs.

The main goal of the cryopreservation procedure is to minimize tissue injury from low, subzero temperatures (Shaw and Jones 2003). The storage at a low temperature can continue for decades and the only theoretical limitation of storage time is influence by cosmic radiation, which over several thousand years would degrade the genome of the cryopreserved material. This factor can be neglected in any practical work of cryopreservation in our society. In other words, by usage of cryopreservation the biological clock can be halted for an unlimited time (Kuwayama 2007).

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The term cryobiology refers to the knowledge and understanding of the effects of low temperature on cellular system and the utilization of this information to develop improved cryopreservation protocols. The science of cryobiology can be considered to have its starting point about 70 years ago (Luyet and Gehenio 1940). At that time, Luyet tried to achieve cryopreservation by cooling epidermal plant cells quickly and published a monograph about his pioneer work in 1940 (Luyet and Gehenio 1940). Three years later a scientist from England succeeded in cryopreservation of human and fowl spermatozoa, using glycerol as the agent that would protect against freezing injury (Polge et al. 1949).

The studies of cells and organ freezing have increased considerably since Prof. Polge's early attempts and it may well be that the technique of cryopreservation will be routinely used in the future to store cells and organs.

1.2 *Fundamental Cryobiology*

Therefore cells are mostly constituted of water, which makes up 60–85 % of the cell volume (Mazur 2004). In addition to free water, biological systems also contain “bound” water molecules. The “bound” water molecules that are intimately hydrogen-bonded to the atoms within molecules are such as proteins, RNA, DNA, or membrane phospholipids head groups. These water molecules form a substantial coat around the biological molecule and are essential to maintain cells structure and function (Shaw and Jones 2003). The “bound” water molecules are incapable of freezing (Sun 1999). Thus, water plays an important role in cryobiology since at cooling to low subzero temperature, 90 % of water in a cell will convert into ice (Mazur 2004) and the 10 % of bound water will not freeze.

Cell is in osmotic equilibrium, which means that concentrations of any solution inside and outside the cell are the same, since the cell membrane is semipermeable. In other words, water moves in or out of the cell depending on changes in solution concentration outside the cell and permeability of the cell membrane, which vary for each specific cell type. In addition to passive diffusion through the membrane lipid bilayer (Solomon 1968), water moves through water transport pores known as aquaporins (Verkman et al. 1996). These active transport proteins can transport water up to 100-fold more efficient than passive diffusion. Also, permeability is temperature dependant (Elmoazzen et al. 2002), so that permeability is higher with increasing temperature. Osmotic stress is defined as shrinkage and swelling of a cell due to osmotic differences between the inside and outside of the cell. When these changes are large it may lead to major damage of the cell and in the worst scenario to cell death.

In pure water, the highest temperature at which ice can form at normal pressure is 0 °C, but ice nuclei form only very reluctantly at this temperature, with the result that nuclei do not usually form until the temperature falls below 0 °C. In fact, ice in general forms at temperatures between –5 and –15 °C; through spontaneous or

induced (by seeding) ice nucleation. Once an ice nucleus has formed, further water molecules can very rapidly bond onto this frozen surface, allowing it to grow in size.

When ice forms there is a release of heat (the latent heat of fusion or crystallization), as the change from liquid to crystal releases energy. The amount of energy released may be considerable and commonly brings the temperature of the whole sample back up to 0 °C. With pure water the temperature will remain at 0 °C until the freezable water has formed ice, after which it will re-equilibrate with the ambient temperature. Following the crystallization of pure water, very little remains unfrozen even at temperatures as high as ± 5 °C (>80 % ice) or ± 10 °C (>90 % ice) state (Shaw and Jones 2003).

To thaw all this ice the temperature has to rise above the freezing point. Pure water therefore melts at 0 °C, the same as the highest temperature at which ice crystals can first form. During the thawing process, the change from crystal to liquid requires energy input. The solution which remains free on ice in a temperature below freezing point is in a supercooled state (Shaw and Jones 2003).

