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S. Samuel Kim *Editor*

Oocyte Biology in Fertility Preservation

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This book is dedicated to Roger Gosden, my mentor and best friend, who has inspired many young scientists with his brilliant scientific mind and visions.

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Chapter 1
The Importance of Oocyte Biology for the Future of Fertility Preservation

S. Samuel Kim

Keywords Fertility preservation • Cancer • Cryopreservation • Ovarian transplantation • Follicle activation • Stem cells • Chemotherapy • DNA repair • Autophagy • In vitro maturation

The demand for fertility preservation in young cancer patients has increased dramatically over the last decade as a result of increased long-term survival after cancer therapy. Currently, more than 13 million cancer survivors are living in the USA, and approximately 450,000 cancer survivors are of reproductive age. Among those, 250,000 are women between the age of 20 and 39 years (Ries et al. 2008). Although advanced cancer treatment is lifesaving for many cancer patients, it can be severely gonadotoxic and lead to infertility and premature ovarian failure. A survey of fertility issues in young cancer patients revealed that fertility after cancer treatment is a major concern (Partridge et al. 2004). As most women of reproductive age wish to preserve fertility, it is important to provide adequate information and offer options for fertility preservation before cancer treatment.

Several strategies for fertility preservation have been developed by applying contemporary cryotechnologies and reproductive technologies; currently available options include the use of gonadotropin releasing hormone agonist (GnRHa), cryopreservation of mature oocytes and/or embryos after ovarian stimulation, cryopreservation of immature oocytes or in vitro maturation (IVM) followed by cryopreservation of MII oocytes without ovarian stimulation, and cryopreservation of ovarian tissue. Some of these options are investigational and may not be clinically applicable, however, the existence of these options itself provides hope to young cancer survivors.

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There has been a marked increase in technical efficiency and improved clinical outcomes with embryo cryopreservation and more recently with oocyte cryopreservation. Indeed, either of these technologies can be offered to post-pubertal women who desire fertility preservation. However, requirement of ovarian stimulation for cryopreservation of embryos or oocytes can be problematic in certain cancer patients since it can delay cancer treatment. In addition, the risk of high estrogen levels with ovarian stimulation should be considered in hormone dependent malignancies. Furthermore, oocyte retrieval can be an issue in patients with low blood counts since complications such as hemorrhage and infection can occur. Alternatively, oocyte retrieval without ovarian stimulation followed by in vitro maturation (IVM) and oocyte cryopreservation can be considered, although it is an investigational technology and the clinical efficacy of this procedure is hard to assess at present.

Cryopreservation of ovarian tissue has several advantages and carries fewer ethical dilemmas (Kim 2006). As ovarian tissue cryopreservation is achieved without the necessity for ovarian stimulation, there is no need to worry about increased estrogen levels and delayed cancer treatment. With cryopreserving ovarian issue, hundreds of immature oocytes can be preserved for future use. Cryopreservation of ovarian tissue has been quite successful (up to 80 % primordial follicle survival) with a slow freezing method, although this method has not been optimized. Recently, vitrification of ovarian tissue has been attempted with good results (Amorim et al. 2012; Sheikhi et al. 2011). Although promising, clinical application of vitrification is currently too premature due to scarcity of solid scientific data supporting its superiority. To investigate tissue damage at the molecular level after vitrification, we conducted a preliminary study using various markers of DNA damage and repair, apoptosis and autophagy. Our results revealed increased DNA damage and apoptosis post-vitrification in bovine and human ovarian tissue (Kim et al. 2010).

Most follicles stored in cryopreserved ovarian tissue are immature, as growing and mature follicles do not survive well after freezing and thawing. Immature oocytes are known to display greater tolerance to cryoinjury. The issue is how to effectively develop those immature oocytes stored in ovarian issue for fertilization. In theory, there are three strategies to grow and mature immature oocytes stored in ovarian tissue that include autotransplantation (either orthotopically or heterotopically), xenotransplantation, and in vitro culture (Kim et al. 2001). To date, orthotopic autotransplantation of frozen-thawed ovarian tissue (which resulted in over 20 live births worldwide) is the only practical and proven technology for restoration of fertility. No live births have been reported after heterotopic autotransplantation of frozen-thawed ovarian tissue, although restoration of endocrine function (even long-term lasting over 7 years) has been established (Kim 2012).

Ovarian tissue cryopreservation followed by transplantation appears to hold promise for fertility preservation in women and children with cancer. However, it is an invasive procedure that can limit the clinical application, and patients (and their parents) as well as health care providers should be aware of the surgical risks and complications. There are many uncertainties with this technology that include safety issues, adverse effects on offspring, and its efficacy (Kim 2006). Of those, two major concerns of autotransplantation are ischemic injury while waiting for revascularization and the risk of cancer cell reintroduction.

The ideal solution for issues with autotransplantation is developing innovative in vitro culture systems that can support the entire follicular growth and maturation process outside the human body. Though the factors and mechanisms involved in folliculogenesis are complex and not fully elucidated in humans, it is encouraging to see some progress in in vitro culture techniques. Most promising are the applications of an encapsulated three-dimensional follicle culture system (Hornick et al. 2012) and a multistep follicle culture system (Telfer and Mclaughlin 2011). Development of a perfect culture system for primordial follicles in stored human ovarian tissue is a truly challenging task, which cannot be achieved without first expanding the robust scientific basis of oocyte biology and ovarian physiology. Even after the successful achievement of an in vitro culture system (for primordial follicles in ovarian tissue), genetic and epigenetic integrity of these in vitro grown oocytes should be thoroughly investigated and secured before clinical applications.

Although current strategies for fertility preservation are useful, future strategies will focus on preventing gonadotoxicity of chemotherapy or radiation and/or protecting germ cells and somatic cells with noninvasive, real time, pharmaco-chemical protective agents. Thus, it is imperative to accumulate advanced knowledge on molecular mechanisms of follicular loss and activation, as well as DNA damage/repair mechanisms of both germ cells and somatic cells in the ovary upon exposure to chemotherapy or radiotherapy. In addition, research on genetic and epigenetic effects of fertility preservation (such as imprinting defects) should be emphasized to secure the health of offspring resulting from various fertility preservation strategies.

It is particularly noteworthy that several signaling pathways for oocyte activation have been investigated and recently discovered. Of those, PTEN/PI3K (phosphatase and tensin homolog deleted on chromosome ten/phosphatidyl inositol 3-kinase) and Tsc/mTORC1 (tuberous sclerosis complex/mammalian target of rapamycin complex 1) signaling are well characterized. PTEN/PI3K signaling is important for maintenance of follicle dormancy. Deletion of PTEN in the oocyte increases Akt phosphorylation and nuclear extrusion of Foxo3 proteins, which leads to activation of dormant primordial follicles (Li et al. 2010). In addition, oocyte specific deletion of either TSC1 or TSC2 leads to increased mTORC1, which causes the activation of primordial follicles. Furthermore, elevation of mTORC1 activities enhances activation of p70 S6 kinase 1 (S6K1)-rpS6 (Adhikari et al. 2012). Therefore, the alteration of these genes (PTEN, Foxo3, TSC, Akt, rpS6, PI3K, and mTORC) can be responsible for premature ovarian failure (POF). One of the goals of fertility preservation is prevention of follicular loss from gonadotoxic cancer treatment. This may be achieved in many ways, as previously mentioned, but fertility preservation through manipulation of activation of signaling pathways without invasive and expensive procedures is very attractive. As primordial follicles are much more resistant to chemotherapy than growing follicles, suppression of follicular activation may prevent a significant portion of follicular loss. Indeed, pharmacological inhibition of mTORC1 with rapamycin prevented over-activation of the primordial follicle pool in response to elevated PI3K signaling (Adhikari et al. 2010, 2012).

It is also possible to activate dormant follicles to generate mature oocytes in the ovary of patients with primary ovarian insufficiency (POI) or cryopreserved ovarian tissue. When the mouse ovary was treated in vitro with a PTEN inhibitor, such as

bpV9(pic) and PI3K activating peptide, the number of secondary and antral follicles increased significantly (about fourfold increase) after transplantation of ovarian tissue (Li et al. 2010).

The process of apoptosis and autophagy following stress and damage to germ cells and somatic cells is an important topic to investigate in conjunction with ovarian physiology. Furthermore, uncovering mechanisms of DNA damage/repair in oocytes after chemotherapy or radiotherapy will aid in developing new strategies for fertility preservation. Cytotoxic mechanisms of each chemotherapeutic agent and radiation have not been completely elucidated. However, it is known that DNA damage is the most common consequence of radiation and chemotoxicity. Most cells are equipped with programmed repair mechanisms for rapid response to DNA damage. Acetylation has now been assigned a role in regulation of the DNA damage response via autophagy. If repair is successful the cell survives. Failure to repair DNA damage leads to cell death, primarily through apoptosis. Autophagy helps to maintain cellular homeostasis and enhances cell survival under stress conditions. Recently, we investigated DNA damage and repair, autophagy, and apoptosis using biomarkers including γH2AX (DNA double strand break sensor), RAD 51 (repair protein), LC3B (autophagy marker), and cPARP (apoptosis marker) in bovine ovarian tissue after exposure to radiation, chemotherapy, and freezing–thawing (Kim et al. 2010; Albertini et al. 2011). The results demonstrated cancer treatment affects the genetic integrity of follicles leading to cell death, unless active repair mechanisms kick in to rescue DNA damage caused by radiation, chemotherapy, or cryoinjury in a timely manner. In addition, significant chromatin condensation was noticed in the germinal vesicle of immature follicles in cryopreserved ovarian tissue (especially with vitrification), which may affect the oocyte maturation and quality.

Lastly, it is hard to ignore one of the new exciting areas of biomedicine: stem cell research and its clinical applications. Future stem cell technologies may alter the landscape of fertility preservation. Indeed, Hayashi et al. demonstrated that embryonic stem cells and induced pluripotent stem cells (iPSC) can be induced into primordial germ cell-like cells (PGCLCs) (Hayashi et al. 2012). If human oocytes can be derived from stem cells, the supply of oocytes will no longer be an issue for women with premature ovarian failure. The concept and technology of gamete production in vitro from pluripotent stem cells is revolutionary. Furthermore, a recent study showed that ovaries of reproductive age women possess rare mitotically active germ cells (White et al. 2012). This report of potential existence of germ line stem cells in the adult ovary is fascinating but functionality of these germ cells should be confirmed.

In summary, fertility can be preserved via protecting oocytes from external and internal stress through minimizing DNA damage, stimulating DNA repair, inhibiting apoptosis, and facilitating autophagy. The next generation of fertility preservation strategies will rely on new scientific discoveries for mechanisms of follicular loss and activation, cell damage and repair responses of oocytes to chemotherapy and radiation therapy, genetic and epigenetic impact of cancer therapy and cryopreservation, and molecular genetic mechanisms of follicle development. In addition, it may be possible to create healthy germ cells from embryonic stem cells or iPSC, and to repopulate germ line stem cells in the ovary. If stem cell technologies to produce gametes can be perfected, the landscape of fertility preservation will be changed forever.

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Chapter 2
The Control of Oocyte Survival by Intrinsic and Extrinsic Factors

Meltem Kurus, Cengiz Karakaya, Mete Hakan Karalok, Grace To, and Joshua Johnson

Capsule Mechanisms that control the survival of oocytes and, by extension, the duration of ovarian function have been identified. However, it is still not clear whether oocyte "quality" is related to survival, nor is the role of the granulosa cells of follicles in follicle survival entirely understood. Here, we consider oocyte-intrinsic and oocyte-extrinsic mechanisms of oocyte loss and argue that developing a better understanding of such physiological events is needed to protect fertility, fecundity, and ovarian function in women.

Abstract The duration that ovaries function is, as is intuitive, controlled by the number of remaining oocytes within follicles. Once the number of follicles drops beneath a threshold number, ovarian function ceases. Thus, understanding mechanisms that control oocyte survival is paramount as we consider strategies to protect or prolong ovarian function in women. It is often assumed that physiological oocyte survival is entirely controlled by "oocyte- intrinsic" factors, such as poor genetic quality or accumulated damage to the oocyte itself. Oocytes that have poor genetic quality due to development or accumulated damage would then die sooner than

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those of higher "quality." Indeed, new data suggest that oocyte-intrinsic genetic quality as determined by the ability to repair double-stranded DNA breaks is a significant contributor to oocyte survival and the duration of ovarian function. However, the nature of the follicle, where the oocyte and surrounding granulosa cells exist in intimate contact and rely upon each other for survival signals and metabolic function, makes it unlikely that oocyte-intrinsic factors entirely control oocyte survival. We and others are assessing the role of adjacent somatic (granulosa) cells in follicle survival, determining the relative importance of "oocyte-extrinsic" factors.

Keywords Oocyte • Ovary • Function • Follicle • Granulosa

2.1 Introduction

2.1.1 CSO: Crime Scene Ovary

The follicles of the mammalian ovary consist of an oocyte and granulosa cell layers, bounded by a basement membrane and theca cells. Although there are many thousands of follicles present during the reproductive years, only one follicle per menstrual cycle will survive to ovulate and be made available for conception. The fate of the vast majority of follicles is instead death (referred to as atresia), and it is the rate of follicle death that determines the duration of ovarian function—the timing of the onset of menopause. Along with the numerical decline of follicles, the ability of human eggs to give rise to offspring declines precipitously around age 35 (Conway 1997; Hunt and Hassold 2008). This decline has been shown to correlate with age-related changes in oocyte meiotic spindle morphology and chromosome alignment (Hunt and Hassold 2008). This means that many oocytes survive for long periods of time that do not have the "quality" to give rise to offspring (below).

While a great deal has been revealed about the nature of follicle death, a precise understanding of the causes and timing of events that activate atresia is lacking. If we could perform an "autopsy" on individual follicles, we could pinpoint the ultimate cause of death. Did the subject die of "natural" causes, the aforementioned physiological atresia that is the fate of the majority of follicles? Certain factors need to be ruled out at the time of autopsy. We would like to use "forensic" techniques to reveal whether the oocyte died first, the granulosa cells, or, perhaps both died at the same time. In a population of follicles, why did one follicle die while its neighbor lived? Could it have been… foul play?

2.1.2 Foul Play: Poisoning and Murder

For ovarian follicles, external sources of foul play are well known. Poisons can be to blame, perhaps heavy metal (Banu et al. 2011) or toxic chemical (Hoyer et al. 2001) exposure. Radiotherapy (Oktay and Sonmezer 2008) and certain chemotherapeutic

agents (Beard et al. 1984; Jurisicova et al. 2006; Mazaud et al. 2002; Oktem and Oktay 2007; Sklar 2005) are remarkable in terms of how potently they damage the different cells of the follicle (Bar-Joseph et al. 2010; Roti Roti et al. 2012) and induce atresia. Or was it perhaps the insidious effects of long-term cigarette smoking? (Matikainen et al. 2001). Determining that the cause of follicle death was due to such exposures has led to major advances in protecting ovarian function from damage due to extra-physiological factors like these. The use of some of these stimuli in experimental models has also generated information about the massive loss of follicles that occurs physiologically over time.

Without evidence of foul play, a challenging question emerges in the autopsy: was there perhaps a hidden defect? Was there a previously hidden defect, akin to a heart anomaly in a human, that resulted in the subject's early demise? Are some oocytes less "fit" than others and thus sentenced to die early without warning? Here, we consider whether the cause of death was absolutely oocyte-intrinsic (Thomson et al. 2010). Was the oocyte itself less fit (or damaged such that it was less fit), and this was the ultimate cause of its demise? If we knew that the oocyte died first while the granulosa cell layers were intact (in the absence of an external toxic influence), we would favor this explanation. Alternatively, was there an oocyte-extrinsic cause of follicle death? Was the development of the granulosa cell layers abnormal, or, did granulosa cells begin to die first, and the oocyte died as indirect consequence of the loss of its supporting granulosa cells? In both cases, there could be genetic underpinnings of the compromise. Below, we will consider some evidence that certain alleles of genes involved in DNA repair can accelerate follicle death due to fostering oocyte or granulosa cell dysfunction.

Last, in insects, we have identified a particularly striking ovarian crime. We have shown that a unique oocyte-extrinsic mechanism exists where the surrounding somatic cells can be stimulated to destroy a perfectly intact oocyte (Thomson and Johnson 2010; Thomson et al. 2012). Data in Fig. 2.1 show that inhibition of the TOR pathway by feeding Drosophila fruit flies the specific inhibitor Rapamycin (RAP) results in a loss of oocyte (and, when mated, embryo) production. In those studies, we showed that this was a result of the somatic cells around the oocyte collapsing and phagocytosing the germ cell(s) before there were any signs of oocyte damage (Thomson and Johnson 2010). If embryos laid by these mothers are moved to food lacking RAP, the embryos essentially all hatch and develop to adulthood. This outcome supports an oocyte-extrinsic mode of oocyte death, the "killing" of the oocyte by a former partner and ally (Sect. 2 2, below). Using the same model, flies were instead treated with the chemotherapeutic agent Cyclophosphamide (CP) (Becker and Schoneich 1982; Hansmann 1974). Here, embryo production was unaffected. However, when embryos laid by flies treated with CP were moved to fresh food lacking drug, significantly fewer numbers of embryos survive even to hatch as larvae. We interpreted this as being the result of damage to the oocyte inside the mother(s), evidence of oocyte-intrinsic damage that did not result in follicle involution and clearance.

Could an equivalent process be activated in mammalian ovarian follicles? Can granulosa cells be activated such that they destroy intact oocytes? We have shown that the same stimulus, RAP treatment, results in the destruction of mouse oocyte within follicles cultured in vitro (Thomson and Johnson 2010). Whether these

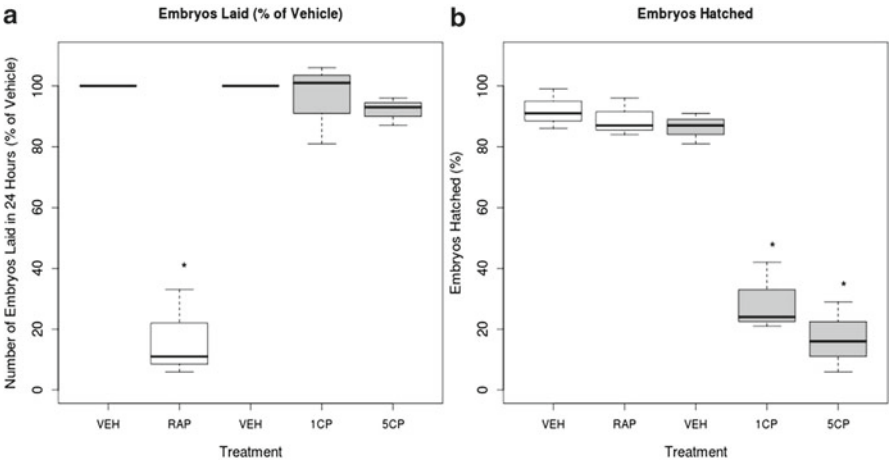

Fig. 2.1 Somatic versus germ line control of oocyte survival. Drosophila fruit flies were housed on food containing 10 mM RAP, 1 or 5 mM Cyclophosphamide (CP), or the respective vehicles for the two drugs. Embryos were moved to fresh food lacking any treatment hourly. As seen previously (Thomson and Johnson 2010), RAP treatment resulted in significantly reduced embryo laying (**a**). However, the embryos laid by RAP-treated mothers hatched and survived to adulthood at normal rates (**b**, *white boxes*). In contrast, CP treatment did not significantly affect embryo laying (and therefore, oocyte development). However, CP treatment did result in a dose-dependent decrease in embryo hatching, suggestive of oocyte damage

oocytes were similarly undamaged by the drug—as seen in the fly—before being destroyed by their surrounding granulosa cells remains unclear.