It is well established that cell survival rate during cryopreservation depends mostly on the cooling rate (Mazur 1970). In extensive studies of survival of yeast cells and human red blood cells, as a function of cooling rate, it was showed that a curve of survival versus cooling rate exhibited the shape of an inverted U. This curve can be interpreted as the resultant of two different mechanisms of which one damages living cells at high cooling rates and the other damages at low cooling rates. If the rate is too fast or too slow, cells do not survive and balancing these two cryoinjuries is the key to success (Mazur 1970).

1.3 Cryoprotectant Solutions

Adding salts and other solutes to pure water lowers the temperature at which ice forms/melts. There is no limit to how low the freezing/melting point can be depressed by solutes, with the result that solutions with very high concentrations may never form ice, rather they will solidify into an ice-free (vitreous) state. Even when salts and solutes are present, they rarely become incorporated into the ice crystals themselves, because of the very specific shape and bonding requirements of the growing ice crystals. Molecules other than water molecules are instead excluded from the growing ice crystals, and tend to accumulate between crystals or are physically pushed ahead of an advancing ice front. The formation and growth of ice crystals can, however, be modified by compounds such as antifreeze proteins (O'Neil et al. 1998), ice blocking agents (Wowk et al. 2000), and cryoprotectants.

Many chemicals have been recognized as having a cryoprotective function and these are known as "cryoprotectant solutions" (CPAs). During the freezing process, CPAs can serve several functions including lowering the freezing point, binding water to prevent it freezing at zero degree and decreasing membrane damages (Farrant 1980). According to their ability to transport through cell membrane, CPAs can be divided into two categories, permeable and non-permeable. Permeable CPAs can diffuse through cell membrane and non-permeable CPAs do not enter the cytoplasm.

Permeable CPAs are small, nonionic molecules with low toxicity and high solubility in water. The rate of CPA permeation and dilution is determined by the species, cell type, and stage of development, solution composition, temperature, and hydrostatic pressure (Liebermann et al. 2002; Shaw and Jones 2003). The most commonly used permeable CPAs are DMSO, propylene glycol (PROH), and ethylene glycol (EG). The exact mechanism by which these permeable CPAs protect living cells from cryoinjury is not completely understood. However, their general mechanisms seem to be by lowering the freezing point by replacement of some of the bound water molecules in and around proteins, deoxyribonucleic acid and head groups of phospholipids (Shaw and Jones 2003). Moreover, the permeable CPAs stabilize cellular proteins in the cytoplasm as well as in the cell membrane (Karlsson and Toner 1996). At entrance into the cell, they also reduce the concentration of electrolytes by lowering the amount of ice formed at a given temperature (Pegg 1984).

Non-permeable CPAs are usually long-chain polymers that are soluble in water and increase the osmolality of the solution. The most frequently used non-permeable CPAs are disaccharides (sucrose, glucose, fructose, sorbitol, saccharose, trehalose), some macromolecules (polyvinylpyrrolidone, polyvinyl alcohol, Ficoll), and proteins (bovine serum albumin; BSA). They contribute to cell dehydration, counteract osmotic stress and reduce the toxicity of permeable CPAs (Muldrew 2004).

1.4 Cryopreservation Techniques

There are two principally different cryopreservation procedures: vitrification and slow freezing. Recently, a new directional freezing technology was introduced (Arav and Natan 2009; Revel et al. 2004) which provides identical cooling rates through the specimen being frozen. All three procedures include four common steps: exposure of the samples to CPA, freezing/cooling to the storage temperature ($-196\text{ }^{\circ}\text{C}$), thawing/warming, and CPA removal. The terms “freezing and thawing” relate to the slow and directional freezing procedures whereas “cooling and warming” are more correct in relation to the vitrification procedure.

1.4.1 Vitrification

The term vitrification originated from the Greek word “vitri,” which means “glass.” Vitrification is a procedure in which solution/specimen solidifies to form a glasslike, or vitreous, state without any ice crystal formation during cooling and remains in this state throughout the warming step (Shaw and Jones 2003).

The main idea of ultrarapid cooling is to pass rapidly through the critical temperature zone where the cells are most sensitive for chilling injury (Liebermann et al. 2002). To achieve this glasslike solidification, high cooling rates in combination with high concentrations of CPA that interact strongly with water preventing water molecules from interaction to form ice, are used. In general, the rate of

cooling/warming and the concentration of the cryoprotectant required to achieve vitrification are inversely related. Typically, the temperature is reduced directly from 0 °C to -130 °C by plunging the sample into LN. Cooling rate at vitrification varies in the range of 2,500–30,000 °C/min or greater. However, one has to take into account that every cell seems to require its own optimal cooling rate. Also, the natural state of liquid water inside living cells is retained during vitrification (Sformo et al. 2010), leading to minimal disturbance within the cryopreserved sample. A sample that will be vitrified is exposed to CPA at the same manner as during slow freezing, but the vitrification procedure requires higher concentration of CPAs to achieve high cooling rate. Warming after vitrification is usually also fast. A major potential drawback of vitrification is the use of high concentration of cryoprotectant, and an unintentional negative impact of these cryoprotectants in turn can be their toxicity, which may affect the cell or tissue.

Vitrification has been recommended as the method of choice for oocytes or embryos. All developmental stages of human embryos cultured in vitro have been successfully vitrified and warmed, with the generation of offsprings. In 1999 and 2000 successful pregnancies and deliveries after vitrification and warming of human oocytes were reported (Kuleshova et al. 1999). Since that time, and because it seems to be that both entities appear to be especially chill-sensitive cells in assisted reproductive technology (ART), oocytes and blastocysts seem to receive a potentially significant boost in survival rates by avoiding ice crystallization using vitrification.

Vitrification is very simple, requires no expensive programmable freezing equipment, and relies especially on the placement of the embryo in a very small volume of vitrification medium (referred also as “minimal volume approach”) that must be cooled at extreme rates not obtainable in traditional enclosed cryostorage devices such as straws and vials. The importance of the use of a small volume, also referred as “minimal volume approach” was described and published in 2005 (Kuwayama et al. 2005) (Kuwayama 2007). In addition, recent publications have shown the dominance of warming rate over cooling rates in the survival of oocytes subjected to a vitrification procedure (Seki and Mazur 2009) (Mazur and Seki 2011). Vitrification has also been proposed for cryopreserving whole organs. However, there is evidence that a large tissue or whole organ will require slow cooling rates (Hubel et al. 1991). In fact, fractures of the organ could be caused by vitrification and warming procedures, and devitrification if the storage temperature is above the glass transition temperature (Fahy et al. 1990). The large problem is eliminating or sufficiently limiting ice formation throughout the large organs without inducing unacceptable toxicity caused by high doses of CPA (Fahy et al. 1990; Gavish et al. 2008)

1.4.2 Conventional Freezing

Convictional slow freezing is a method which aims to have a protocol that is slow enough to dehydrate the cells in order to prevent intracellular crystallization and fast enough to minimize osmotic stress to the cells (Mazur 1990). The convictional cooling procedure is usually ended when the temperature lies between -30 °C and

-80°C and after the sample can be plunged into LN (Shaw and Jones 2003). When applied on cells the convectional freezing method reduces the likelihood of intracellular ice formation by initiation of extracellular ice crystal formation at a high sub-zero temperature. The freezing rate is then responsible for how fast the extracellular ice crystals will grow. The extracellular ice draws water out of the cell until little amount of free water remains and only small (nonlethal) ice crystal has been formed (Shaw et al. 2000). The best outcome of this cryopreservation method is obtained when the rate of freezing allows equilibrium between cell dehydration and the rate at which water is integrated into extracellular ice crystals.

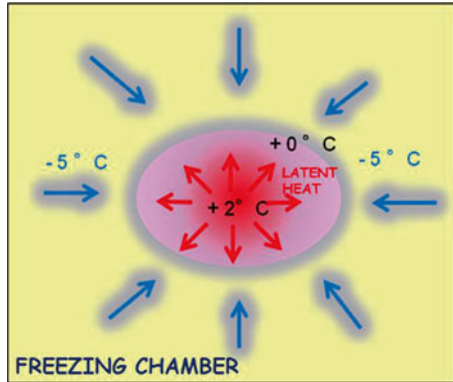
The rate of dehydration, which is the movement of water outward across the cell membrane, depends on the cooling rate that is determined by temperature drop in relation to time. Thus, the movement of water through the cell membrane decreases as a consequence of lower temperature (Mazur 1984).