In this chapter, we consider the data about follicle death so that we might one day identify better strategies to protect the ovarian reserve of follicles. We first provide a more detailed discussion of follicle development, and consider how tightly intertwined oocytes' fate is with their attached granulosa cells. This is followed by a reconsideration of the concept of "oocyte quality," and finally, the forensic examination of factors that result in ovarian follicle atresia.

2.2 The Victims: Oocytes and Granulosa Cells Have a Dependent Relationship

During postnatal life, most oocytes exist in dormant primordial follicles within a single layer of a few "flat" squamous, non-proliferating granulosa cells. Upon growth activation, the granulosa cells switch to a cuboidal morphology and begin to proliferate while the oocyte initiates a growth phase. Follicle development consists of continued proliferation and differentiation of the granulosa layers, acquisition of an additional cell layer outside the basement membrane termed the theca, and continued oocyte growth. Eventually, a fluid-filled cavity forms within the granulosa layers termed the antrum, giving the follicle an asymmetrical character.

By this time, the granulosa cells in closest association with the oocyte are physically separate from those that make up the follicle wall; the granulosa cells in these locations are a separate functional lineage, the steroidogenic mural population (in the wall) and the cumulus population (surrounding the oocyte) (Chaffin et al. 2012; Johnson et al. 2001). Should the follicle survive to finish the journey of growth and maturation, the egg within its cumulus layers is expelled from the surface of the ovary during ovulation.

During the early stages of follicle growth, specialized gap junctions form between the oocyte and granulosa cells (Albertini and Rider 1994; Moor et al. 1981; Simon et al. 2006). These gap junctions are referred to as transzonal processes due to their crossing the zona pellucida. Proteins (Cieniewicz and Woodruff 2010; Curran and Woodruff 2007) and other critical macromolecules like cyclic AMP (Kalma et al. 2004; Sela-Abramovich et al. 2006) can transit these processes. Metabolic processes can be shared, or can be compartmentalized into the oocyte, or, granulosa cells (Eppig et al. 2005; Sugiura et al. 2005) meaning that they truly cannot develop without one another. Specifically, oocytes regulate glucose metabolism and the TCA cycle in granulosa cells by regulating the expression of glycolytic enzymes. A further example of this is the regulation of oocyte pH by granulosa cells (FitzHarris et al. 2007); immature oocytes lack pH-regulatory HCO(3)(−)/Cl(−) exchange factors and thus rely on the granulosa cells to maintain their physiological pH for normal function and development, and possibly survival.

We highlight the tight developmental interdependence of the oocyte and its attached granulosa cells to emphasize the difficulty of identifying the cause of death at the time of "autopsy." When considering the fate of the follicles in the ovary and the rate at which they die, it's important to know who to try to protect. Establishing that so much critical metabolic function (e.g., glycolysis and the TCA cycle) has been assigned to the granulosa cells, though, also means that granulosa cells, and not the oocyte, are the major source of metabolites that can cause oxidative stress during early follicle development. Does this also mean that reducing the amount of reactive oxygen species produced by granulosa cells will prevent damage from occurring in the oocyte? The accumulation of damage, including DNA breaks, due to oxidative stress over time has long been hypothesized to be a cause of ovarian aging and oocyte loss. A very recent paper provides both model-based basic information and clinical data from patients suggesting that it is the ability to repair DNA damage that declines with age.

2.3 Autopsy Results: Evaluation of Compromised DNA Repair Reveals an Oocyte-Intrinsic Mechanism

Accumulating damage with age is an attractive model for accelerating oocyte loss. Primordial follicles are thought to be present for the postnatal lifetime of most female mammals, and if this is true, the enclosed oocytes must experience a lifetime of potential oxidative "hits." DNA double-stranded breaks (DSBs) are one consequence of oxidative stress, arising due to interactions between the DNA and reactive oxygen species that result from normal metabolism. Titus et al. (2013), (see Johnson

and Keefe 2013, for a commentary) evaluated the role of genes known to be involved in the repair of DSBs, hypothesizing that reduced BRCA gene function would correspond to accelerated oocyte loss. The BRCA1 and 2 proteins function in the well-characterized general ATM (ataxia telangiectasia mutated) DNA repair complex (Gage et al. 2012; Kitagawa and Kastan 2005). DNA DSBs attract a complex of BRCA1, Rad50, Mre11, and ATM proteins, which in turn attract BRCA2 and Rad51 in order to elicit repair. If the lesion cannot be repaired, or, if the number of lesions overwhelms the available repair machinery, apoptotic cell death can be activated in a p53-dependent fashion.

Titus et al.'s data can be briefly summarized as follows. First, aged oocytes and follicles were found to have more histone H2A.X-positive lesions (marking DSBs) than their respective controls. The expression of Brca1 and Brca2 were each found to decrease with age. Heterozygous Brca1 mutant mice were found to have significantly reduced numbers of follicles at all stages of development, and increased H2A.X-positive lesions. This was paralleled by the finding that the indirect measure of the number of immature follicles, Anti-Müllerian hormone (AMH) levels, was significantly reduced in women harboring BRCA1 mutations, while women with BRCA2 mutations had a slight, but nonsignificant reduction in AMH levels. The data were bolstered by a direct look at the consequences of dysregulating DNA repair, by the delivery of siRNA targeted to individual molecules of the ATM repair complex into mouse oocytes. Knockdown of any of Brca1, Mre11, Rad51, or Atm resulted in significantly increased numbers of DSBs, an induction of the apoptosis marker active caspase 3, and decreased oocyte survival. In sum, these data reveal the potent role(s) for ATM-mediated DNA repair in oocyte survival, and also how that role changes during aging.

We can make several inferences from these data. We can infer that death susceptibility due to DNA DSBs begins with follicle growth activation (e.g., when primordial follicles begin to grow). This is because meiotic entry itself consists of the programmed generation of DNA DSBs. Progression past prophase did not appear to be affected by the level of DNA damage (Marangos and Carroll 2012). Further, it seems that this effect may be specific to DNA double-stranded breaks as at least one other type of lesion does not prevent progress through meiosis (Yuen et al. 2012). Last, we can infer that an oocyte's "quality" may also be highly dependent upon the capabilities of its DNA repair pathway, as summarized in the following model.

The cartoon in Fig. 2.2 uses stoplight colors to denote thresholds for oocyte survival and offspring-competence. After attempting to repair damage, oocytes that both survive and achieve the ability to give rise to offspring reach the green threshold on the graph. A separate pool of oocytes survives, but does not reach the threshold for being able to give rise to an offspring (yellow). Last, oocytes that inefficiently repair DSBs or other types of damage do not reach the survival threshold and follicle atresia is activated. In the case of BRCA mutations, the lack of efficient DNA repair reduces the number of oocytes that can efficiently achieve the threshold of repair required for survival, and perhaps the threshold for offspring production. Extremely exciting questions for the field to address are (1) is this truly an

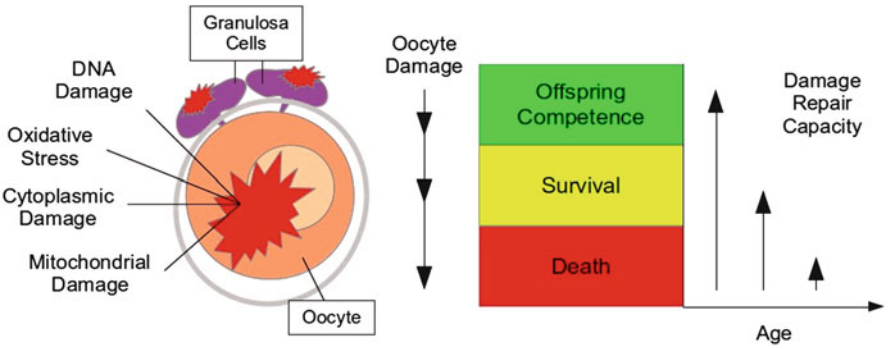

Fig. 2.2 Oocyte-intrinsic versus -extrinsic mechanisms of oocyte loss and thresholds of oocyte repair. The cartoon shows the two cell types considered as targets responsible for oocyte loss. Either the oocyte or a threshold number of granulosa cells is damaged (*red bursts*, *down arrows*), activating damage repair mechanisms. A model is supported, most strongly in the case of double-stranded DNA breaks (Titus et al. 2013), where the capacity to repair damage decreases with age (*graph at right*). Interestingly, oocytes can be repaired to the point that they survive (*yellow*), but cannot support the development of healthy offspring (*green*). The acceleration of oocyte loss with age then occurs when the ability to repair damage cannot reach the survival threshold. It remains unclear whether damage to granulosa cells (*purple*) in the absence of damage to the oocyte can result in follicle death by itself

oocyte-intrinsic outcome, where oocyte loss can occur in the absence of granulosa cell damage? (2) How are these theoretical threshold levels of DNA damage set? Are there specific numbers of DNA DSBs that can be tolerated? Last, (3) do the thresholds for survival and offspring competence depend on a woman's genetic background? Since these factors appear to change with age, do the age-related changes arise solely from declining DNA repair complex expression and activity? The deceptively simple concept of oocyte "quality" is at the heart of these critical forensic questions.

In our view, the jargon terms oocyte "quality" or "health" refer to "…the intrinsic genetic, epigenetic and cytoplasmic characteristics required for the completion of development and capability of producing a normal offspring." (Thomson et al. 2010). If a mechanism is present that ensures the selection and survival to ovulation of high-quality oocytes at the expense of those that cannot give rise to offspring, how might it work? If a quality control mechanism is present that selects for the eggs of highest quality, it must be that in young mammals, the threshold for survival is very stringent, with only few eggs incapable of producing offspring escaping the mechanism. The threshold must become less stringent with age, because the fraction of incompetent eggs that survive to ovulation increases. The identification of the role of DNA repair in the duration of ovarian function provides a basis for investigating these forensic questions and clarifying these concepts more precisely.

2.4 Recreating the Crime Scene: Models Used Dissect the Control of Oocyte Survival

Both in vitro and in vivo models have been used to address the questions of oocyte quality and the factors involved in oocyte death. Most of the time, these models are evaluated in terms of their ability to support follicle survival. In our case, however, we are interested in why, how, and at what rate follicles die.

In 1994, Spears et al. reported the generation of live pups after the two-dimensional culture of primary (small preantral) follicles to maturity, in vitro fertilization, and transfer of embryos to pseudopregnant females (Spears et al. 1994). Starting with these follicles, the group found that without EGF supplementation, 47 % of follicles survived ($n=240$) for 5 days in culture, and none of those produced eggs capable of early embryogenesis after fertilization. With EGF supplementation, nearly 70 % of follicles survived ($n=60$), but only 9 % of fertilized eggs from this group developed into 2-cell embryos. Additional serum supplementation increased the percentage of oocytes that could develop into 2-cell embryos to 41 % ($n=60$), and all of those developed into blastocysts. It is interesting that in the best case of follicles cultured identically, about 30 % of follicles died in culture, and under the best circumstances, approximately 60 % could not develop into even 2-cell embryos. These attrition rates are informative when considering the situation in vivo where only fractions of eggs would be selected to survive to ovulation.

In an attempt to generate mature eggs from primordial follicles, Eppig and O'Brien (1996) started by culturing the ovaries of newborn mice that would have contained many thousands of primordial follicles. After 8 days of culture, oocytes were isolated and cultured further. 192 two-cell embryos were generated after fertilizing the hundreds of mature eggs produced. Of those 192 embryos, only a single live pup was generated after transfer into mothers. A short time later, an improved culture method was provided by the same group (O'Brien et al. 2003). Here, again beginning with many thousands of primordial follicles, thousands of oocytes were produced in vitro that gave rise to 1,160 two-cell embryos; from those, 59 pups were produced after transfer. Pup survival was slightly lower than a control group using mature eggs that developed in vivo, but the survivors themselves were reported to be normal. More recent work has shown that offspring-competent oocytes can be generated from follicles that were cryopreserved and thawed (dela Pena et al. 2002; Kagawa et al. 2007; Liu et al. 2002).

The reason that these models are relevant to the questions of oocyte survival and competence to produce offspring, is that there is a high level of attrition at every step, in what appear to be equivalent follicles. What is killing off these thousands of follicles? Is it possible, that as seen through the course of reproductive life, that some follicles are doomed to atresia, and this cannot be corrected with ideal culture systems under any circumstances? This is highly reminiscent of the clinical situation, where a fraction of oocytes retrieved after gonadotropic stimulation seems always incapable of supporting blastocyst development and the generation of healthy babies (Patrizio et al. 2007). Accordingly, in vitro culture results in a high level of follicle attrition by atresia.

In our hands using a two-step culture method developed in the laboratory of Dr. Evelyn Telfer (McLaughlin and Telfer 2010; Smitz et al. 2010; Telfer et al. 2008), the attrition of human follicles occurred similarly (McLaughlin et al. 2011). Not only did about one-third of follicles die and disappear from strips of human ovarian cortex in the 6 days of culture, the addition of RAP again resulted in increased follicle attrition, such that about two-thirds of follicles went missing. What is the difference between those follicles that died and those that survived? We hypothesize, consistent with Titus et al.'s data, that individual follicles have differential abilities to repair damage induced during handling and culture. Some were able to repair this damage above the survival threshold, and some were not. The remaining question is whether anything can be done about this attrition, within the functioning ovary, or in in vitro models perhaps most relevant to fertility preservation strategies (see Johnson and Patrizio 2011, for a review).

2.5 Using Postmortem Examinations to Reduce Deaths in the Future

It is important to remember that the threshold of follicle survival does not always—and does not always need to—meet the threshold of offspring production. Supporting ovarian function, and potentially extending it so that menopause is delayed, can be considered as a powerful way to increase health and wellness parameters in aging women. In terms of developing techniques to offset DNA damage, one must contrast the situation with that seen in cancer patients. In cancer, many chemotherapeutic agents rely on their genotoxicity (e.g., induction of DNA double-stranded breaks) for their deleterious action upon rapidly dividing cancer cells. There, we seek in increase DNA double-stranded breaks and decrease the cells' ability to repair them. In the case of oocytes and follicles, however, we must decrease those breaks and foster DNA repair, a challenge that is beginning to be addressed.

So how might we reduce the rate of oocyte loss during the aging process? As seen with more general parameters like total lifespan, and global metabolic function, ovarian function has been extended in mouse trials of caloric restriction (Selesniemi et al. 2011), and treatment with different dietary agents. For example, resveratrol (Bentov et al. 2010; Kong et al. 2011; Liu et al. 2013) and dietary omega-3 fatty acids supplementation (Nehra et al. 2012) each increase the duration of ovarian function and extend the window of fertility in mice beyond the limits of controls. The common theme seems to be the ability to offset oxidative damage that would otherwise accumulate. This would seem to be the same target as that of therapies that target mitochondria (Barritt et al. 2001). Overall, dietary and/or pharmacological interventions are in development that could have similar effects upon the thresholds for oocyte survival and offspring competence (Fig. 2.2) in women. The more crime scenes we analyze, and the more basic and clinical forensic data that we generate, the more we will be able to protect follicles and potentially help them "cheat death."

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Chapter 3
Effects of Fertility Preservation on Oocyte Genomic Integrity

David F. Albertini and Rachel Olsen

Abstract An important adjunct to the field of fertility preservation is cryobiology. At present, the long-term storage of oocytes, embryos or ovarian tissues relies upon cryopreservation technologies that fall into roughly two different modalities: traditional slow freeze (SF) or rapid cooling, often invoking the process of vitrification. Unlike most cells in the body, female germ cells or oocytes present unique biophysical constraints as either isolated entities or within the context of ovarian follicles. Especially relevant is the fact that the oocyte nucleus, often referred to as the germinal vesicle, is highly hydrated and presents a voluminous non-chromatin occupied space that undergoes significant alterations in chromatin organization during its development. While the impact of cryopreservation on the integrity of the oocyte plasma membrane, organelles, and spindle cytoskeleton have been the focus of most studies to date, the short-term and long-term consequences of chilling and cryoprotectants on the chromosomal and genomic integrity has received much less attention. This chapter reviews the topic of genomic integrity at the level of the oocyte and provides guidelines for the design and implementation of strategies that will permit objective assessment of current and future protocols applied in the field of fertility preservation.

Keywords Chromatin • Chromosomes • Spindle • DNA damage • DNA repair • Nuclear architecture

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3.1 Introduction

Cryobiological methods are well established, generally in medicine, and have assumed an increasingly prominent role in the practice of reproductive medicine. Despite the many challenges that attend the perturbations in cell and tissue physiology implicit in low temperature storage, remarkable improvements in technology have resulted in the nearly routine use of cryopreservation for gametes, embryos, and most recently ovarian or testicular tissue. As with all physical or chemical insults, it is imperative to seek out improvements in technology that will minimize long and short range damage incurred by the freezing process or reactive events triggered by cryoprotectant exposure (Meirow 2000). The recovery of physiological homeostasis, whether at an individual cell or tissue level, eventually depends on the conditions used to restore the physical environment to a state identical to that from which the materials were derived. Thus, an additional challenge must be confronted when biological materials are thawed and re-equilibrated to restore ionic and osmotic conditions consistent with survival and continued functionality (Borini et al. 2010; Coticchio et al. 2006).

One of the least studied compartments of the cell in the field of cryobiology is the nucleus. This is surprising in view of the fact that cryostorage is most often performed with subsequent propagation and expansion of cells as an ulterior motive. Therefore, with respect to genetic integrity, it seems of utmost importance that this technology be designed to preserve nuclear and genomic structure in a manner consistent with faithful DNA replication and chromosome segregation so as to avoid at all costs the generation and possible expansion of mutations or unbalanced chromosome numbers (Ganem et al. 2009; Giunta et al. 2010). It is with this backdrop that the present chapter will consider recent advances in the subject of genomic integrity especially as it applies to the mammalian oocyte. Further topics to be covered will include appropriate methodologies for the assessment of DNA damage and the relationship of the DNA damage and repair response in the context of fertility preservation.

3.2 Genomic and Nuclear Integrity Defined

Far from the traditional views of nuclear and genome architecture of 25 years ago, the nucleus in general and the genome in particular are not only subjects of intense investigation, but have taken on a complex and dynamic character given the many advances in the fields of cell and molecular biology (Burke and Ellenberg 2002). From the perspective of genomics, and well beyond the realm of sequencing, the organization of genes and the regulation of their activation or repression have been revealed within the context of a dynamic landscape whereby chromatin domains between individual chromosomes assume spatial positions according to the specific needs of the cell. Whether recovering from single or double-strand breaks in DNA, or strand replication, or in anticipation of progressing through the cell cycle, energy consuming processes that direct enzymatic and structural modifications in

chromatin are tightly coupled to redox metabolism and cell signaling pathways to insure the propagation a healthy genome to the next generation (Agarwal et al. 2005). Moreover, superimposed on the chromatin-intrinsic and highly regulated changes in chromatin that occur during the cell cycle, differentiation, or death for that matter, are so-called epigenetic modifications such as DNA methylation and posttranslational alterations in chromatin-associated proteins. Many recent reviews have covered these topics in detail (Bromfield et al. 2008; Santos et al. 2002).

More to the point of this chapter are the unique attributes of the mammalian oocyte that make the regulation of its genetic integrity central to the field of fertility preservation (Adriaens et al. 2009). From the above discussion it should be appreciated that defining genetic and nuclear integrity is not a straightforward task. As shown in Table 3.1, the loss of genetic integrity can arise from perturbations at many levels of genome organization. For example, base substitutions account for many kinds of transmissible mutations. In addition, exposure to chemicals or radiation of various kinds is widely implicated in the formation of single or double-strand breaks in DNA. At the level of the chromosome, breaks and subsequent translocations are a common and well-known form of rearrangement accounting for many disease states. But several other lesions are known to occur with varying frequencies. Here the more common and better-studied cases of telomere shortening and chromosome malsegregation have been implicated in the loss of genetic integrity in human oocytes that occurs upon advancing maternal age (Hassold and Hunt 2001; Liu et al. 2002). Most recently, a process known as chromothripsis, originally thought to be an unusual form of chromosome rearrangement restricted to cancer cells, has been described in the female germ line of humans and sheep (Chiang et al. 2012). Chromothripsis involves the reformation of whole chromosomes after they have been pulverized, typically in response to a cell cycle delay imposed by environmental stress (Stephens et al. 2011).

Finally, there is growing appreciation that nuclear architecture itself can contribute to the loss of genetic integrity. Modifications in the nuclear lamina, and attendant defects in nucleo-cytoplasmic trafficking, have been implicated in genetic aging disorders such as progeria and a growing body of evidence supports the notion that a nucleo-skeleton imparts a structural backbone to the organization of nucleosomes and chromatin (Moir et al. 2000). Add to this the widely recognized function of lamin B in the nuclear matrix, long considered to be a fundamental linker between DNA and the nuclear envelope, and one can readily appreciate that many factors contribute to the maintenance of genetic integrity, as gleaned from somatic cells in most cases. So what makes the oocyte worthy of special attention over and above the obvious need to obtain healthy oocytes in the practice of reproductive medicine?

First and foremost, the nucleus of oocytes is among the largest of all cells. Known historically as the germinal vesicle, the chromatin within the oocyte nucleus is largely retained in an open configuration with heterochromatic foci until gene expression subsides and the chromatin becomes transcriptionally silent (Fig. 3.1). This is particularly evident in the oocytes of rodents. In contrast, many species exhibit true chromosomal patterns of diplotene during and following transcriptional silencing. A common feature of oocyte nuclei is the condensation of chromatin

Table 3.1 Factors that contribute to the loss of genetic integrity

Level of organization	Lesion	Damage Source	
Genomic			
	DNA strand breaks	*Endogenous*	
	Single strand breaks, SSB	DNA replication error	
	Double-strand breaks, DSB	DNA replication stress	
		Oxidative stress	
		Respiration	RNS
		Phagocytosis	ROS[a]
		Necrosis	RNS[b]
		Inflammation	
		Exogenous	
		Ionizing radiation	
		UV	
		γ-ray	
		X-ray	
		Chemotherapy	
		Mutagens	
		Toxins	
		Viruses	
Chromosomal			
	Rearrangements	DSB[c]	
	Translocations		
	Telomere shortening	Aging	
	Chromothripsis	Unknown	
	Malsegregation	Unknown	
Nuclear			
	Altered nuclear/cytoplasmic transport (Nuclear envelope permeability)	Aging	
	Intranuclear skeleton (Actin)	Unknown	
	DNA anchoring	Unknown	
	To nucleolus		
	To lamin B		

[a]Reactive oxygen species
[b]Reactive nitrogen species
[c]Double-strand break

around the nucleolus, another conspicuous feature of the mammalian oocyte. This arrangement reflects a spatial remodeling of chromosomes bearing the nucleolar organizing genes. Thus, variations exist in oocyte chromatin between species and according to the degree of transcriptional activity (De La Fuente et al. 2004).

Second, the volume of the mammalian oocyte nucleus is far greater than that found in somatic cells. Given the chromatin/DNA content is no different from a somatic cell that has completed the S phase of the cell cycle, this feature underscores the high degree of hydration that must exist even before an oocyte has started to grow within the confines of the ovarian follicle.

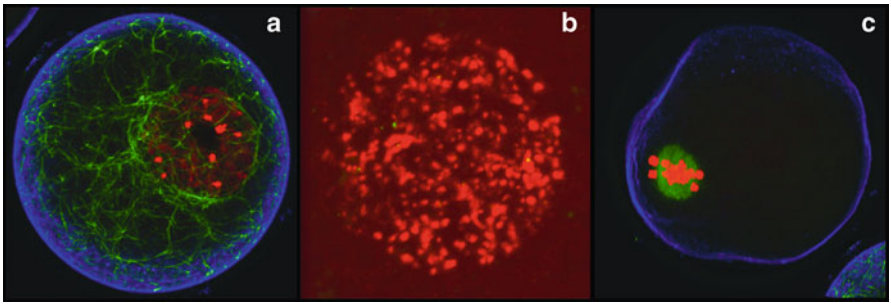

Fig. 3.1 Fluorescence micrographs of oocyte chromatin in diffuse (**a**), condensed (**b**), and chromosomal (**c**) states. All samples were stained with ethidium homodimer to label DNA and microtubules (*green*). Mouse oocytes are shown in G2 (**a**) and metaphase-1 (**c**) states while (**b**) is a bovine oocyte GV illustrating diplotene chromosomes

Table 3.2 Genetic plasticity during oogenesis

Stages	Meiotic status	Process	Mechanism	Consequence
Early				
	Prophase	Homologous recombination	DDR[a]	Asynapsis Dys-synapsis
Diapause				
	Diplotene	Transcriptional regulation Activation Repression	DDR (?)	DSB[b] retention
		Chromatin remodeling	Histone modifications	
Late				
	M phase I	Homologue segregation	Spindle cohesins	Aneuploidy
	M phase II	Chromatid segregation	Spindle cohesins	Aneuploidy

[a]DNA damage repair
[b]Double-strand break

It is important to emphasize in closing this section that the mammalian oocyte nucleus undergoes a series of profound modifications in chromatin organization at various stages of oogenesis (Mattson and Albertini 1990). Accordingly, we recommend subdividing further discussions of oocyte genetic integrity according to the sequence of processes that define the oocyte's developmental history. This categorization will also be useful as it allows for a separation of functions relevant to the handling and utility of human oocytes in a clinical setting. Moreover, it highlights the changing character and relative risks for genetic damage that might be expected depending on patient age and or treatments, as summarized in Table 3.2.

The unique process of homologous recombination and synapsis is confined to the germ line and represents the earliest event in the life history of the oocyte requiring major chromosome reorganization (Burgoyne et al. 2009). The process is mediated by a highly conserved DNA damage and repair response (DDR) and affects the

exchange of genetic materials between adjacent chromatids within homologous chromosomes. The reparation of DSBs at crossover sites sometimes fails, resulting in asynapsis of homologues that can ultimately lead to nondisjunction at metaphase of meiosis 1. Oocytes that fail early in meiotic prophase are eliminated by apoptosis. Those that survive become enclosed within primordial follicles where, in humans, they can spend up to 40 years arrested in meiotic prophase. This constitutes the second event in oogenesis, which we refer to as diapause since these oocytes remain in a precarious state for extended periods of time (Albertini et al. 2008; Hutt and Albertini 2007).

The diapause condition defines oocytes in diplotene of meiosis 1. Here again, chromatin remodeling is pronounced as noted above due to changes in transcriptional activity associated with the growth phase of oogenesis. We know little about how genetic integrity is maintained at this stage but hypothesize that an active DDR must exist to repair DNA strand breaks that would otherwise accumulate over time and in response to cumulative oxidative insults.

The final and defining event in oogenesis has involves the choreographing of chromosome segregation during the process of ovulation. Two successive M phases of the meiotic cell cycle direct the physical dissociation of first paired homologues, and later sister chromatids, in the process of reduction to a haploid state. Both spindle assembly checkpoints for monitoring chromosome alignment and maintenance of cohesins have been implicated during this process to assure equivalent segregation. Interestingly, several reports now indicate that deficiencies in these surveillance mechanisms may underlie the increase in aneuploidy associated with human oocytes from older women (Hassold and Hunt 2001).

3.3 Methods for Assessing Genomic Integrity

At present, most methods used to detect genomic integrity rely upon traditional chromosome spreads and karyotyping, although the increased adoption of both FISH and more recently comparative genomic hybridization has both materially extended the resolution and clinical utility for studying this problem (Dyson et al. 2001). In particular, genetic diagnosis of human embryos is now being used widely and the implementation of CGH for reading first or second polar genetic composition allows for a direct analysis and diagnosis of oocyte genetic integrity prior to in vitro fertilization (Fragouli et al. 2010).

But these technologies remain impractical for assessments of genetic integrity in oocytes that have yet to undergo meiotic maturation or remain in an ovarian context. Thus, for both oocyte cryopreservation and ovarian tissue freezing, there is a clear need to develop new technologies that could allow for a more subtle evaluation of genetic integrity prior to and following cryopreservation. The most common assay used to date is the TUNEL assay for the detection of apoptosis. This provides a reasonable approximation of the incidence of cell death by apoptosis and, when coupled with the immunohistochemical detection of other biomarkers such as

Annexin V, cleaved PARP, and fractin, can be helpful as an initial screen when evaluating novel freezing protocols and variations on the theme of cryoprotectant choice and re-equilibration conditions. The shortcoming of this approach is that it cannot evaluate other mechanisms of cell death and survival, including autophagy.

In conclusion, developing methodologies that will permit a more discriminating analysis of cell and tissue viability, and especially oocyte genomic integrity, is much needed. Ideally, the adoption of vital staining techniques that would allow for the detection and quantitation of pathways involved in oocyte activation and genetic alterations would materially extend the field of fertility preservation (Amorim et al. 2012). However, caution is warranted due to the possible effects of fluorescent dyes and their excited state actions in the generation of free radicals, itself possibly inducing genetic damage (Agarwal et al. 2005).

3.4 The Impact of Current Technologies

The main focus for oocyte evaluation in fertility preservation is founded in the areas of oocyte (OC) and ovarian tissue cryopreservation (OTC). In the case of OC, the most widely adopted technology was originally that of slow freeze protocols although vitrification has taken on an increasing role due to the demonstration of improved survival upon thawing. It should be noted that nearly all studies in the human have been performed using metaphase-2 arrested oocytes. The reasons for this are manifold and include the fact that spindle integrity and chromosome alignment are readily achieved after thawing using slow freeze or vitrification protocols with the higher concentrations of cryoprotectant used in vitrification believed to effect great stability of the meiotic spindle microtubules (Bromfield et al. 2009). Other variables have been identified that have been well characterized in the case of slow freezing of human oocytes, but less so for vitrified oocytes. These include, but are not limited to patient age and the timing during re-equilibration required to achieve optimal restoration of the spindle (Borini et al. 2010; Coticchio et al. 2009).

It should be apparent that OC will continue to be widely used in fertility preservation strategies for patients who avail mature oocytes following routine cycles of controlled ovarian hyperstimulation (COH). The situation is, however, very different for younger patients and for patients whose cancer treatments contraindicate the use of COH (Amorim et al. 2009). In both of these cases, the more plausible strategy has been to cryopreserve ovarian tissue for subsequent transplantation. While the number of successful transplant recipients has increased over time, the one remaining problem has been the progressive loss of primordial follicles that compromises fecundity chances or the need to maintain ovarian function for extended periods of time (Donnez et al. 2010). Here, more questions arise. Is OTC itself altering the follicle reserve by predisposing follicles to premature activation or by damaging somatic cell or oocyte genetic integrity? What are the short-term and long-term consequences of thawing and transplantation given inflammatory conditions that could occur under in vivo or in vitro conditions? These issues and others pose serious

obstacles for the field and prompt further inquiry into the genetic integrity of oocytes and somatic elements of the ovary following OTC, culture of ovarian tissues, or transplantation (Mattson and Albertini 1990; Wickramasinghe and Albertini 1992).

3.5 Directions for the Future

In conclusion, the subject of oocyte genetic integrity remains poorly understood with exception to those factors that mediate the final segregation of chromosomes during the latest stages of oogenesis. As the field of fertility preservation moves forward, it is clear that a deeper understanding of oocyte genetic integrity is required in the context of the many levels of chromatin organization that will contribute to the genome provisions inherited by the embryo. To achieve this, many of the tools and approaches that have been used to evaluate genetic integrity in cancer biology now need to be applied in a vigorous fashion before technological improvements can be realized for purposes of fertility preservation.

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Chapter 4
In Vitro Activation of Dormant Follicles for Fertility Preservation

Deepak Adhikari

Abstract Recent advances in radiotherapy and chemotherapy have led to higher cure rates for female children and adolescents with cancer. However, these treatments adversely affect germ cell survival, and ovarian failure is thus a probable side effect of these anticancer therapies. Moreover, an increasing number of women are choosing to postpone childbearing until later in life, but their primordial follicle reserves degenerate with advancing age. Thus there is a pressing need for the development of fertility preservation methods for these individuals. Ovarian tissue cryopreservation prior to loss of the primordial follicle population either due to cancer treatments or normal aging is a promising option for safeguarding fertility. A complete in vitro maturation (IVM) system could help generate mature eggs for later use without the patient having to undergo the cumbersome process involved in current assisted reproduction methods to generate mature eggs. Cryopreserved ovarian cortical tissues have attracted the attention of reproductive biologists and clinicians because of the large number of safely frozen primordial follicles in them, and it is theoretically possible to use these follicles for in vitro activation (IVA) and subsequent IVM. Ovarian tissue collection is independent of patient age and social or personal conditions. Despite being widely accepted potential techniques for fertility preservation, IVA and IVM of human primordial follicles to obtain fertilizable eggs remains far from reality. This chapter highlights the current achievements and obstacles in obtaining growing follicles through activation of dormant follicles.

Keywords Primordial follicle • Fertility preservation • In vitro activation

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4.1 Introduction

4.1.1 The Importance of Primordial Follicles for Female Fertility

Primordial follicles are the first small follicles to appear in the mammalian ovary. To produce a mature oocyte, a primordial follicle emerges from its pool to become a primary follicle, and this transition is known as primordial follicle activation. The majority of primordial follicles are maintained in a dormant state to be used in later reproductive life (Adhikari and Liu 2009; Reddy et al. 2010). A limited number of primordial follicles are recruited into the growing follicle pool through follicular activation, which is followed by subsequent follicular development and the production of fertilizable eggs. The remainders of the primordial follicles remain dormant and can survive for months, years, or even decades depending on the species (Hirshfield 1991; McGee and Hsueh 2000).

Once formed, the pool of primordial follicles serves as a source of developing follicles and oocytes, and this pool size declines with age (Broekmans et al. 2007; Hansen et al. 2008). Thus, the steady and highly regulated decline in the primordial follicle population determines the time span of female fertility. However, primordial follicles are vulnerable to ionizing radiation and to the alkylating chemotherapeutic agents that are used for the treatment of various cancers (Meirow et al. 2010; Meirow and Nugent 2001; Wallace et al. 1989). Such therapies lead to the loss of primordial follicles and cause accelerated depletion of the ovarian reserve leading to premature ovarian failure (Byrne et al. 1992; Kim 2006; Lobo 2005). Moreover, it is increasingly common for many women to choose to delay childbearing until later in life. With advancing age, the primordial follicle reserve degenerates (Gurtcheff and Klein 2011; Hansen et al. 2008) and the eggs that are produced are of lower quality (Broekmans et al. 2007; Jessberger 2012) leading to a decline in fertility. Thus, there is an increasing need for preserving fertility in both cancer patients and healthy women.

Primordial follicles are located in the cortical region of the ovaries (Gougeon and Chainy 1987) and they can withstand freezing and thawing processes. This is possibly due to their relatively high surface area-to-volume ratio, their low metabolic rate, and the absence of the zona pellucida in the oocytes of primordial follicles (Gosden 2005; Gosden et al. 1994; Oktay et al. 1997a). Cryopreservation of ovarian cortical tissue can thus save a large population of primordial follicles for later use (Hovatta 2004). Although different options of fertility preservation are available (Table 4.1), ovarian tissue cryopreservation remains the only probable fertility-preserving option for children because neither ovarian stimulation and collection of mature oocytes nor collection of fertilized embryos is feasible (Davis 2006; Jeruss and Woodruff 2009; Kim 2006). Regardless of the age of an individual, primordial follicles are the most predominant type of follicle among the follicles at various stages of development. Thus, over the past decade more and more fertility centers

Table 4.1 A comparison of in vitro activation (IVA) and in vitro maturation (IVM) of dormant follicles with other available methods of fertility preservation

Method	Advantages	Disadvantages
Protection of ovaries during anticancer treatment	Feasible in practice Does not require a male partner or donor spermatozoa	Efficacy is not well documented Side effects and complications
Ovarian tissue cryopreservation and transplantation	Suitable for prepubescent females, single women, and patients who need immediate cancer treatment Ovarian stimulation not required; safe for hormone-sensitive breast cancer patients	May contain tumor cells that may be inadvertently reimplanted Controversy as to its potential Low success rate
Embryo cryopreservation	The most reliable and clinically available technique for fertility preservation in women	Not suitable when urgent cancer treatment is required Not suitable for hormone-sensitive breast cancer patients Requires a male partner or donor spermatozoa
Oocyte cryopreservation	Does not require a male partner or donor spermatozoa	Low success rate Not suitable when urgent cancer treatment is required. Not suitable for hormone-sensitive breast cancer patients
IVA and subsequent IVM of dormant follicles	Has the potential to provide a large number of eggs for in vitro fertilization (IVF) Suitable for prepubescent females, single women, and patients who need immediate cancer treatment Ovarian stimulation not required; safe for hormone-sensitive breast cancer patients	Still experimental in humans due to lack of a good IVM method to culture primordial follicles

have turned to cryopreservation of ovarian tissues prior to gonadotoxic therapies, especially in prepubescent patients (Anderson et al. 2008; Hovatta 2004; Rosendahl et al. 2011). The hope is that these cryopreserved cortical tissues might possibly be used for generating mature, fertilizable oocytes.