It is important to emphasize that in this procedure, the cooling rate is dependent on the size and permeability of the specific cells/tissue to be cryopreserved. When trying to freeze a large tissue or a whole organ with convectional freezing, one of the major problems is that cooling rates will vary across the samples depending on the geometry (surface, depth, and volume) and heat conductivity. In fact, the conventional slow-freezing method involves lowering the temperature of the chamber in a controlled stepwise manner. This method is based on using multidirectional (equiaxial) heat transfer to achieve a rate of temperature change in the sample that depends on the thermal conductivity and geometrical shape of the container and of the biological material within it (Armitage 1987) (Bischof 2000; Karlsson and Toner 1996; Maas et al. 2000). The thermal gradient within the sample is determined implicitly by the temperature of the chamber and the thermal conductivity of the materials of the sample, and is not directly controllable. Furthermore, the ambient temperature gradients within the freezing chamber and the unreliability of temperature recording measurements add to the difficulty of achieving the optimal cooling rate.

One of the major problems that occurs during cryopreservation of large tissue using convectional freezing is the release of latent heat when crystallization occurs. There are two problems regarding latent heat release. The first problem is that the heat is transferred to colder areas in which the ice is just formed and so it could possibly re-warm the ice and cause local melting (Fig. 9.1).

The second problem is the isothermal period. When samples of large volume with relatively surface/volume are frozen, the release of latent heat may cause a long isothermal period in the material being frozen. At the same time, the temperature in the freezing chamber or on the surrounding medium is lowered, increasing the temperature difference between the sample and its surrounding (Fig. 9.1). Consequently, since the thermal conductivity of water (0.6) is lower than that of ice (1.6), when latent heat is no longer released, the temperature in the material being frozen will drop very quickly to a temperature close to the temperature of the surrounding environment. This might lead to a non-optimal cooling rate and possibly to cellular damage through intracellular crystallization (Petersen et al. 2006) (Armitage 1987) (Bischof 2000; Maas et al. 2000).

Fig. 9.1 Release of latent heat. See the temperature differences between the core and the surface of the sample and between sample and the freezing chamber



The current approach to overcome the problem of cell damage due to release of latent heat is the removal of the excessive heat through the adjustment of the cooling rate at specific time points and ensuring uniform freezing by maintaining a high ratio of surface to volume. In other words, the sample to be frozen is made as thin as possible, whereupon heat from the inner part of the sample, which is being released through the surface of the sample, will be removed faster due to the steep temperature gradient. In this way, it is possible to apply the optimal cooling rates for each sample while providing a heat sink for rapid absorption of the released latent heat.

1.4.3 Directional Freezing

Recently, a new directional freezing technology (MTG) was introduced (Arav and Natan 2009; Revel et al. 2004) which provides identical cooling rates through the specimen being frozen and it is aimed at cryopreserving whole organs (Fig. 9.2).

This technology is based on a series of heat conductive block (usually built of brass or aluminum) arranged in a line, with a straight track running through the blocks. Along the blocks, different temperatures (T1, T2, and T3, Fig. 9.2) can be set, in order to obtain a temperature gradient along the blocks (G1, G2, and G3). The blocks are separated by gap and the temperature of the block on one side of the gap (T1) is above the freezing point temperature and on the other side of the gap (T2) is below the freezing point temperature, thereby imposing a temperature gradient across the gap (G1). Biological samples to be frozen are placed inside test tube and are moved along the track at a certain velocity (V). The samples are frozen at rates according to the specific protocols of the samples; the rate of freezing is given by the temperature gradient multiplied by the velocity ($CR_{1,2,3} = G_{1,2,3} * V$).

The MTG can generate a linear thermal gradient in an organ/tissue to be cooled. It is possible to change heat transfer in a directional watt according to the heat transfer in the sample. The movement of the sample in the thermal gradient does not exceed this velocity in a way that heat will remain directional. In this way, heat



Fig. 9.2 The Multi-Thermal-Gradient (MTG) freezing device. (A) cooling block; (B) entrance of the sample's glass tube; (C) metal rod, which advances the sample's glass tube forward into the cooling channel in the cooling block; and (D) electric motor

transfer will propagate in the tissue in a linear way and the cooling rate for each point in the organ will propagate the same.

Heat transfer in directional solidification is opposed to the direction of sample movement. This is when the velocity is slower than the speed at which the heat is removed from the center of the sample toward the direction of the liquid and toward the conductive material of the device. In directional freezing the ice formation occurs according to the solution's freezing point temperature. Therefore supercooling, a non-equilibrium thermodynamic state in which a solution is cooled below its freezing point without crystallization, is avoided completely.

The freezing technology is based on directional freezing in which the biological material is transferred through a linear temperature gradient so that the cooling rate and ice front propagation is precisely controlled. Thus maximizing the survival rate of cells subjected to freezing and thawing requires the careful control of the freezing process. Although this method is rather new, results showed that directional freezing is a promising method for cell, tissue, and entire organ cryopreservation (Arav et al. 2005; Revel et al. 2004; Saragusty and Arav 2011).

1.4.4 Thawing

The thawing process and the removal of cryoprotectant are also important steps for a successful procedure. There is an opinion that the ice formed during thawing is less dangerous (Shaw et al. 1991), although some authors recognize slow thawing as a prime destructive factor (Arav and Natan 2009). The longer the duration of the thawing period, the greater the damage occurring to the cells. The increase of the

solute effects and the maximal growth of ice crystals are responsible for this damage. The large crystals have an abrasive action producing a mechanical disruption of cells. Crystal growth is maximal at the range of 0 °C and -40 °C. The disruptive ultrastructural changes in cells are increased with recrystallization. The thawing is most destructive if it is done completely, so that all of the frozen tissue is thawed, which takes full advantage of recrystallization. For these authors, basically two types of damage occur during slow thawing. The first is recrystallization, which is the growth of ice that was formed during the freezing process, and the second is the refreezing of the solution that occurs after complete thawing.

1.4.5 Cryoinjury

All cells and tissues that are subjected to freezing may be damaged during the freezing procedure or during thawing. This is referred to as cryoinjury and includes several types and causes of injuries. It may be a result of excessive cell dehydration (Mazur et al. 1972), intracellular ice formation (Acker et al. 2001), CPA toxicity (Fahy 2010), or a combination of these. It is well known that formation of ice inside the cell is lethal (Muldrew and McGann 1990).

This event occurs when a cell is unable to maintain equilibrium with the extracellular space during slow freezing or when the critical cooling rate is not obtained by vitrification methods. A recently introduced factor is the possible protective effect of the so-called “innocuous” intracellular ice formation (Acker and McGann 2003), which may reduce osmotic stress. The survival of a cell at freezing can also be affected by the specific forms of ice crystals that are present and mechanical deformation of cells caused by large extracellular ice crystals have been reported (Hubel et al. 1992). Moreover, a phenomenon known as devitrification results in recrystallization (Rall et al. 1984), that is a formation of ice crystals during warming. Despite the intended protective effect of CPAs, they may also be toxic and their presence may contribute to osmotic stress (Pedro et al. 1997). However, decreasing the time and temperature of cell exposure to CPAs can reduce the toxicity while stepwise addition and removal of CPAs decreases osmotic stress and excessive volume change of the cells (Wowk 2010). It should be pointed out that the exact mechanism of cell damage during cryopreservation and CPA toxicity has not yet been elucidated (Fahy 2010; Karlsson and Toner 1996).

2 Organ Banking

The narrow time frame between harvesting of organs and transplanting the donated organs area and the ever-growing need for organs, has motivated intensive research in the organs preservation. At present, solid organs, such as liver, are preserved *ex vivo* under hypothermic conditions during which the organ remains viable for only a short period of time that is limited to 12–24 h.

Because hypothermic preservation failed to provide a solution for the problem of long-term preservation of organs, alternative approaches were sought, among them storage at subzero conditions.