One way of using the cryopreserved ovarian cortical tissues is by transplanting them back into the patient after thawing. First attempted in 2004 (Donnez et al. 2004), ovarian tissue reimplantation has so far led to the birth of 13 healthy babies from 10 different women (reviewed in: Donnez et al. 2011). However, ovarian tissue transplantation is technically demanding and only a few centers have reported success with this technique. Additionally, it is not conclusively known whether the restored ovarian function is due to residual or transplanted tissue (Hubinont et al. 2012; Oktay and Tilly 2004). Considering the number of patients whose ovarian tissues have been

frozen and banked at various centers around the world, the efficacy of ovarian tissue transplantation in achieving successful pregnancies has not been very significant. Moreover, transplanting ovarian tissue back into cancer survivors runs the risk of reintroducing cancer cells and this method is contraindicated in cases of ovarian disease and blood-borne illnesses (Shaw and Trounson 1997; Shaw et al. 1996).

Research on IVA and subsequent IVM of primordial follicles in cryopreserved ovarian tissue has recently gained enormous attention as a potential means of obtaining mature eggs for IVF. Fertilizable eggs and live pups have already been obtained from cultured primordial follicles in mice (Eppig and O'Brien 1996; O'Brien et al. 2003), but similar success in humans has not yet been achieved.

4.1.2 Understanding the Mechanisms of Primordial Follicle Activation

The activation of primordial follicles is a highly regulated process, but its underlying mechanisms are not fully understood (Adhikari and Liu 2009). For this reason, we do not know the exact culture conditions that should be used to enhance the activation of primordial follicles while at the same time enhancing their survival.

4.1.3 Suppressors of Primordial Follicle Activation

Several molecules that suppress the growth of primordial follicles have recently been discovered in mice. These include oocyte-specific phosphatase and tensin homolog deleted on chromosome ten (PTEN) (Reddy et al. 2008), tuberous sclerosis complex 1 (Tsc1) (Adhikari et al. 2010), tuberous sclerosis complex 2 (Tsc2) (Adhikari et al. 2009), Foxo3a (Castrillon et al. 2003), and p27 (Rajareddy et al. 2007). Similarly, anti-Müllerian hormone (AMH) (Durlinger et al. 1999, 2001, 2002a, b) that is produced by the growing follicles has been found to inhibit subsequent primordial follicle activation. In animal models in which the genes for these proteins have been deleted, the pool of primordial follicles is prematurely activated showing that these molecules suppress the growth of primordial follicles and maintain them in a quiescent state.

4.1.4 Enhancers of Primordial Follicle Activation

Likewise, in vitro experiments and studies with genetically modified mice have led to the discoveries of several signaling molecules and growth factors that enhance primordial follicle activation (Table 4.2).

Table 4.2 Molecules/pathways involved in the activation of primordial follicles

Molecules/pathways	Description
Kit/Kit ligand (KL)	Kit receptor protein tyrosine kinase is expressed on the surface of oocytes and its ligand, KL (also called stem cell factor, SCF), is produced by the surrounding granulosa cells. Together these facilitate the development of primordial follicles in mouse and rat ovaries (Driancourt et al. 2000; Huang et al. 1993; Kuroda et al. 1988; Packer et al. 1994; Parrott and Skinner 1999; Vanderhyden 2002; Yoshida et al. 1997). Addition of KL to the in vitro culture medium can also enhance primordial follicle activation in dissected pieces of bovine ovarian cortex (Fortune et al. 2011)
Phosphatidylinositol 3 kinase (PI3K) signaling	Oocyte-specific deletion of *Pten* causes premature activation of the entire pool of primordial follicles (John et al. 2008; Reddy et al. 2008). In *Pten*-null mouse oocytes, PI3K becomes constitutively activated, which can be efficiently abolished by the specific PI3K inhibitor LY294002 (Reddy et al. 2008). Moreover, when ovaries from neonatal mice or cortical pieces from human ovaries are cultured in vitro with a PTEN inhibitor and a PI3K activating peptide activation of primordial follicles is significantly enhanced (Li et al. 2010)
Mammalian target of rapamycin complex 1 (mTORC1) signaling	Oocyte-specific deletion of either *Tsc1* (Adhikari et al. 2010) or *Tsc2* (Adhikari et al. 2009) leads to a global activation of all primordial follicles. Intra-oocyte mTORC1 activation is high in *Tsc1* and *Tsc2*-null oocytes. However, treatment with the mTORC1-specific inhibitor rapamycin halts the excessive activation of primordial follicles in Tsc deletion mutant mice (Adhikari and Liu 2010; Adhikari et al. 2010)
Insulin	Primordial follicle activation in the bovine ovarian cortex takes place in vitro only if the culture medium contains insulin; otherwise such activation is halted (Fortune et al. 2011; Wandji et al. 1996). Insulin also promotes the activation of primordial follicles in cultured fetal hamster ovaries (Yu and Roy 1999) and neonatal rat ovaries (Kezele et al. 2002)
Other growth factors	Basic fibroblast growth factor (Nilsson et al. 2001; Nilsson and Skinner 2004), keratinocyte growth factor (Kezele et al. 2005), platelet-derived growth factor (Nilsson et al. 2006), glial-derived neurotrophic factor (Dole et al. 2008), leukemia inhibitory factor (Nilsson et al. 2002), bone morphogenic protein 7 (BMP-7) (Lee et al. 2001, 2004), BMP-4 (Nilsson and Skinner 2003), and growth differentiation factor 9 (GDF-9) (Hreinsson et al. 2002; Martins et al. 2008; Vitt et al. 2000) enhance the activation of primordial follicles in vitro. Similarly, Smad3 (Tomic et al. 2004) and growth hormone (Slot et al. 2006) also enhance follicular activation in mice

4.2 In Vitro Activation of Dormant Follicles

4.2.1 Organ or Cortical Strip Culture

The initial activation and the sustained in vitro growth of the most abundant dormant follicles are crucial to maximizing the reproductive potential of cryopreserved or fresh ovarian tissue. Very exciting results were obtained by culturing whole newborn

mouse ovaries in serum-containing medium that did not contain exogenous gonadotropins. During an 8-day culture of the neonatal mouse ovaries, primordial follicles were successfully activated with a similar activation pattern that is observed during the first wave of in vivo follicular development (Eppig and O'Brien 1996; O'Brien et al. 2003). Further culturing of the isolated granulosa–oocyte complexes in a different culture medium generated mature, fertilizable oocytes (Eppig and O'Brien 1996; O'Brien et al. 2003). These experiments demonstrated for the first time the feasibility of generating mature follicles by culturing primordial follicles in vitro.

For obvious reasons, studies using primate (human and nonhuman) ovarian tissues for in vitro follicle activation are extremely limited. However, ovarian tissues obtained from domestic animals such as cows, sheep, goats, cats, and pigs have found widespread use for studying in vitro activation of dormant follicles. Generally, whole ovaries from small animals such as neonatal rats and mice but only cortical tissues that are rich in primordial follicles from species with large ovaries, such as humans and cattle, are used for in vitro culture. A significant difference has been observed in the extent of primordial follicle activation between the culture of intact rodent ovaries and ovarian cortical pieces from the ovaries of other animals. Most of the primordial follicles in bovine (Wandji et al. 1996) and baboon (Wandji et al. 1997) cortical slices become activated during in vitro culture, while only limited numbers of primordial follicles are activated in cultured mouse ovaries (Eppig and O'Brien 1996).

These disparities in the patterns of primordial follicle activation between intact mouse ovaries and cortical pieces of bovine and baboon ovaries led to the hypothesis that the primordial follicles may be subject to an inhibition of growth initiation, possibly secreted from the medullary region of the ovary (Fortune et al. 2000). Although in vitro culture of bovine cortical pieces leads to spontaneous activation of primordial follicles (Wandji et al. 1996), such activation is halted if the culture medium lacks insulin (Fortune et al. 2011). Insulin has also been found to promote the activation of primordial follicles in cultured fetal hamster ovaries (Yu and Roy 1999) and neonatal rat ovaries (Kezele et al. 2002). Thus, insulin appears to be essential to activate primordial follicles at least in vitro.

The precise culture conditions that mimic the natural in vivo conditions in terms of nutrients, growth factors, pH, temperature, and oxygen concentration, however, have yet to be defined for the in vitro culture of human ovarian tissues. It has been demonstrated that primordial follicles within the cortical strips could be cultured in serum-free media up to the stage of pre-antral follicles. Upon subsequent isolation from the cortical tissue, such pre-antral follicles have been grown to the antral stage in the presence of follicle stimulating hormone and activin A (Telfer et al. 2008). In these experiments, the cortical strips were initially cultured for 6 days and the pre-antral follicles that developed were dissected from the strips and individually cultured for an additional 4 days in a medium that also contained activin A (Telfer et al. 2008).

Although this observation is encouraging, the findings have yet to be repeated. Moreover, there remains a concern regarding the quality of the oocytes generated in such a short duration of just 10 days of in vitro culture (Telfer et al. 2008). In the human ovary, the time required for the primary follicle to reach the secondary stage has been estimated to be greater than 120 days, and an additional 71 days are needed to grow from the secondary to the early antral stage (McGee and Hsueh 2000).

We (our unpublished data) and others (Li et al. 2010) have also noticed that it takes about 6 months for the development of primordial follicles to the antral stage after transplanting human ovarian cortical strips under the kidney capsules of SCID (severe combined immunodeficient) mice.

The results obtained from studying various molecules and growth factors in genetically modified mice are immensely encouraging (Table 4.2), and this knowledge can be used to design conditions for the in vitro culture of human primordial follicles. With our current understanding of the molecules and signals that maintain the primordial follicles in a dormant state, it might be possible to inhibit these suppressor molecules and thereby trigger activation of the primordial follicles in culture. In vitro culture of ovarian cortical tissues in the presence of small molecule chemical inhibitors of PTEN, Tsc1, Tsc2, p27, or Foxo3a could possibly trigger the activation of primordial follicles and increase the survival rate of cultured follicles.

As a proof of principle, a PTEN inhibitor has been successfully used in vitro to activate primordial follicles in both mouse and human ovaries (Li et al. 2010, 2011). Because the original protocols for the in vitro culture of neonatal mouse ovaries (Eppig and O'Brien 1996; O'Brien et al. 2003) do not efficiently generate germinal vesicle breakdown (GVBD)-competent oocytes, these protocols have recently been modified to also include a PTEN inhibitor and a PI3K activator in the first step of ovarian culture (Li et al. 2011). After 13 days of culturing neonatal ovaries in this modified medium, the isolated pre-antral follicles were cultured on an ovarian stromal cell feeder layer. This led to the generation of a significantly higher number of GVBD-competent oocytes (Li et al. 2011) than the original culture protocol (Eppig and O'Brien 1996; O'Brien et al. 2003).

These results suggest that PTEN inhibitors could potentially be used in human ovarian culture medium to trigger the activation of primordial follicles. However, one must be extremely cautious while using PTEN inhibitors because PTEN has been shown to be a tumor suppressor (Cully et al. 2006; Salmena et al. 2008) and metabolic regulator (Ortega-Molina et al. 2012) in various tissues.

To study the safety and efficacy of the use of a PTEN inhibitor, primordial follicles from neonatal mouse ovaries were activated by transient treatment with an inhibitor. The treated ovaries were then transplanted under the kidney capsules of recipient mice to generate mature oocytes. The mature oocytes were fertilized in vitro and progeny mice were obtained after transfer of the embryos back into female mice. Long-term monitoring up to the second generation of progeny mice showed that the mice were reproductively active and were free from any overt signs or symptoms of chronic illnesses (Adhikari et al. 2012). These results suggest that PTEN inhibitors could also be safely and effectively used for generating mature human oocytes for use in novel IVF techniques.

4.2.2 Culture of Isolated Primordial Follicles

It has been observed that although the freezing and thawing during ovarian tissue cryopreservation is well tolerated by primordial follicles, the stromal cells are less

tolerant to such cryopreservation and undergo necrosis (Keros et al. 2009). Culture of live follicles together with dead stromal tissue might not be an ideal situation. When the ovarian cortical tissue is taken as the starting material for in vitro culture, one cannot be certain of culturing only the primordial follicles because small growing follicles can very well be present in the tissue. Moreover, the density of primordial follicles in the cortical tissues varies between individuals and within individual human ovaries and each tissue fragment might have a different follicular distribution (Schmidt et al. 2003). It is also very difficult to follow the development of one particular follicle when an entire piece of cortical tissue is cultured. These factors might contribute to the inconsistencies in the results that are usually observed during ovarian tissue cultures.

To avoid such issues, primordial follicles can be separated from the pool of various types of follicles by enzymatic digestion of the ovarian tissues. Primordial follicles have been isolated from fresh and cryopreserved human cortical tissues by partial disaggregation followed by microdissection without affecting follicle viability (Oktay et al. 1997b). By using these isolated primordial follicles as the starting material for culture, growth of an individual follicle can be followed during the culturing process (Abir et al. 1999; Dolmans et al. 2006; Hornick et al. 2012).

Unfortunately, isolated human follicles rapidly degenerate during in vitro culture and lose their three-dimensional structure (Abir et al. 2001). When the isolated primordial follicles were cultured in vitro, the pre-granulosa cells detached from the oocytes and the follicles collapsed (Picton et al. 2008). To prevent this from happening, primordial follicles isolated from rhesus macaque ovarian cortex were encapsulated in alginate hydrogels before culturing them in vitro (Hornick et al. 2012). These hydrogels mimicked the rigid physical environment of the ovarian cortex and supported the survival and growth of primordial follicles in culture (Hornick et al. 2012). This finding indicates the importance of mimicking the physiological environment of the ovary for the maintenance of follicular integrity and growth promotion during in vitro culture. Combination of this novel technique with the modified culture media that exploits the known regulators of primordial follicle activation (Table 4.2) could be useful in achieving healthy, growing human follicles.

4.3 Assessment of In Vitro Follicle Activation

4.3.1 Morphology

Morphological and histological examination of the ovarian tissues before and after the in vitro culture has been the major way of assessing the success of follicular activation. A fresh (uncultured) cortical fragment is fixed in a fixative for histological analysis. Paraffin sections stained with Hematoxylin and Eosin have been widely used to evaluate the morphology of the follicles. Follicular development during the culture can be monitored under a dissecting microscope where growing follicles can be visualized as follicular swellings (Telfer et al. 2008). After staining the sections, a more detailed evaluation of follicular size, granulosa cell proliferation, and oocyte

health can be made. Because the outcome of the culture largely depends upon the nature of the starting cortical tissue, the comparison between fresh and cultured ovarian tissue is crucial for the evaluation of the efficacy of the culture conditions. A good in vitro culture system should lead to a decrease in the number of primordial follicles and a proportionate increase in the primary and further developed follicles compared to the fresh tissue. However, a small fragment may not truly represent the primordial follicle density in another fragment from the same ovary. Because follicles are not homogenously distributed within the ovarian cortex of an individual (Schmidt et al. 2003), simple comparison of follicles within two different cortical fragments is not a reliable criterion for the evaluation of an in vitro culture system.

4.3.2 Immunohistochemistry and Immunofluorescent Techniques

The expression of various markers in the oocyte and granulosa cells is used to assess the activation of primordial follicles in culture. Proliferating cell nuclear antigen (PCNA), a marker of cell growth and proliferation, has frequently been used to assess follicular activation. For instance, PCNA staining is not detected in the pre-granulosa cells or the immature oocytes from bovine (Wandji et al. 1996), rat (Oktay et al. 1995), or mouse (Sobinoff et al. 2011) ovaries. However, intense PCNA staining is observed in the granulosa cells and oocytes of growing primary follicles (Oktay et al. 1995; Sobinoff et al. 2011; Wandji et al. 1996). Similarly, follicles that are undergoing atresia can be identified by staining for activated Caspase-2 and Caspase-3, which are early markers of apoptosis in oocytes and granulosa cells, respectively. TUNEL assays can be used to detect those follicles that are undergoing the final stages of atresia (Sobinoff et al. 2011). The TUNEL assay is a technique used to detect DNA strand breaks indicative of DNA degradation (Negoescu et al. 1996). Bromodeoxyuridine incorporation into granulosa cells also indicates cell proliferation and is, therefore, an indicator of growing follicles (Li et al. 2010). AMH is another growth factor that is commonly used for the characterization of activated follicles. AMH and its mRNA are not found in pre-granulosa cells of primordial follicles. Once activated, however, the granulosa cells begin expressing AMH (Durlinger et al. 1999, 2002a). GDF-9 and zona pellucida proteins are expressed in the oocytes once the follicles become activated. Thus, their expression in the oocyte is an indicator of primordial follicle activation (Durlinger et al. 2002a).

4.4 Conclusion

The human ovarian cortex contains numerous primordial follicles that can be cryopreserved without compromising their viability. For the primordial follicles to be useful for fertility treatments, however, methods to activate them need to be developed. The ability to activate and eventually mature these follicles in vitro could be

of great importance for infertility treatments. Although fertilizable eggs and live mouse pups have already been obtained from cultured primordial follicles, similar success in humans still awaits an experimental breakthrough. The molecular networks that govern the activation of dormant follicles have just begun to be understood, and much more needs to be learned about the genes and growth stimulators that activate dormant follicles in vivo in humans. The main research focus until now has been trying different growth factors in the culture media as a way of improving the success rate of IVA. However, it has been realized that providing an in vivo-like physical environment for the growing follicles is equally crucial, and emerging three-dimensional culture systems have been shown to maintain intact follicular structures during culture. A more dynamic culture system that constantly changes according to the needs of a growing follicle at different developmental stages would better mimic in vivo growth conditions. Thus, further research into the signaling pathways that regulate primordial follicle activation and technological developments in in vitro culture systems show great promise for finding ways of culturing dormant follicles as novel methods of fertility preservation in women.

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Chapter 5
Primate Follicular Development and Oocyte Maturation In Vitro

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Abstract The factors and processes involved in primate follicular development are complex and not fully understood. An encapsulated three-dimensional (3D) follicle culture system could be a valuable in vitro model to study the dynamics and regulation of folliculogenesis in intact individual follicles in primates. Besides the research relevance, in vitro follicle maturation (IFM) is emerging as a promising approach to offer options for fertility preservation in female patients with cancer. This review summarizes the current published data on in vitro follicular development from the preantral to small antral stage in nonhuman primates, including follicle survival and growth, endocrine (ovarian steroid hormone) and paracrine/autocrine (local factor) function, as well as oocyte maturation and fertilization. Future directions include major challenges and strategies to further improve follicular growth and differentiation with oocytes competent for in vitro fertilization and subsequent embryonic development, as well as opportunities to investigate primate folliculogenesis by utilizing this 3D culture system. The information may be valuable in identifying

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optimal conditions for human follicle culture, with the ultimate goal of translating the experimental results and products to patients, thereby facilitating diagnostic and therapeutic approaches for female fertility.