There are also numerous problems associated with freezing and thawing large vascularized organs, including the difficulty of generating heat transfer within a large thermal mass with a geometry that has little malleability, the packing density of cells within the organ, and the presence of many different cell types, each with its own requirements for optimal freezing and thawing conditions. Another problem associated with freezing large biological samples is the isothermal period caused by the massive release of latent heat during the crystallization stage of the process.

2.1 Cryopreservation of Whole Ovary

Current cancer treatments improve the long-term survival rates of young women suffering from malignancies. However, many of these therapies have lasting effects on fertility because they cause severe injuries to the ovarian reserve, which may lead to consequent premature ovarian failure, with a negative impact on the life quality of young cancer survivors (Maffei et al. 2013a, b).

At the present, one of the future strategies to preserve fertility is the transplantation of the cryopreserved whole intact ovary. Whole ovary transplantation with vascular anastomosis was proposed as a mechanism to reduce ischemic damage and, in theory, prolong the longevity of the graft (Jeremias et al. 2002). In this technique, the whole ovary with its vascular pedicle, is removed, cryopreserved, thawed, and then transplanted using a microvascular anastomosis into a heterotopic or orthotopic site (Jadoul et al. 2007).

Whole frozen ovary transplantation with microvascular anastomosis was first described in rats by Wang et al. (Wang et al. 2002). They described successful vascular transplantation of frozen–thawed rat ovaries and reproductive tract in four of seven (57 %) transplants, which survived for ≥ 60 days, were ovulatory and resulted in one pregnancy. Chen et al. (Chen et al. 2006) showed that frozen–thawed rabbit ovaries remained functional for at least 7 months after microvascular transplantation in 13 of 15 (86.7 %) animals.

The challenge of whole ovary cryopreservation and transplantation technology is not only the surgical technique but the cryopreservation protocol for an entire organ. It appears that, in large mammals and humans, cryopreserving such a large-sized intact ovary may prove more problematic than in small animals, largely due to the physical constraints that limit an appropriate heat transfer between the core and the periphery of the organ (Arav and Natan 2009). In addition, the large volume of the whole organ poses some limitation to the perfusion and diffusion of cryoprotectants (Falcone et al. 2004; Torre et al. 2013) (see Chap. 1.1.4.2). Both are essential for preventing intravascular ice formation which would irreversibly compromise a rapid and efficient resumption of the blood supply (Pegg 2010).

Nevertheless, data obtained from experiments mostly performed in sheep show some positive results (Grazul-Bilska et al. 2008) and pregnancies (Salle et al. 2002) but also highlight stromal and vascular damage following either slow freezing (Onions et al. 2009; Wallin et al. 2009) or vitrification (Courbiere et al. 2009; Salle et al. 2003).

Imhof et al. reported that 18 months after transplantation of whole ovary frozen with slow freezing, the follicular survival rate was less than an 8 % (Imhof et al. 2006). Other authors reported an even lower follicular survival rate (6 %) and the depletion of the entire follicular population after whole ovary vitrification and transplantation (Courbiere et al. 2009). Bedaiwy et al. (2003) reported the restoration of ovarian function after autotransplantation of intact frozen–thawed sheep ovaries with microvascular anastomosis, but it should be noted that 8 of 11 ovaries were lost due to thrombotic events in the reanastomosed vascular pedicle.

Although transplantation of the whole cryopreserved–thawed ovary was not performed in humans, cryopreservation of a whole ovary using a slow freezing protocol has been successfully attempted.

Recently, Martinez-Madrid et al. (Martinez-Madrid et al. 2007) described a cryopreservation protocol for intact human ovary with its vascular pedicle and proved high survival rates of follicles (75.1 %), small vessels and stroma, and a normal histological structure in all the ovarian components after thawing.