Keywords Folliculogenesis • In vitro follicle maturation • Three-dimensional follicle culture • Fertility preservation • Primate

5.1 Introduction

Ovarian follicular development is a dynamic process that is regulated by complex interactions between gonadotropic hormones and local paracrine/autocrine factors (Gougeon 1996). Although progress in understanding early folliculogenesis has been made, particularly in mice through gene manipulation (Matzuk 2000; Drummond 2006), the regulation and dynamics of primate folliculogenesis, aside from phenotypic analysis in women (Chand et al. 2010; Ewens et al. 2010), remain poorly understood due to the lack of adequate in vitro models. Two general approaches of follicle culture have been pursued with dissected follicles attaching to the culture plate and growing two-dimensionally (2D), or follicles encapsulated in a matrix that maintains their intact three-dimensional (3D) structure. Secondary follicles from marmosets produced metaphase II (MII) oocytes following 2D culture and oocyte in vitro maturation (IVM; Nayudu et al. 2003). Human preantral follicles encapsulated in agar (Roy and Treacy 1993) or collagen (Abir et al. 1997, 1999, 2001) gels maintained their morphology and grew to the early antral stage. Recently, early antral follicles have been obtained from ovarian cortical strip culture (Telfer et al. 2008).

Biomaterials have been applied to 3D follicle culture, which maintain the cell–cell and cell–matrix connections important in regulating follicle development in vivo (West et al. 2007). Alginate was successfully used for the culture of murine follicles, and its application to nonhuman primates resulted in the growth of small preantral follicles through the antral stage with production of ovarian steroids and local factors, as well as oocyte maturation (Xu et al. 2009a, 2010, 2011a, b). This in vitro follicle maturation (IFM) technique is a powerful instrument for monitoring the endocrine and paracrine/autocrine function of individual follicles, as well as manipulating regulatory factors or signaling pathways, which is essential to obtain knowledge of their role(s) and importance in follicular and oocyte development in primates.

Besides the research relevance to basic ovarian biology, IFM, combined with advances in ovarian tissue cryopreservation (Ting et al. 2011, 2012), may be applied to fertility preservation in women, including cancer patients (Jeruss and Woodruff 2009). Although ovarian cortex transplantation using fresh tissue in monkeys (Lee et al. 2004), as well as using fresh and cryopreserved tissue in women (Dittrich et al. 2012; Donnez et al. 2004; Silber et al. 2008), yielded viable offspring, the IFM approach has the advantage of eliminating the reintroduction of cancer cells into the patients and providing a way to harvest more mature oocytes (Woodruff 2007). Live offspring were generated in mice through IFM (Xu et al. 2006a), and studies

demonstrated the potential application of IFM in humans (Xu et al. 2009b; Smitz et al. 2010). Even though the meiotic competence and developmental capacity of human oocytes grown from preantral stages in vitro have not yet been reported, animal studies indicate that ovarian tissue storage followed by IFM is a valid prospect for clinical translation to humans to prevent the destruction or damage to ovarian germ line cells caused by radiotherapy and/or chemotherapy (Woodruff 2007). Information obtained by growing rhesus macaque and baboon follicles during 3D culture may be valuable in identifying the optimal conditions for primate follicle culture prior to human application.

Thus, the current status of efforts to study primate follicular development during IFM are summarized to (1) consider the characteristics and regulation of the survival and growth of primate preantral follicles during encapsulated 3D culture; (2) review the endocrine (ovarian steroid hormones), paracrine/autocrine (local factors), and gametogenic (oocyte maturation) function of primate follicles prior to and during antral development in vitro; and (3) discuss the challenges and opportunities for further advances using primate follicles and cumulus–oocyte complexes (COCs) for fertility preservation.

5.2 3D Culture of Macaque Follicles

5.2.1 General Techniques and Variables

The Old World monkey is a valuable model for studying the primate ovary, since characteristics and regulation of cyclic ovarian function are comparable to those in women. It is possible to address issues in macaques that can only be indirectly studied with human tissue due to practical and ethical reasons. Thus, the main focus of this review is encapsulated 3D culture of small preantral follicles obtained from ovaries of rhesus macaques (*Macaca mulatta*), as well as baboons (*Papio anubis*; see later section).

Secondary (125–225 μm in diameter) follicles can be mechanically isolated from monkey ovarian cortex, encapsulated into alginate hydrogel matrix, and cultured individually for several weeks for further development to the antral stage (~1 mm in diameter). Alpha minimal essential medium (αMEM) has been used as basal culture media which is supplemented with transferrin and sodium selenite. Bovine serum albumin used in early studies (Xu et al. 2009a) was replaced by human serum protein supplement (SPS; Xu et al. 2010; 2011a) for relevance to future clinical application. The culture media also contains fetuin, a major glycoprotein in serum and follicular fluid, which reportedly prevents "hardening" of the zona pellucida in a serum-free culture environment (VandeVoort et al. 2007; Schroeder et al. 1990). Bovine fetuin is used currently due to unavailability of human protein. Follicles are cultured in 5 % oxygen (O_2) (v/v) (Xu et al. 2011a), which mimics the partial pressure of O_2 in the peritoneal cavity where the ovaries are located (Tsai et al. 1998), in contrast to early primate follicle culture studies where 20 % (v/v) atmospheric O_2

tension was used (Xu et al. 2009a; 2010). Although full knowledge of the milieu for follicle growth in vivo is still lacking, there is evidence that adequate levels of certain hormones are essential for the growth of healthy follicles in vitro (Picton et al. 2008). The pituitary gonadotropin, follicle stimulating hormone (FSH) promotes follicle survival and growth in a 3D matrix (Xu et al. 2006a, 2009a, b), but there is concern that a supraphysiological level of FSH can alter the expression of oocyte and cumulus cell transcripts in cultured murine follicles (Sánchez et al. 2010). Prolonged high-dose FSH exposure may also disturb oocyte control of granulosa cell proliferation and differentiation, as well as cumulus cell function, during primate follicle growth in vitro (Xu et al. 2011a). Luteinizing hormone (LH) enables preantral follicles to respond to later LH-dependent growth during murine follicle culture (Cortvrindt et al. 1998; Wu et al. 2000). There is also an increasing body of evidence that insulin stimulates growth and improves the overall viability of human follicles in vitro (Wright et al. 1999; Louhio et al. 2000). In contrast, insulin can have profound detrimental effects on oocyte developmental competence during the culture of murine COCs (Eppig et al. 1998). Therefore, studies included treatment groups to evaluate the effects of media components, as well as hormones, on parameters of primate follicles during IFM.

Maternal age and the stage of the menstrual cycle at which follicles are collected also need to be considered. Oocyte quality and fecundity, either spontaneous or assisted, decline by 30 years of age in premenopausal women according to clinical observations (Gougeon 2005). This feature of ovarian aging is relevant to maternal age-effect on developmental competence of in vitro-developed primate follicles. It takes approximately 90 days for a small preantral follicle that has entered the growing pool to become a preovulatory follicle in women (Gougeon 1996). The hormonal and local milieu around follicles in the follicular versus luteal phase may have differentially effect on follicle potential.

5.2.2 Follicle Survival

Follicle "health" can be grossly identified using an inverted microscope, with viable follicles exhibiting an intact basement membrane, 2–3 layers of granulosa cells, and a round and centrally located oocyte with a visible zona pellucida, versus atretic follicles having dark granulosa cells and/or a dark oocyte. Approximately 50–200 "healthy" secondary follicles can be isolated from a pair of monkey ovaries. Initial studies indicated that not all "healthy" follicles survive during 3D culture in the presence of gonadotropic hormones (Xu et al. 2009a). However, FSH is a critical hormone for survival of macaque secondary follicles in the alginate-based 3D culture system. In the absence of exogenous FSH, all secondary follicles undergo atresia within 2 weeks regardless of animal or culture variables (Xu et al. 2010). Survival rate is lower for follicles cultured with low-dose (0.3 ng/ml) compared to high-dose (15 ng/ml) FSH (Xu et al. 2011a). Likewise, FSH acts as a survival factor for human preantral follicles during ovarian tissue culture (Wright et al. 1999). These data are

consistent with evidence that FSH receptors are expressed in preantral follicles of various species, including primates (Gougeon 1996; Findlay and Drummond 1999). Folliculogenesis is blocked at the early preantral stage in FSH receptor knockout mice (Dierich et al. 1998). However, an essential role for FSH in survival of preantral follicles in vitro is counter to evidence in vivo that human follicles survive and some grow to the small antral stage in conditions of low-to-nondetectable FSH levels, e.g., in infancy (Peters 1979), in women during pregnancy (Khattab and Jequier 1979), in disorders of hypogonadotropic hypogonadism (Goldenberg et al. 1976), and selective FSH (Rabin et al. 1972) or FSH receptor (Aittomäki et al. 1996) defects. Perhaps the ovarian milieu/tissue in vivo, in the absence or presence of very low levels of gonadotropins, can promote survival of small growing follicles. In contrast to FSH, neither LH, added from the beginning of culture or on day 30, nor varying doses of insulin had any effect on follicle survival during culture (Xu et al. 2009a, 2010).

The age and menstrual cycle stage of the animal providing secondary follicles, as well as alginate gel rigidity, influence follicle survival during 3D culture. In the presence of high-dose FSH, there is no difference in follicle survival during 5-week culture between prepubertal (1–3 years of age) and young, adult (4–11 years of age) monkeys. However, survival rate decreases for follicles from older adult animals (13–16 years of age) (Xu et al. 2010), which is consistent with the observation in murine follicle culture (Hirshfeld-Cytron et al. 2011). The mechanisms resulting in lower survival rate of follicles obtained from older adult monkeys remain unclear. Unknown factors responsible for development of preantral follicles may protect them from programmed cell death, as shown for growth differentiation factor 9 in murine follicles (Orisaka et al. 2006). Preantral follicles from older animals may lack these apoptosis-inhibiting factors and not survive in culture. Alternatively, local factors inducing follicle atresia during early follicular development, such as prohibitin identified in mice (Thompson et al. 2004), may increase during aging and result in decreased survival rate of preantral follicles from older animals. Factors influencing autophagy may also be involved (Rodrigues et al. 2009), though this process has not been studied in primate preantral follicles. Follicles isolated during the early follicular phase of the menstrual cycle have a higher survival rate during initial culture than those from the luteal phase. This may due to the absence of a dominant structure (follicle destined to ovulate or corpus luteum); thus more of the follicle cohort is healthy and nonatretic during the early follicular phase.

Although initial studies suggested that 0.5 % alginate was beneficial to follicle survival, the morphology of resultant small antral follicles (SAFs) was atypical (Xu et al. 2009a). More recent experiments demonstrated that 0.25 % alginate promoted follicle survival (Xu et al. 2010, 2011a) and resulted in SAFs with similar morphology to that observed in in vivo-developed SAFs in primates (unpublished data). It is suggested that the microvilli and transzonal projections between the oocyte and somatic cells (Albertini et al. 2001) are maintained in murine follicles grown in alginate (Pangas et al. 2003). The physical properties of the alginate matrix have a significant role in supporting follicle development. This hydrogel has sufficient rigidity to maintain the 3D structure of the follicle, and also allows for expansion due to oocyte growth, granulosa cell proliferation, and antrum formation (West et al. 2007).

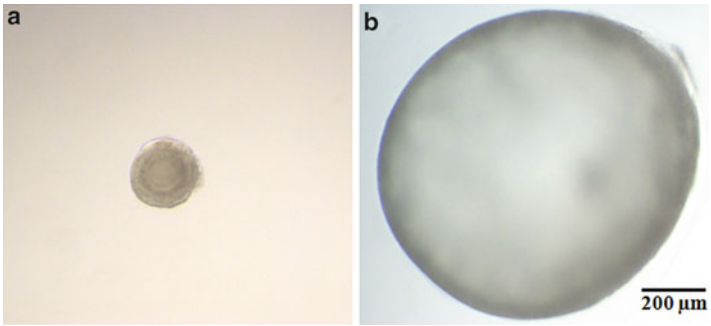

Fig. 5.1 Alginate-encapsulated monkey follicle at day 0 (*panel a*, secondary stage), that displayed "fast growth" by day 35 (*panel b*, small antral stage) of culture. Scale bar = 200 µm

A more physiological level of O_2 (5 %) and fetuin supplementation are beneficial for macaque follicle survival during encapsulated 3D culture. In the presence of high-dose FSH, the survival rate is higher at week 5 when follicles were cultured with fetuin at 5 % O_2, relative to those without fetuin or in 20 % O_2. Previous studies indicated that fetuin is present in the ovarian follicular fluid of the mouse (Høyer et al. 2001), horse (Dell'Aquila et al. 1999), and human (Kalab et al. 1993). A variety of cell types in culture respond to fetuin in promoting cellular attachment, growth, and differentiation (Demetriou et al. 1996; Nie 1992). One unexpected observation is that alginate beads become nontransparent, brittle, and fragmentary after 2 weeks of culture without fetuin, though this is not observed during mouse follicle culture. The mechanism whereby fetuin maintains alginate gel integrity is unknown. Since fetuin is currently not a recombinant protein, additional complexity caused by fetuin impurities cannot be ruled out.

5.2.3 Follicle Growth

At the beginning of the culture, diameters of collected secondary follicles range from 125 to 225 µm, and care is taken to ensure that this range of sizes is represented among experimental groups. However, not all surviving follicles grow at the same rate during culture with three distinct cohorts observed based on their diameters at week 5 (Xu et al. 2010, 2011a). The cohort that remains similar in size to the initial secondary follicles without significant change in diameter (<250 µm) is termed "no-grow" follicles. Another cohort doubles their diameters (250–500 µm) and is termed "slow-grow" follicles. Finally, another group of follicles increases their diameters by a minimum of threefold (>500 µm, in some instances over 1 mm) and is termed "fast-grow" follicles (Fig. 5.1). An antral cavity is evident within 3–4 weeks of culture for all the growing (slow- and fast-grow) follicles. In initial studies on human secondary follicles (Xu et al. 2009b), such differences in follicle growth rate were

not reported. However, it was noted that 75 % of the surviving human follicles developed visible antrum, while others remained at multilayer stage through 30 days of culture. These data suggest that the population of secondary follicles in the primate ovary at early follicular phase of the cycle is heterogeneous in their capacity to grow in an FSH-replete milieu. Their growth rate may depend upon their ability to recognize or respond to FSH (Kreeger et al. 2005) or other hormones (Xu et al. 2010), or to synthesize and respond to other local factors that modulate follicular growth.

The dose of FSH influences the growth rate of slow-grow, but not no- and fast-grow follicles. In the presence of low-dose FSH, slow-grow follicles have lower growth rates compared to high-dose FSH group at weeks 2 and 3. Nevertheless, follicle diameters are larger in the presence of low-dose FSH than those of high-dose FSH cultures at week 5. Low-dose FSH may promote further growth of slow-grow follicles by preventing high-dose FSH effects that disturb oocyte control of granulosa cell proliferation and differentiation during primate follicle growth in vitro (Xu et al. 2011a). LH supplementation at day 30 has no effect on the distribution of surviving follicles, and does not promote further growth of follicles regardless of animal and culture conditions (Xu et al. 2010, 2011a). This is consistent with in vivo evidence that antral follicular development can occur in the presence of minimal (hypogonadal) levels of LH in monkeys (Zelinski-Wooten et al. 1995) and women (Schoot et al. 1994; Kumar et al. 1997). The addition of LH throughout the culture period decreases monkey follicle diameters, which may be related to either the larger size of preantral follicles utilized, or due to the use of high-dose FSH (Xu et al. 2009a).

Insulin affects fast-grow follicles, but not slow- and no-grow follicles. The insulin dose does not alter the growth rate of no- and slow-grow follicles. However, even though fast-grow follicles maintain a similar growth rate in the presence of either low- or high-dose insulin for the first 3 weeks of culture, follicle diameters become larger in high-dose, compared to the low-dose, insulin at weeks 4 and 5 (Xu et al. 2010). Insulin receptor mRNA and protein are located in theca, granulosa, and stromal cells of antral follicles in women (el-Roeiy et al. 1993; Samoto et al. 1993). Insulin may mimic local insulin-like growth factor (IGF) activity, which is suggested to improve viability of cultured follicles in primates (Louhio et al. 2000). Further studies regarding the effects of insulin and IGFs, both of which are present at appreciable levels in follicular fluid of macaque antral follicles (Brogan et al. 2010), on primate follicular development in vitro are warranted.

Maternal age of the monkeys has a significant impact on IFM outcome. During culture with high-dose FSH, follicles collected from older adult monkeys display a greater proportion of no-grow follicles. Also, the percentage of slow-grow follicles is greater, and the diameters at week 5 are smaller in follicles from prepubertal monkeys compared to those from young adults. Notably, fast-grow follicles are only observed when culturing secondary follicles from young adult, not prepubertal or older adult, animals (Xu et al. 2010). In different species, including primates, FSH receptor expression increases during early folliculogenesis and the receptors remain on granulosa cells of healthy follicles until they become atretic or luteinize (Findlay and Drummond 1999). It is possible that, compared with young adults, the FSH receptor expression and signaling pathway are not fully developed in follicles of

prepubertal ovaries or decrease in function through aging. The basis for these differences in preantral folliculogenesis between ages remains an intriguing question with relevance to the age at ovarian tissue collection for future fertility preservation in cancer patients.

The effect of fetuin on in vitro follicle growth seems to be related to FSH levels. During culture with high-dose FSH and fetuin, more than 50 % of surviving follicles fall into the growing follicle category. However, when fetuin is absent, the majority of surviving follicles are no-grow follicles, despite the presence of high FSH. When cultured with low-dose FSH, similar proportions of growing follicles are observed with or without fetuin, and fast-grow follicles are only obtained from culture without fetuin (Xu et al. 2011a). Whether cultured follicles require exogenous fetuin to promote further growth after antrum formation is unclear. Alternatively, endogenous fetuin production by cultured follicles could be inhibited by high-dose FSH. The specific effects of fetuin on follicle cell proliferation and differentiation remain to be determined.

Oxygen tension influences the growth rate of slow-grow follicles, but not no- and fast-grow follicles. Follicles cultured at 20 % O_2 are less likely to grow compared to those cultured at 5 % O_2. Follicles maintain similar growth rates during the first 3 weeks in 5 % and 20 % O_2. However, diameters increase and become larger at week 5 for follicles cultured at 5 % O_2 than those of 20 % O_2. This observation is consistent with follicle culture studies using domestic animals, where low O_2 concentration (5 %) stimulated follicle growth with a high proportion of them developing an antral cavity (Cecconi et al. 1999; Silva et al. 2010). It is hypothesized that the ovarian follicle, in a low-oxygen environment, is often challenged by hypoxia (Redding et al. 2008), and antrum formation provides a way to support further growth by avoiding hypoxia in the follicle wall (Redding et al. 2007). The effects of O_2 tensions less than 5 % (hypoxic, i.e., 1–3 % O_2) on macaque follicles during 3D culture have not been tested.

5.2.4 Follicle Maturation

The in vitro-developed follicles produce local and endocrine factors, and mature oocytes, as a function of their growth rate and developmental stage during culture.

5.2.4.1 Steroidogenesis

Individually cultured macaque follicles differed in their ability to produce ovarian steroids, such as progesterone (P4), androstenedione (A4), and estradiol (E2), which are detectable in the culture media (Xu et al. 2009a, 2010, 2011a). Their steroid production correlates positively with follicle growth rate and is promoted by exogenous FSH levels. For no-grow follicles, media P4, A4, and E2 levels remain at baseline throughout culture and are not influenced by any experimental variables.

For growing follicles, ovarian steroids start increasing at week 3–4 and are higher at week 3–5 compared to those observed in the beginning of culture. Steroid concentrations of fast-grow follicles are higher than those of slow-grow follicles at week 3–5. Steroid levels of growing follicles cultured with low-dose FSH are lower than those of high-dose FSH treated follicles at week 3–5. The E2-to-P4 ratio at week 5 is higher in low-, relative to high-, dose FSH cultured follicles (Xu et al. 2011a). Thus, increased steroidogenesis coincides with antral development that occurs at week 3–4 of 3D follicle culture. High-dose FSH, while optimal for promoting follicle survival in the first weeks of culture, may cause premature differentiation of granulosa cells following antral formation that results in their luteinization to generate high levels of P4, which also serves as substrate for A4 and E2 (Fauser and Van Heusden 1997).

Notably, FSH alone is sufficient to support steroidogenesis in antral follicles during IFM. For growing follicles cultured with FSH and high-dose insulin, P4, A4, and E2 concentrations increase after antrum formation during in vitro follicle growth. Steroid levels from slow-grow follicles cultured with low-dose insulin stay at baseline and are lower than those of the high-dose insulin treated follicles at weeks 4 and 5 (Xu et al. 2010). Steroid production by fast-grow follicles cultured with low-dose insulin has yet to be examined. In mammals, insulin has been shown to promote theca and granulosa cell steroidogenesis (Barbieri et al. 1984; Erickson et al. 1990; Langhout et al. 1991). Thus, insulin/IGFs could also be an important regulator of primate thecal-granulosa differentiation and steroidogenesis in vitro.

Although steroidogenesis can be achieved by cultured follicles with FSH alone, LH-regulated steroid production is also observed during IFM. The addition of LH at day 30 increases P4, A4, and E2 production of slow-grow follicles between pre-LH (week 4) and post-LH (week 5) exposure in the presence of high-dose FSH. LH treatment increases P4 and A4, but not E2, levels with low-dose FSH and are higher at week 5 than those without LH administration (Fig. 5.2). The increased androgen and estrogen levels are consistent with the 2-cell, 2-gonadotropin model wherein LH-receptor signaling promotes A4 production by theca cells which allows steroidogenic maturation of the follicles by providing A4 as substrate for E2 production in the granulosa cells (McNatty et al. 1980). This LH responsiveness suggests the presence of theca cells in in vitro developed primate follicles, which has been conclusively demonstrated recently (Xu et al. 2013). In low-dose FSH cultured follicles, well-developed theca cells may be stimulated by LH to produce P4 and A4, while granulosa cells undergoing appropriate proliferation may utilize A4 efficiently to synthesize high levels of E2. LH supplementation at day 30 has no effect on the patterns or levels of steroids during week 5 for fast-grow follicles regardless of culture conditions (Xu et al. 2010, 2011a). This may be due to the high steroid production prior to the LH addition which prevents further stimulation. During 3D monkey follicle culture, continuous LH exposure decreased P4, without having effect on A4 and E2, production (Xu et al. 2009a). This may result from the desensitizing of LH receptors by continuous LH exposure or not distinguishing slow-grow from fast-grow follicles.

The impact of maternal age on steroid production is observed during IFM. When cultured with high-dose insulin, slow-grow follicles from young adult animals display higher P4, A4, and E2 concentrations than those from prepubertal and older

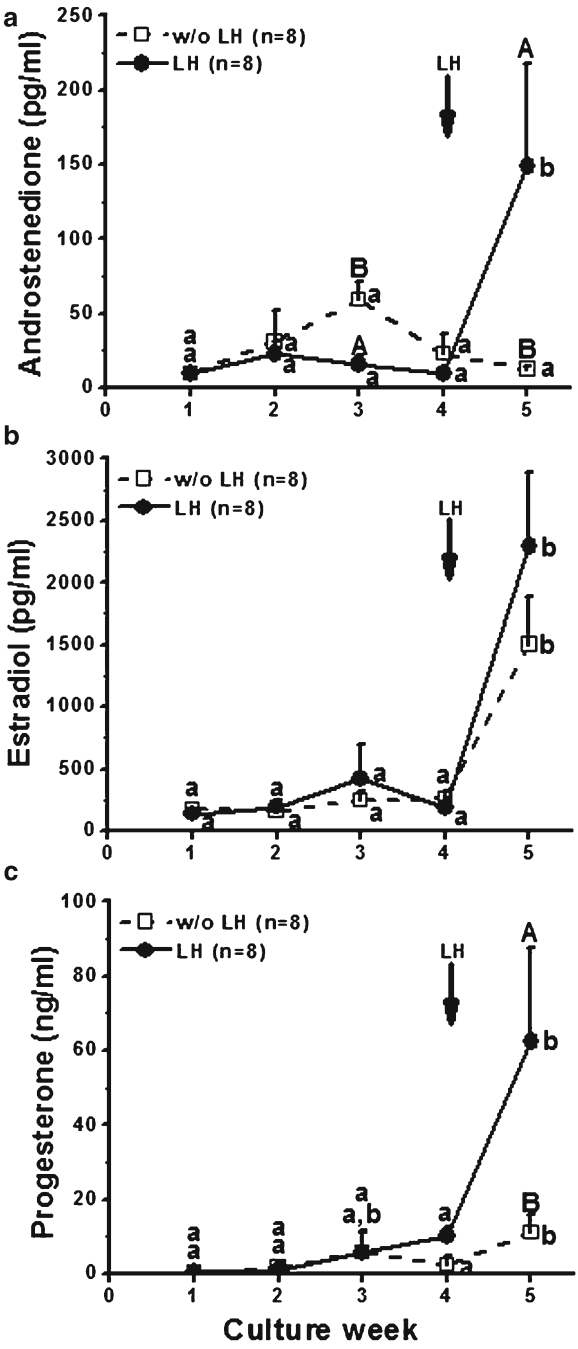

Fig. 5.2 Luteinizing hormone (LH) effect on androstenedione (*panel a*), estradiol (*panel b*), and progesterone (*panel c*) production by slow-grow follicles during in vitro follicle maturation. *Arrow*, LH supplementation at day 30. Significant differences over time (*lowercase*) or between the LH treatment groups (*uppercase*) are indicated by different letters ($P<0.05$). Data are presented as the mean ± SEM. *N*, number of follicles (This figure was originally published in and reproduced with permission from Xu et al., Hum Reprod 2011a)

adult monkeys at week 5. Follicles from prepubertal animals produce less E2 and more P4 compared to follicles from older adults (Xu et al. 2010). This may due to less FSH sensitivity of the follicles as described above (Davoren and Hsueh 1984). Follicle function may be suboptimal in prepubertal and older adults if insulin enhanced FSH-stimulated steroidogenesis is lacking.

Fetuin exposure and O_2 tension (5 or 20 %) do not alter steroid levels in culture media of in vitro-developed macaque follicles (Xu et al. 2011a).

5.2.4.2 Paracrine/Autocrine Factor Production

The quantitative assessment of the production of paracrine/autocrine factors, e.g., anti-Müllerian hormone (AMH) and vascular endothelial growth factor (VEGF), by individual primate follicles is possible in the IFM system. Follicles secrete detectable levels of AMH and VEGF into the culture media, in distinct temporal patterns, as a function of growth rate, developmental stage, and culture milieu (Xu et al. 2010, 2011a).

AMH production by in vitro-developed follicles correlates positively with growth rate. When follicles are cultured with high-dose FSH at 5 % O_2, AMH levels produced by no-grow follicles do not change throughout the culture. Although no difference between mean diameters of no-, slow-, and fast-grow follicles could be distinguished until 2–3 weeks of culture, levels of AMH produced by growing follicles at week 1 are significantly higher than those of no-grow follicles. Moreover, AMH levels of fast-grow follicles increase at week 2 and remain at high levels until declining at week 5. AMH levels during weeks 3 and 4 are distinct among all three follicle categories. By week 5, all cultured follicles return to basal levels of AMH (Xu et al. 2011a). The heterogeneity of the small preantral follicle pool, in terms of their ability to produce AMH, is supported by the differences between size-matched preantral follicles in the marmoset ovary that immunolocalized AMH (Thomas et al. 2007). Previous studies in marmoset and human ovaries localized AMH mRNA or protein to granulosa cells of preantral and SAFs, which diminished in the subsequent stages of follicle development (Durlinger et al. 2002; Weenen et al. 2004; Thomas et al. 2007). Thus, AMH production in vitro during 3D IFM may mimic in vivo processes and be an early marker for predicting further development of individual preantral follicles with different growth rates during further culture, prior to any differences in follicle diameter and steroidogenic capacity. AMH is also an important local factor regulating follicle growth in encapsulated 3D culture systems. The addition of AMH into cultures of human or rat ovarian cortical strips reportedly improved the recruitment, survival and growth of primordial follicles (Schmidt et al. 2005; McGee et al. 2001) suggesting a stimulatory role of AMH on very early follicular development. However, the numbers of preantral follicles of different classes were not known at the start of culture in the pieces of tissue exposed to AMH in vitro. AMH may also act as a paracrine factor to modulate FSH-regulated folliculogenesis (Durlinger et al. 2002; La Marca et al. 2009). Nevertheless, direct actions of AMH during follicular development in primates have yet to be demonstrated.

In macaque follicles during 3D culture, FSH promotes AMH production in vitro in a dose-dependent manner. When cultured with low-dose FSH, growing follicles produce higher levels of AMH during week 2–4 than no-grow follicles. However, the levels are lower than those of high-dose FSH culture (Xu et al. 2011a). Based on data from human (Weenen et al. 2004), marmoset (Thomas et al. 2007), and rodent (Salmon et al. 2004) ovaries, AMH production by cultured follicles after antrum formation may originate from mural or cumulus cells. Other factors that regulate AMH expression in primate follicles remain unclear, but oocyte–granulosa cell coculture experiments determined that *AMH* mRNA expression within granulosa cells of mouse preantral follicles is regulated by signals from the oocyte (Salmon et al. 2004).

Also, there is the evidence that the production of AMH, like other potential growth factors (e.g., VEGF; Shweiki et al. 1992), is regulated by O_2 tension in vitro. A physiological level of O_2 (5 %) is beneficial for AMH production by macaque follicles during encapsulated 3D culture. Similar patterns are obtained for AMH produced by follicles cultured at 20 % O_2, except that AMH declines to basal levels earlier at week 3 (Xu et al. 2010).

Unlike AMH, VEGF levels do not increase until antrum formation. However, VEGF production also correlates with the growth rate of monkey follicles during IFM. In the presence of FSH, VEGF levels produced by no-grow follicles do not change throughout 5 weeks of culture. In contrast, VEGF concentrations of growing follicles increase markedly at weeks 4 and 5, with distinct levels among all three follicle categories. The pattern is consistent with previous in vivo studies that VEGF mRNA (Ravindranath et al. 1992) and protein (Yamamoto et al. 1997) were expressed in the theca cells of antral follicles and granulosa cells nearest the oocyte in the preovulatory follicle of primates, but not in granulosa cells of primordial and preantral follicles. VEGF likely plays an angiogenic role during antrum development, when the thecal layer acquires a vascular sheath, to provide an increased supply of gonadotropins, growth factors, oxygen, and steroid precursors to the growing follicle (Stouffer et al. 2001). Besides VEGF, other vascularization-related factors, e.g., angiopoietin 2 (ANGPT2), also follow a similar pattern as VEGF production with elevated levels after antrum formation in growing follicles during monkey IFM (unpublished data). ANGPT2 action may be important during angiogenesis to destabilize existing vessels for further growth (Xu et al. 2005). Increased VEGF and ANGPT2 production by growing follicles in encapsulated 3D culture may indicate achievement of a size and maturation state in the follicle, at the antral stage, that requires vascularization to achieve further development in vivo with additional substrates and release of hormones. In addition to angiogenic action, VEGF may also be a cytoprotective factor in the extravascular granulosa cell compartment. Co-expression of VEGF and its receptor reportedly protects bovine granulosa cells from apoptotic cell death and follicle atresia (Greenaway et al. 2004). VEGF may also promote nuclear and cytoplasmic maturation of bovine oocytes in vitro (Luo et al. 2002). Thus, VEGF may play a role during follicle development and/or be a marker of follicle quality in vitro.

The patterns of AMH and VEGF production by macaque follicles in 3D culture remain similar throughout 5 weeks regardless of LH addition, insulin dose, animal age, and the presence of fetuin (Xu et al. 2010, 2011a, unpublished data).

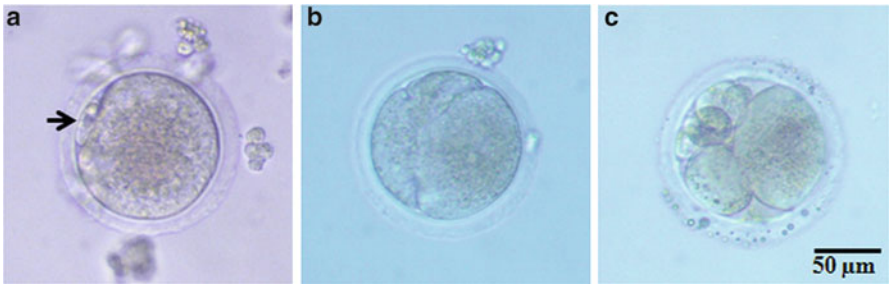

Fig. 5.3 Metaphase II oocyte at retrieval (*panel a*) and after (Day 1, *panel b*; Day 3, *panel c*) insemination using intracytoplasmic sperm injection. The fertilized MII oocyte cleaved to two cells (*panel b*) and arrested with uneven cleavage (*panel c*). *Arrow*, polar body. Scale bar=50 µm (This figure was originally published in and reproduced with permission from Xu et al., Hum Reprod 2011a)

5.2.4.3 Oocyte Maturation

In vitro developed follicles that reach the antral stage can be treated with recombinant human chorionic gonadotropin (hCG) in the media for 34 h to examine reinitiation of meiotic maturation of the oocyte within the follicle (Xu et al. 2011a).

Healthy, as well as degenerate, oocytes are obtained from cultured macaque follicles that achieve the small antral stage (~1 mm in diameter). Most of the healthy oocytes remain at the germinal vesicle (GV) stage following hCG exposure. However, for the first time, MII stage oocytes have been retrieved from hCG-treated antral follicles (Fig. 5.3a), following growth from preantral follicles under chemically defined conditions. In GV-intact oocytes, the diffuse chromatin is evident as a perinucleolar ring. In MII oocytes, the chromatin is condensed and reflects the chromosome organization for meiosis. A spindle and the first polar body are observed with normal sizes and positions in MII oocytes. MII oocytes can be fertilized following insemination by intracytoplasmic sperm injection (ICSI). The fertilized oocytes cleave to two cells within 24 h after ICSI (Fig. 5.3b). Subsequent cleavage is observed, but embryonic development arrests by day 3 post-ICSI (Fig. 5.3c; Xu et al. 2011a). In rhesus monkeys, the transition from maternal to embryonic genome occurs at the 6–8-cell stage (Schramm and Bavister 1999). Therefore, the oocyte must contain the appropriate instructions, involving expression of new proteins from maternal mRNAs/genes (Kocabas et al. 2006), to drive the first few divisions and the awakening of the embryonic genome. Thus, a few macaque oocytes obtained from current IFM experiments are capable of reinitiating meiosis and fertilization, i.e., achieving nuclear maturation, but do not complete cytoplasmic maturation. It was noticed that MII oocytes have larger diameters (>110 µm) than GV oocytes (~100 µm) (Xu et al. 2011a). The current culture systems need to be optimized to promote further growth of oocytes. Additional indices of oocyte quality also need to be examined, such as cumulus–oocyte communication (Kimura et al. 2007), to monitor the competence to undergo maturation. Further studies are warranted to improve oocyte cytoplasmic and nuclear maturation, as well as the developmental competence of embryos produced from oocytes retrieved from primate follicles after encapsulated 3D culture.

Oocyte growth and maturation appear related to FSH exposure. Healthy oocytes are retrieved from follicles cultured with either high- or low-dose FSH. However, the diameters of GV oocytes from follicles cultured at low-dose FSH are larger than those of high-dose FSH group (Xu et al. 2011a). High FSH negatively impacted preantral follicle development in mice wherein the increase in FSH dose changed both oocyte and cumulus cell transcript levels during mouse follicle culture (Kreeger et al. 2005). Conversely, a decrease in FSH dose seemed to limit inappropriate gene expression (Sánchez et al. 2010). Prolonged high FSH exposure may disturb oocyte control of granulosa cell proliferation and differentiation, as well as cumulus cell function, thus favoring somatic cell function, i.e., steroidogenesis, over oocyte developmental competence during primate follicle growth in vitro.

Oocyte parameters are also influenced by age of donor monkeys and O_2 milieu. The majority of oocytes retrieved from antral follicles that developed in vitro from secondary follicles obtained from young adult monkeys are healthy. In contrast, fewer oocytes retrieved from in vitro-developed antral follicles from prepubertal animals are healthy, and only a few healthy oocytes are retrieved from antral follicles grown in older adults. To date, MII oocytes are only obtained from fast-grow follicles of young adult monkeys. The oocyte diameters of fast-grow follicles from young adults, are larger than those from prepubertal macaques (Xu et al. 2010, 2011a). This may due to the difference in FSH receptor expression and signaling pathway between follicles of various age groups as described above. More healthy oocytes are also retrieved from the follicles cultured at 5 % than 20 % O_2. It is well established that high O_2 tension is associated with higher levels of reactive oxygen species, and oxidative stress plays a role in cytotoxic activity (Evans et al. 2004; Devine et al. 2012). As such, low O_2 tension (5 %), compared to 20 %, reduced cumulus cell apoptosis in canine COCs during culture (Silva et al. 2009).

LH addition has no effect on oocyte maturation and size. While insulin was reported to have deleterious effects on oocyte development in mouse follicle culture (Eppig et al. 1998) and mouse oocyte competence for embryonic development (Acevedo et al. 2007), the lower insulin concentration in encapsulated 3D culture system does not improve oocyte quality in macaques (Xu et al. 2010, 2011a).

5.3 3D Culture of Baboon Follicles

The baboon is another nonhuman primate model used for studies related to women's reproductive health in the areas of contraception, reproductive aging, infertility, implantation, and endometriosis (D'Hooghe et al. 2004). Only a few reports have applied the baboon model to understand early events of folliculogenesis (Fortune et al. 1998; Wandji et al. 1997). In a recent study, baboon preantral follicles were cultured in a semidegradable 3D matrix to investigate the effect of gonadotropin on follicle survival, growth, and oocyte maturation (Xu et al. 2011b).

Primate ovarian tissues have denser connective tissue than rodents, which renders the isolation of individual follicles somewhat difficult without enzymatic treatments. However, collagenase digestion not only loosens the connective tissue surrounding the follicle, but may also disrupt the basement membrane and remove most, if not all, theca-interstitial cells of follicles. Whether and how the digestion itself or loss of the basement membrane and theca-interstitial cells, or combined forces would impact follicle growth in vitro is open for debate (Abir et al. 1997; Roy and Treacy 1993). Initial studies on macaque (Xu et al. 2009b) and baboon (Xu et al. 2011b) follicles indicate that preantral follicles can survive and grow after collagenase treatment. However, due to variation among animals, it was difficult to uniformly control the level of stromal digestion that many times led to secondary follicle damage during isolation (unpublished results). Whether and how theca-interstitial cells promote primate follicle growth and oocyte maturation during culture awaits further study.