Most recently, a multigradient freezing device was used with promising results (see Chap. 1.1.4.3). Very recently Maffei et al. compared this new freezing method with the conventional freezing demonstrating that directional freezing allows good preservation of whole ovaries (Maffei et al. 2013a, b). In the follow-up study, they also showed that directional freezing improves the viability of cryopreserved ovarian tissue not only when used with whole organs but also with ovarian fragment (Maffei et al. 2013a, b). Furthermore, Arav et al. (Arav et al. 2005) reported progesterone activity 36 months after vascular transplantation of frozen–thawed sheep ovaries in three of eight transplants, and retrieval of six oocytes, resulting in embryonic development up to the 8-cell stage after parthenogenic activation. Even after 6 years the cryopreserved and transplanted tissue appeared functional as was indicated by histological examination. Normal tissue architecture, healthy blood vessels, and follicles at different stages were all observed in these ovaries. Even after 6 years the cryopreserved and transplanted tissue appeared functional as was indicated by histological examination. Normal tissue architecture, healthy blood vessels, and follicles at different stages were all observed in these ovaries (Arav et al. 2010). These results are promising in finding an optimal method for the cryopreservation of whole ovaries, so that in the future it will be possible to restore fertility long-term and to prevent premature menopause in young female cancer patients.

2.2 Cryopreservation of Kidney and Liver

Currently there are over 100,000 patients awaiting liver transplants with transplant rates of only 38 %. There is clearly a need to improve both preservation of the organs and to expand the donor pool.

Hypothermia was employed for organ preservation to reduce the kinetics of metabolic activities that would otherwise lead to cellular degradation when oxygen is removed from the donor organ. Simple cold storage (SCS) is a process by which the cryoprotectant is infused into the organ and then stored at hypothermic temperatures.

Currently there are two clinical methods for preserving livers: SCS and hypothermic machine perfusion (HMP). All other methods, such as normothermic machine perfusion (NMP) and oxygen persufflation (OP), are in various stages of preclinical and early clinical studies.

Hypothermia was employed for organ preservation to reduce the kinetics of metabolic activities that would otherwise lead to cellular degradation when oxygen is removed from the donor organ. SCS is a process by which the preservation solution is infused into the organ and then stored statically at hypothermic temperatures.

For the liver, prior to the discovery of the University of Wisconsin (UW) solution, preservation by SCS was limited to 6 h. With the UW solution, preservation improved to 16 h and allowed long distance procurement of the donor organ and this UW solution is currently used for other donor organs including kidneys, hearts, pancreas, intestine, and lungs.

The other method for preserving livers is the HMP. This method of preservation was developed for kidney to extend both preservation time and quality. HMP is able to supply oxygen to the tissue for ATP synthesis via perfusion of fluids that can carry oxygen. The oxygen requirements of cold tissues are low so the demand for oxygen is also low. This allows for slow flow rates during hypothermia and the relatively low oxygen carrying capacity of most crystalloid perfusates are adequate at low temperatures. However, perfusing organs at low temperatures cause side effects from both hypothermia and perfusion. Mitigating these side effects is a main function of most modern day perfusion solutions. HMP preservation has proven to be a reliable method for preserving good renal function in explanted cadaveric kidneys for transplantation (Moers et al. 2009). This method was used also for the liver preservation. In large animal studies, Piennar (Pienaar et al. 1990) showed that HMP with the UW solution could be used for 72 h preservation of the liver in the canine transplant model. In human, the first clinical trial was described in 2010 (Guarrera et al. 2010) demonstrating that HMP for donor livers provided safe and reliable preservation method.

3 Preservation of Blood Cells

3.1 Freeze-Drying

Water is essential to life, providing a universal solvent supporting bio-chemical activities within cells, which enables metabolism to continue and sustains all living processes. In the absence of water, life will cease, resulting in a state of death or

dormancy in live cells. Water also plays a major role in the degradation of stored material, providing conditions that potentiate autolysis, or promote the growth of spoilage organisms.

In order to stabilize labile products, it is therefore necessary to immobilize or reduce the water content of stored samples.

Vaccines, other biological materials, cells, and tissues can be stabilized by chilling or freezing. However, maintaining and transporting samples in the frozen state is costly, whereas freezer breakdown may result in the complete loss of valuable products (Fanget and Francon 1996).

Alternatively, bioproducts can be dried in air using high processing temperatures. Freeze-drying or lyophilization describe precisely the same process.