Soft hydrogels provide a more permissive environment for follicle growth relative to rigid hydrogels (Xu et al. 2006b). Alginate hydrogels, which are not degradable and thus have a relatively stable elastic modulus, may resist the large deformations associated with significant increases in follicle diameter, which could result in a nonpermissive condition for primate follicles, as they need to grow to much larger sizes than mouse follicles. A semidegradable matrix containing fibrin, alginate, and Matrigel (FAM) was employed to grow baboon preantral follicles because a previous study indicated that it provided a dynamic mechanical environment that promoted mouse follicle growth and increased the number of meiotically competent oocytes relative to alginate (Shikanov et al. 2009). Indeed, the FAM matrix facilitated baboon follicle expansion while maintaining antral follicle architecture. Moreover, compact COCs isolated from baboon antral follicles underwent IVM to yield oocytes that reinitiated meiosis (MII stage) with a normal appearing spindle structure (Fig. 5.4).

A necessity for FSH in mouse (Abir et al. 1997), macaque (Xu et al. 2011a), and human (Adriaens et al. 2004) preantral follicle development in vitro has been established. Interestingly, in the baboon, the transition from preantral to small antral follicles in vitro appears to be FSH-independent under certain culture conditions. The absence of exogenous FSH did not affect follicle survival and health in the baboon (Xu et al. 2011b), while exogenous FSH did impact follicle growth rate, particularly in the beginning of culture. With a higher dose of FSH (100 mIU/ml), follicles increased from an average diameter of 288 ± 9 μm to 439 ± 24 μm in 4 days, while it took 8–10 days to reach an equivalent size when follicles were grown in the absence of or with a lower dose of FSH (10 mIU/ml). Although follicles exposed to a higher dose of FSH showed a faster growth rate in the beginning of culture, growth plateaued after antral formation. On the other hand, the follicles cultured without FSH steadily grew to an equivalent diameter and formed an antral cavity. Whether the FSH-independent growth of baboon preantral follicles, unlike that of macaque or human follicles, is due to species differences or different culture techniques (e.g., presence of Matrigel, and its associated growth factors, as an extracellular matrix) is unknown.

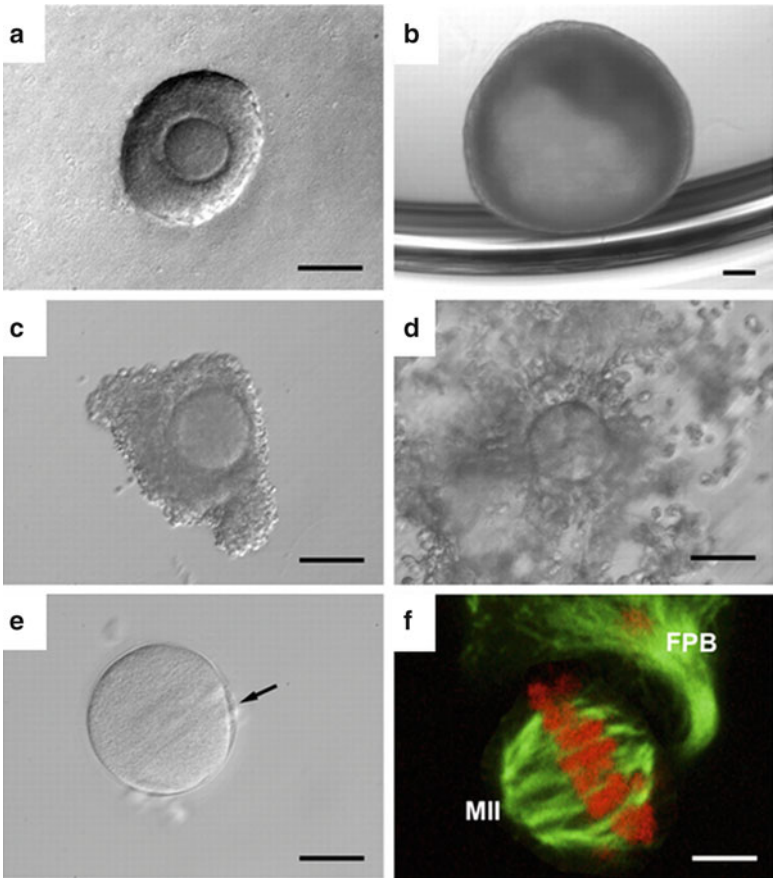

Fig. 5.4 In vitro follicle growth of a baboon preantral follicle. A preantral follicle isolated from baboon ovary was encapsulated in fibrin, alginate, and Matrigel (FAM) matrix (*panel a*). After 14 days of culture without FSH, the follicle developed to the small antral stage (*panel b*). A compact cumulus–oocyte complex (COC) was recovered from the follicle for in vitro maturation (IVM; *panel c*). Cumulus cells expanded after 24 h of IVM (*panel d*). The oocyte resumed meiosis, reached the metaphase II (MII) stage within 48 h (*panel e*), and displayed a normal spindle structure (*panel f*). *Arrow*, polar body (PFB). Scale bar = 100 μm (*panels a–d*), 50 μm (*panel e*), 10 μm (*panel f*) (This figure was originally published in and reproduced with permission from Xu et al., Biol Reprod 2011b)

5.4 Comparison of Gene Expression Between In Vivo- and In Vitro-Derived Macaque SAFs

Pilot studies were conducted, using the rhesus macaque as a model, to compare gene expression profiles between SAFs (~1 mm in diameter) derived from encapsulated 3D culture and those developed in vivo during the early follicular phase of

spontaneous menstrual cycles (unpublished data). Preliminary data, generated from Affymetrix microarray assays, indicated that the mRNA levels from genes of major steroidogenic enzymes did not differ (e.g., steroid 17-alpha-hydroxylase/17,20 lyase and aromatase), except that low density lipoprotein receptor (*LDLR*) was up-regulated in cultured SAFs compared to those developed in vivo (Table 5.1). The increase in *LDLR* mRNA expression may be due to the prolonged exposure of cultured follicles to exogenous FSH, which is consistent with the observation that FSH increased both LDLR mRNA (LaVoie et al. 1999) and protein (Veldhuis 1988) expression in cultured porcine granulosa cells. The mRNAs for some local factors secreted by SAFs, e.g., *AMH* and *AMH* receptor, were not expressed differently between in vitro- and in vivo-developed SAFs. However, mRNAs for the angiogenic factor *VEGF* and its receptors were down regulated in cultured SAFs (Table 5.1), which indicates that, though with similar sizes, the cultured SAFs may not achieve the same maturation state as in vivo-derived SAFs that requires vascularization for further development (Stouffer et al. 2001). When analyzing factors involved in cell death, the mRNAs from genes encoding caspases or autophagy-related proteins (e.g., Autophagy related 7 and Beclin 1) did not differ between in vitro- and in vitro-developed SAFs. In contrast, mRNA expression for anti-apoptosis factors increased in cultured SAFs compared to in vivo-derived SAFs, including glutamate-cysteine ligase catalytic subunit (*GCLC*) and epidermal growth factor receptor (*EGFR*) (Table 5.1). Exogenous FSH in the culture media may promote the *GCLC* and *EGFR* mRNA expression, as reported in rat SAF and granulosa cell culture (Hoang et al. 2009; Fujinaga et al. 1994).

Thus, macaque SAFs derived from encapsulated 3D culture exhibited some similarities as well as differences in gene expression compared to those of in vivo-developed SAFs. Further experiments are warranted to validate the microarray results, and to consider the causes and effects of altered gene expression as clues to improve coordinated follicular development leading to oocyte competence.

5.5 Future Studies

Advances in the 3D culture allow primate secondary follicles to grow to the small antral stage and yield mature oocytes. The following conditions to optimize culture are now being employed: (1) a higher dose (3 ng/ml) of FSH for the first 3 weeks to support follicle survival, followed by a low dose (0.3 ng/ml) to avoid premature differentiation (luteinization); (2) a low concentration (0.5 mg/ml) of fetuin to maintain alginate gel integrity; and (3) low O_2 tension at 5 % to limit detrimental effects of high oxygen on follicle survival and mimic the follicular environment in vivo. In the presence of a higher dose of FSH, growing follicles reach the multilayer stage and then form an antrum. After switching to low-dose FSH, fast-grow follicles continue to grow until the diameters are over 1 mm, when some can respond to hCG to yield MII oocytes. AMH production increases when follicles are at the multilayer stage. Steroid and VEGF levels are elevated around or after antrum formation while

Table 5.1 Comparison of mRNA levels for selected genes between in vitro- and in vivo-developed small antral follicles of rhesus monkeys

Function	Gene name	Synonym	Gene symbol	NCBI accession number	Fold change (in vitro/in vivo)[a]
Steroidogenesis	Low density lipoprotein receptor	LDL receptor	LDLR	NM_001195800	2.4 ↑
	Cytochrome P450 family 11 subfamily A polypeptide 1	Cholesterol side-chain cleavage enzyme	CYP11A1	NM_000781	NC
	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	3 beta-hydroxysteroid dehydrogenase/ Delta 5→4-isomerase type 1	HSD3B1	NM_000862	NC
	Cytochrome P450 family 17 subfamily A polypeptide 1	Steroid 17-alpha-hydroxylase/ 17,20 lyase	CYP17A1	NM_000102	NC
	Cytochrome P450 family 19 subfamily A polypeptide 1	Cytochrome P450 aromatase	CYP19A1	NM_000103	NC
Transcription regulation	Anti-Müllerian hormone	Müellerian-inhibiting factor	AMH	NM_000479	NC
	Anti-Müllerian hormone receptor, type II	Anti-Müellerian hormone type-2 receptor	AMHR2	NM_020547	NC
Angiogenesis	Vascular endothelial growth factor A	Vascular endothelial growth factor	VEGFA	NM_001025366	4.2 ↓
	Vascular endothelial growth factor receptor 1	Fms-related tyrosine kinase 1	VEFGR1/FLT1	NM_002019	158.3 ↓
	Vascular endothelial growth factor receptor 2	Kinase insert domain receptor	VEGFR2/KDR	NM_002253	17.9 ↓
Apoptosis	Glutamate-cysteine ligase, catalytic subunit	Gamma-glutamylcysteine synthetase heavy subunit	GCLC	NM_001498	5.8 ↑
	Epidermal growth factor receptor	Epidermal growth factor receptor	EGFR	NM_005228	2.5 ↑
Autophagy	Autophagy related 7	Ubiquitin-like modifier-activating enzyme	ATG7	NM_006395	NC
	Beclin 1, autophagy related	Beclin-1	BECN1	NM_003766	NC

↑/↓, Gene expression levels increased/decreased ($P<0.05$) in in vitro-, compared to in vivo-, developed follicles

[a]Fold changes in mRNA levels, assayed by Affymetrix macaque gene arrays; follicles from $n=3$ animals

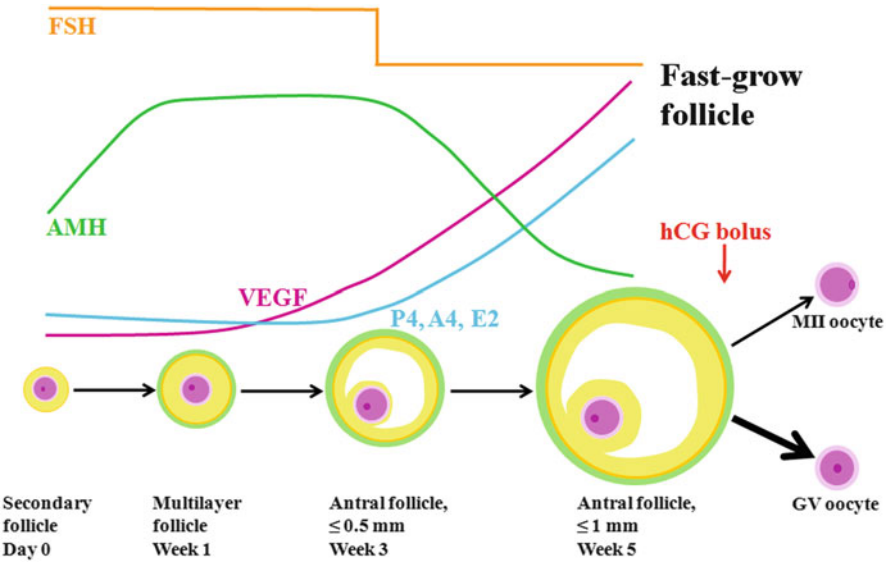

Fig. 5.5 Conceptual graph of macaque follicle development during encapsulated three-dimensional culture, from a secondary follicle at collection to a small antral follicle at week 5. The FSH pattern denotes a higher dose to support follicle survival, followed by a low dose after antrum formation to continue follicle growth. The steroid (progesterone, P4; androstenedione, A4; estradiol, E2), AMH, and VEGF patterns denote production as a function of follicular development. After the hCG bolus, a few oocytes mature to the metaphase II stage, while most of the oocytes remain at the germinal vesicle stage. This graph depicts the development and activities of fast-grow follicles

AMH level decreases (Fig. 5.5). But under the best case scenario, some follicles do not survive, and those that survive vary in growth potential. Healthy oocytes are obtained from in vitro-developed antral follicles, but few mature to the stage of spontaneous reinitiation of meiosis after removal from the follicle, or in response to hCG. The IFM protocol needs further improvement to produce more meiotically and developmentally competent oocytes for subsequent embryonic development after fertilization.

Studies can be conducted using nonhuman primates to compare the structure and function between SAFs or their COCs derived from culture in various matrices and those developed in vivo during spontaneous menstrual cycles. These studies will be valuable for assessing whether the encapsulated 3D system allows coordinated development of granulosa and theca cells, plus cumulus cells and oocytes, similar to that in vivo, and if not, will help define cellular functions that require further optimization in the culture system. This culture system also provides a way to examine the function of endocrine/paracrine factors during folliculogenesis in primates, including gene and protein expression, as well as metabolic pathways. This information can be used to discover biomarkers that predict or monitor follicle and/or oocyte condition during IFM.

Since tissue resources from nonhuman primates or women are limited, efforts are warranted to more efficiently use the entire follicle pool. Smaller resting primordial and early growing primary follicles represent a larger follicle population than secondary follicles. Primordial follicles within pieces of the baboon (Wandji et al. 1997) or human (Telfer et al. 2008) ovarian cortex can survive and develop to the secondary stage in serum-free culture. Human (Vanacker et al. 2011) and macaque (Hornick et al. 2012) primordial follicles can be isolated and maintain their viability when cultured in groups. To date, efforts to grow and mature individual primordial follicles in vitro have not been reported in primates, especially under chemically defined conditions. Experiments conducted in rhesus macaques indicate that it is possible to grow individual primary follicles (80–120 μm in diameter) in vitro to the small antral stage, which function in steroidogenesis, local factor production, and oocyte maturation (Xu et al. 2013). However, the culture interval required to reach the small antral stage is longer when starting with primary verse secondary follicles (13 vs. 5 weeks). There are also SAFs that range in size from 0.5 to 1.5 mm in diameter in the medullary region of the ovary. COCs obtained from these follicles are able to achieve cumulus expansion and oocyte meiotic maturation after IVM in both rhesus macaques (Peluffo et al. 2010) and baboons (Xu et al. 2011b), with demonstration of fertilization and early embryonic development in vitro to the expanded blastocyst stage (Peluffo et al. 2012).

IFM in nonhuman primates is a powerful tool to improve the understanding of the basic biology of primate follicles, such as the heterogeneity of the preantral follicle pool, role(s) of ovarian steroids and local factors on folliculogenesis and oocyte developmental capacity. Once achieved, this knowledge may be valuable in identifying optimal conditions for human follicle culture, with the ultimate goal of translating the experimental results and products to patients, thereby facilitating diagnostic and therapeutic approaches for female fertility.

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Chapter 6
Vitrification of Oocytes: From Basic Science to Clinical Application

Amir Arav and Yehudit Natan

Abstract Vitrification is a physical process by which a liquid is transformed into a solid of amorphous glass form. It was only at the end of the nineteenth century (1898) that Gustav Heinrich Johann Apollon Tammann pointed out that a large number of substances can be obtained as glasses and suggested that this property might be universal (Tammann, Zeitschrift for Physikalische Chemie; 25: 441–479, 1898). Basically, vitrification is the supercooling of a liquid to a temperature at which the viscosity is so high that it can be defined as being at a solid state. The understanding of the vitrification process has been deepened over the years and has been applied for cryopreservation and currently is the method of choice for preserving oocytes and embryos.

Keywords Vitrification • Oocytes • Embryos • Cryopreservation • Application

6.1 The Evolution of Vitrification

When I started working on this chapter, I looked again into the book of "Life and Death at Low Temperatures" by Basile Luyet published in 1940 (Luyet and Gehenio 1940). Imagine my surprise when I found out that the small volume vitrification was already thought at the beginning of the nineteenth century. It was done by the great French chemist and physicist Joseph Louis Gay-Lussac. He is known mostly for his two laws of gases and for his work on alcohol–water mixtures. Gay-Lussac found that water can be cooled to −12 °C without freezing, finding with this discovery the basis of vitrification (von Humboldt and Gay-Lussac 1807).

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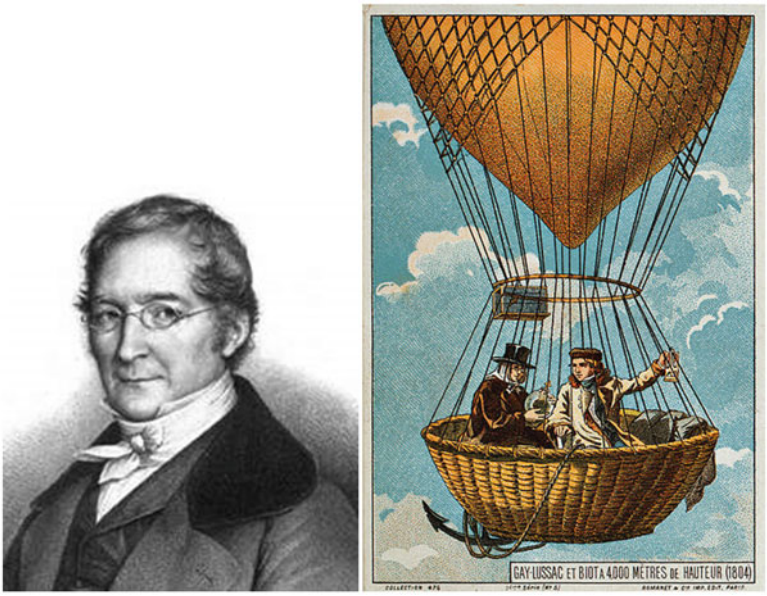

Fig. 6.1 Joseph Louis Gay-Lussac (1778–1850) and portrait of an illustration from 1804 of Gay-Lussac and Jean-Baptiste Biot in the hot air balloon at 4,000 m

In 1804 Gay-Lussac when ascending in a hot air balloon (Fig. 6.1) noticed that the drops in the clouds are not frozen despite the subzero temperatures. He published later the discovery of the effect of small volume of water droplets on supercooling. And indeed the size of the water drops in the cloud is around 8–10 μm which maintains them at a liquid state at a subfreezing temperature of −5 °C.