Freeze-drying can be technically defined as a controllable method of dehydrating labile products by vacuum desiccation and can be summarized in two steps:

1. Cooling of the liquid sample, followed by the conversion of freezable solution water into ice
2. “Evaporation” of water from the amorphous matrix

Freeze-drying has been used in the preparation of pharmaceuticals and vaccines as one of the most important processes for the preservation of heat-sensitive biological materials. Compared to other techniques, freeze-drying has some well-known advantages, including sample stability at room temperature, defined porous product structure, easy reconstitution by the addition of water or aqueous solution, and easy transportation (Xiao et al. 2004). Freeze-drying of cells used to be limited to prokaryotes. Over recent years, a few types of mammalian cells have been successfully freeze-dried, such as human erythrocytes and human platelets (Han et al. 2005; Wolkers et al. 2001). A study carried out in mouse demonstrated that freeze-dried platelets can be stored at room temperature for several months while their physiological viabilities remain (Wolkers et al. 2002).

3.2 Freeze-Drying of Human Blood Cells

Hematopoietic as well as other somatic cells are currently cryopreserved and stored in liquid nitrogen tanks, in nitrogen vapor phase or in 280 °C freezers (Choi et al. 2001; Halle et al. 2001; Rogers et al. 2001).

This conventional mode of cryopreservation is prone to transient warming events and various other hazards, such as cross-contamination (Bielanski et al. 2003; Morris 2005), and results in cell losses of 20–30 % (Laroche et al. 2005).

The possibility of storing blood cells and other cell lines in a dry state through the process of lyophilization could be of immense benefit for clinical application especially when long-term preservation is required.

In addition, freeze-drying does not involve the thawing process to which cell damage is attributed, particularly at large volumes (e.g. from recrystallization). However, the use of freeze-dried blood is still far from clinical application.

In order to freeze-dry the cells, researchers had to develop a method that would enable freezing in the absence of permeating cryoprotectant agents (CPAs) such as DMSO, glycerol or ethylene glycol, to name a few, and would enable the use of additives that have a high glass transition temperature (T_g) and that are solid at room temperature.

The major damaging factor associated with freeze-drying liposomes is lipid-phase transition, it is the main obstacle for successful cryopreservation of many cell types, including sperm (Drobnis et al. 1993) and oocytes (Arav et al. 1996). Chilling injury can be overcome by stabilizing the membrane phospholipids using disaccharides such as sucrose or trehalose (Crowe and Crowe 1991). Other approaches have involved changing the lipid composition of the membrane by using liposomes *in vitro* (Zeron et al. 2002a, b) or dietary additives *in vivo* (Zeron et al. 2002a, b).

The second factor is membrane fusion, liposomes stored above the high glass transition temperature have been shown to rapidly fuse and become damaged, and it was therefore concluded that glass transition or vitrification is an important factor in decreasing the chances of fusion upon drying (Crowe et al. 1998).

As described above, the freeze-drying of cells can be divided into two steps: the first is the freezing process during which large ice crystals are formed, pushing the cells into an area defined as the unfrozen fraction. The unfrozen fraction incorporates the cells that have dehydrated and vitrified in an amorphous matrix during the freezing process. The second step is sublimation of the ice crystals, which occurs in two stages, termed primary and secondary drying.

Recently, an Israeli group tried to freeze human red blood with a new device (Arav and Natan 2011). In this study, human blood freezing was performed by the MTG-1314 apparatus (see chapter 1.4.2). Lyophilization was performed by putting the frozen samples in the commercial lyophilizer near the condenser (Freezone Plus 6, Labconco), which reaches a temperature of $-80\text{ }^{\circ}\text{C}$, for 3.5 days. Thawing was performed by immersing the frozen samples in a water bath heated to $37\text{ }^{\circ}\text{C}$. At the end of the lyophilization process, samples were immediately rehydrated by adding 2.4 mL double-distilled water that had been pre-warmed to $37\text{ }^{\circ}\text{C}$.

Currently, Arav and his group working on determining the optimal conditions for sublimation and storage which they believe will result in further increasing the survival of cells after freeze-drying.

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