Luyet described it in his book: "Some of the oldest investigations on subcooling were made by Gay-Lussac (1836) who observed that water can be subcooled to −12 °C when it is enclosed in small tubes" (Luyet and Gehenio 1940).

Already in 1858 Johann Rudolf Albert Mousson sprayed droplets of water less than 0.5 mm in diameter on a dry surface and observed that the smaller the drops the longer they stayed subcooled (Mousson 1858). Not only the volume was important to achieve supercooling, but also among other factors which might have an influence in inducing crystallization as was mentioned by Luyet are the cooling velocity and the concentration of the supercooled or supersaturated solutions. Luyet wrote "To avoid freezing, the temperature should drop at a rate of some hundred degrees per second, within the objects themselves" and also "The only method of vitrifying a substance is to take it in the liquid or gas state and cool it rapidly so as to skip over the zone of crystallization temperatures in less time than is necessary for the material to freeze". Luyet also wrote "It is evident that when the crystals grow faster one must traverse the crystallization zone more rapidly if one wants to avoid crystallization" (Luyet and Gehenio 1940).

6.2 A Better Understanding of the Vitrification Process

The velocity of cooling also depends on the thermal mass of the sample and on its surface area. To achieve rapid cooling we should use material with the lowest heat mass and maximum surface to volume ratio.

Indeed Gregory M. Fahy and William F. Rall published in 2007 the critical cooling rates needed to vitrify aquatic solutions which contain different concentrations of cryoprotectants (CPs) (Fahy and Rall 2007), as we can see it was extrapolated that for pure water over 100×10^6 °C/min is needed to form a glass state without crystallization. Also, it is interesting to note that for 15 % (v/v) of most CPs almost 1×10^6 °C/min is needed which is also very hard to achieve. We have shown that 15 % (v/v) of CPs can be vitrified at a relatively slow cooling rate when volume of the drop is 0.07 μl (Arav 1992). Therefore, it is much more possible to achieve vitrification by lowering the volume than by increasing the cooling rate.

James H Walton and Roy C Judd measured the velocity of ice crystal growth and found that it is in the range of 65 mm/s (Walton and Judd 1914). This means that if we want to avoid crystallization in a drop which is placed on a cool metal plate and has a diameter of 0.01 mm, we will need a velocity of 1/6,500 mm/s which is 0.0001 s. If we cool from RT to −180 °C this means we need to reduce 200 °C at a rate of 0.1 μs or at 78×10^6 °C/min. This is actually the cooling rate that was estimated by Balds and by Bruggeler (Fig. 6.2) (Luyet and Gehenio 1940; Fahy and Rall 2007); however, because this cooling rate is impossible to achieve the ability to reach vitrification of pure water in a small drop can be achieved in relatively slow

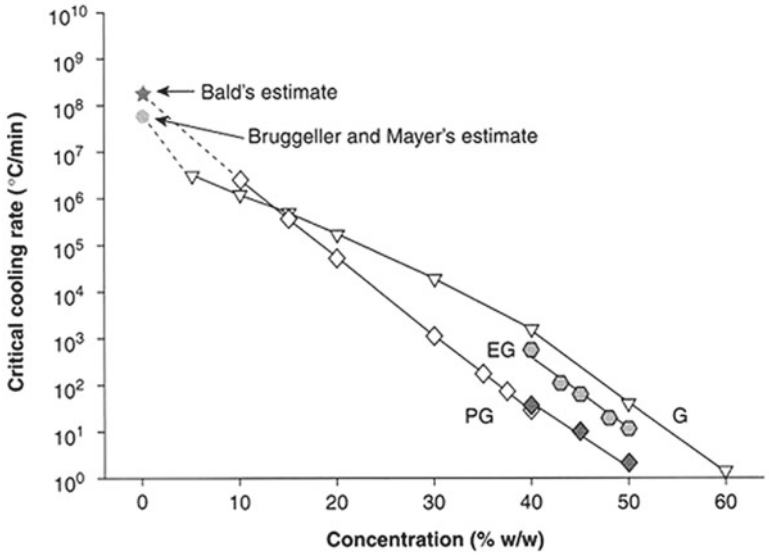

Fig. 6.2 Critical cooling rates of different solutions with different CPs concentration (Fahy and Rall 2007)

cooling rates which indicate that the small volume has an independent effect on the probability of vitrification.

Later attempts at vitrifying pure water were made by a few investigators; L Hawkes (1929) published an experiment in which a drop of solid amorphous water was obtained, by chance, during rapid cooling. EF Burton and WF Oliver obtained, from steam, some solid water in which X-ray analysis did not reveal any crystalline structure (Burton and Oliver 1935). As we can see these achievements were mainly due to the samples' small volume and not the velocity of cooling.

6.3 The First Survival of Cells After Vitrification

The year 1938 was the most important for cryobiology as Basile J. Luyet and Eugene L. Hodapp published the first successful vitrification of sperm (Luyet and Hodapp 1938); Luyet started his research with colloids (i.e., gelatin, milk, or agar) (Luyet 1937) and have found that their water content determines the possibility or impossibility of vitrification. In general, with 50 % gelatin solutions, they had vitrified layers of 0.3 mm thickness (by the method of immersion in liquid nitrogen), while with solutions containing 90 % water, they could vitrify only smears of a few micron thickness (Luyet and Gehenio 1940). They were the first to demonstrate successful cryopreservation of frog sperm by vitrification using 2 M of sucrose and small drops.

Few years later, in 1949, Christopher Polge et al., when trying to repeat Luyet results, discovered the cryoprotective property of glycerol and so opened the field of slow freezing.

Currently, there are two methods for gametes cryopreservation: slow freezing and vitrification. Slow freezing has the advantage of using low concentrations of CPs. On the other hand, vitrification requires high concentrations of CPs, which are associated with chemical toxicity and osmotic shock. Vitrification is a rapid method, which reduces chilling sensitivity and crystallization damage to cells.

6.4 Applying Vitrification for Oocytes and Embryos

For many years slow freezing, and not vitrification, was the method of choice. This was because vitrification was not achieved easily due to the need of high CPs concentrations and relatively high volume samples. Only in 1985 the first successful vitrification of mouse embryos using a relatively large volume sample was done (Rall and Fahy 1985). They vitrified mouse embryos with 6.5 M of glycerol and in a large volume inside a 0.25 ml straw plunged into liquid nitrogen. At that time I was a veterinary student at the University of Bologna, Italy and I met Bill Rall who told me about the exciting work he had done on mouse embryos. Two years later I started to work with Boris Rubinsky on cryomicroscopy of oocytes and embryos

and as in our lab we used to prepare oocytes for histology evaluation by fixing them with a small drop over a microscopic slide. I had the idea of using the same technique for vitrification in a small drop which I later called the "minimum drop size" (Arav 1989; Arav 1992; Arav and Zeron 1997; Arav et al. 2002).

As it was noted, the probability of vitrification increases as the volume of the sample decreases. Pure water is vitrified only in very small droplets obtained from aerosols. Vitrification of thin layers (<1 μm) of viruses in water suspension was achieved in rapid cooling for the purposes of electron microscopy (Adrian et al. 1984). In 1989 we introduced a new method, called the "minimum drop Size (MDS)" (Arav 1989, PhD thesis), for the vitrification of oocytes and embryos using directional freezing cryomicroscopy. The volume we used for the vitrification was in the range of 0.07 μl and the concentration of the vitrification solution (VS) was about 50 % lower than that of the VS used for large volume vitrification (Arav 1992; Rubinsky et al. 1991).

We called it the MDS as this was the minimal size that allows keeping oocytes or embryos without damage due to desiccation.

Vitrification of embryos, on the other hand, although initially attempted in the late 1980s, has not been clinically applied until recently. Vitrification is currently producing very satisfactory outcomes by means of methodologies that use a minimum volume (Kuwayama et al. 2005a, b; Cobo et al. 2008).

Three important factors should be considered:

1. *Cooling rate and warming rate*—high cooling rate is achieved with liquid nitrogen or liquid nitrogen slush and a warm water bath for warming. When using liquid nitrogen, the sample is plunged into liquid nitrogen resulting in cooling rates of hundreds to tens of thousands degrees Celsius per minute, depending on the container, the volume, the thermal conductivity, the solution composition, etc. (Yavin and Arav 2007). To achieve liquid nitrogen (LN) slush, the LN needs to be cooled close to its freezing point (−210 °C). Slush is generated by the VitMaster (IMT Ltd, Ness Ziona, Israel), a device that reduces the temperature of the LN to between −205 °C and −210 °C by applying negative pressure. LN slush is then formed, and the cooling rate is dramatically increased. The cooling rate is especially enhanced in the first stage of cooling when cooling down from room temperature to 0 °C. The cooling rate is enhanced two to six times more than that by plunging into LN (−196 °C) with 0.25 ml straws or any other device such as open-pulled straws (OPS) or electron microscope (EM) grids (Arav and Zeron 1997). It was shown that for oocytes and embryos that increasing cooling rates improved survival rates by up to 37 % (Saragusty and Arav 2011). Recently, it was shown that warming rate is an important factor for successful vitrification of mouse oocytes (Seki and Mazur 2012).
2. *Viscosity* of the medium in which the embryos are suspended, or the glass transition coefficient of the solution at low temperatures. This is defined by the concentration and behavior of various CPs and other additives during vitrification. The higher the concentration of CPs, the higher the glass transition temperature (Tg), thus lowering the chance of ice nucleation and crystallization. Different CPs and other additives have different toxicity, penetration rate, and Tg.

The viscosity of the medium in which the embryos are suspended is defined by the concentration and behavior of various CPs and other additives used during vitrification. The combination of different CPs is often used to increase viscosity, increase Tg, and reduce the level of toxicity. In the cattle industry, so as to avoid handling of the post warmed embryos and allow direct transfer, ethylene glycol (EG) is often used as the permeating CP because of its high penetration rate (Saha et al. 1996).

3. *Volume*—the smaller the volume, the higher the probability of vitrification (Arav 1989; Arav 1992; Arav and Zeron 1997; Arav et al. 2002). Smaller volumes allow better heat transfer, thus facilitating higher cooling rates. However, small volume has an independent effect on the probability of nucleation as was discovered 200 years ago by Gay-Lussac. Many techniques have been developed to reduce sample volume, with an explosion of methods appearing in the literature during the last decade.

These techniques can generally be divided into two categories: surface techniques and tubing techniques (Saragusty and Arav 2011). The surface techniques (Fig. 6.3) include electron microscope grid (Martino, 1996), minimum drop size (MDS; Arav 1992; Arav and Zeron 1997; Yavin and Arav 2001), Cryotop (Hamawaki et al. 1999; Kuwayama and Kato 2000), Cryoloop (Lane et al. 1999a, b), Hemistraw (Vanderzwalmen et al. 2000), solid surface (Dinnyes et al. 2000), nylon mesh (Matsumoto et al. 2001), Cryoleaf (Chian et al. 2005), direct cover vitrification (Chen et al. 2006), fiber plug (Muthukumar et al. 2008), vitrification spatula (Tsang and Chow 2009), Cryo-E (Petyim et al. 2009), plastic blade (Sugiyama et al. 2010), and Vitri-Inga (Almodin et al. 2010). To the tubing techniques (Fig. 6.4) belongs the plastic straw (Rall and Fahy 1985), OPS (Vajta et al. 1997, 1998), closed pulled straw(CPS)(Chen et al. 2001), flexipet-denuding pipette (Liebermann et al. 2002), superfine OPS (Isachenko et al. 2003), CryoTip (Kuwayama et al. 2005a, b), pipette tip (Sun et al. 2008), high-security vitrification device (Camus et al. 2006), sealed pulled straw (Yavin et al. 2009), Cryopette (Portmann et al. 2010), Rapid-i (Larman and Gardner 2010), and JY Straw (RC Chian, personal communication). Each of these two groups has its specific advantages. In the surface methods, the size of the drop (0.1 μl) can be controlled, high cooling rate is achieved because these systems are open, and high warming rates are achieved by direct exposure to the warming solution. The tubing systems have the advantage of achieving high cooling rates in closed systems, thus making them safer and easier to handle. Decreasing the vitrified volume and increasing the cooling rate allow for a moderate decrease in CP concentration so as to minimize its toxic and osmotic hazardous effects (Yavin et al. 2009). Combining these three factors can result in the following general equation for the probability of vitrification:

Arav Equation:

$$\text{Probability of vitrification} = \frac{\text{Cooling} \times \text{warming rate} \times \text{viscosity}}{\text{volume}}$$

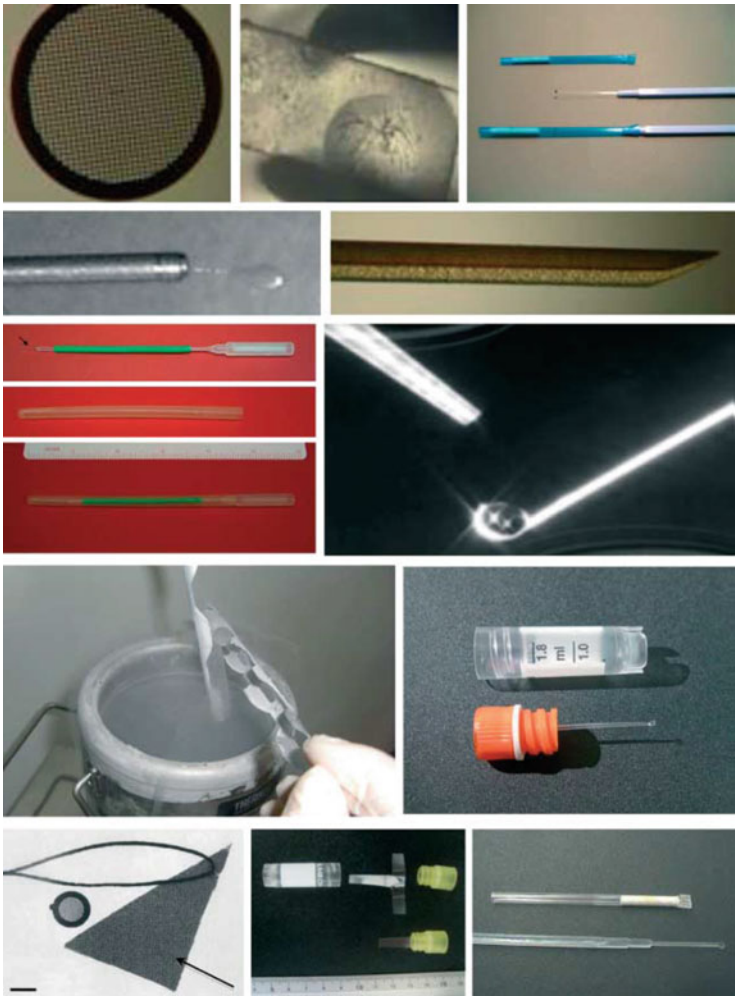

Fig. 6.3 Vitrification surface techniques

Oocytes are very different from sperm or embryos with respect to cryopreservation. The volume of the mammalian oocyte is in the range of three to four orders of magnitude larger than that of the spermatozoa, thus substantially decreasing the surface-to-volume ratio. However, this is not the reason why the oocytes are sensitive to low temperature and to slow freezing, i.e., mature oocytes are very sensitive to slow freezing; however, after fertilization the volume of the oocytes remains the same but their sensitivity is reduced to minimum and in fact this is the best stage for freezing human oocytes (2PN freezing—Testart et al. 1986). The reason why many oocytes are non freeezable is due to their chilling sensitivity which occurs at different cellular levels: the zone pellucida (ZP), plasma membrane, meiotic spindles, cytoskeleton, etc.

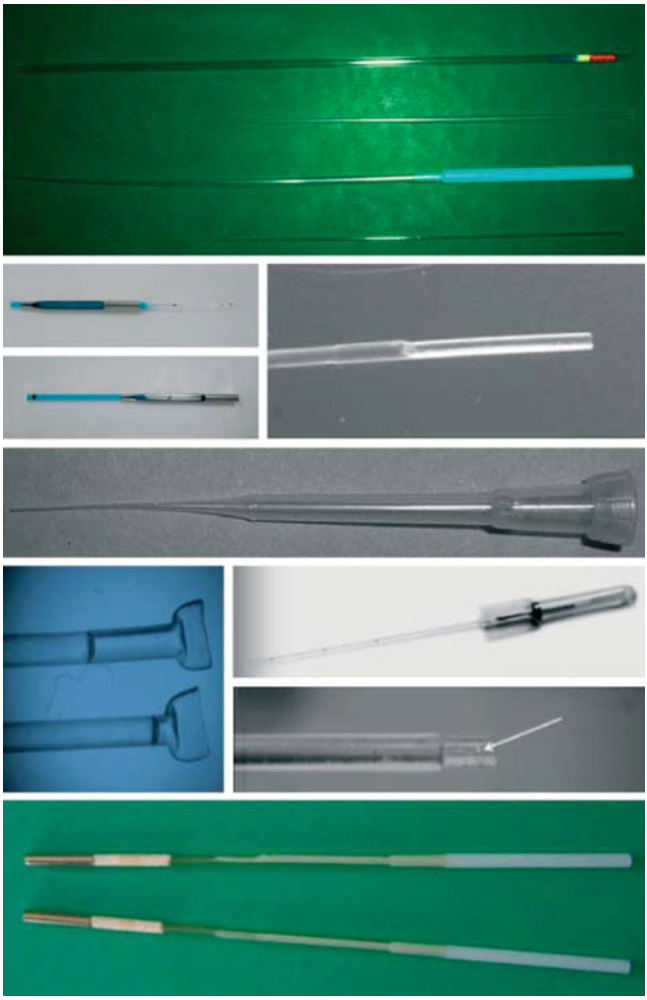

Fig. 6.4 Vitrification tubing techniques

The plasma membrane of oocytes at the MII stage has a low permeability coefficient, thus making the movement of CPs and water slower (Ruffing et al. 1993). In addition freeze–thaw process causes premature cortical granule exocytosis, leading to hardening of zona pellucida and thus making sperm penetration and fertilization impossible (Carroll et al. 1990; Mavrides and Morroll 2005), a process that can be overcome by the use of ICSI or subzonal sperm insertion. Oocytes also have high cytoplasmic lipid content that increases chilling sensitivity (Ruffing et al. 1993). They have less submembranous actin microtubules (Gook et al. 1993), making their membrane less robust. Cryopreservation can cause cytoskeleton disorganization, and chromosome and DNA abnormalities (Luvoni 2000). The meiotic spindle, which has been formed by the MII stage, is very sensitive to chilling and may be

compromised as well (Ciotti et al. 2009). It does, however, tend to recover to some extent after thawing or warming and IVC, a recovery that is faster following vitrification than that following slow freezing (Ciotti et al. 2009). Oocytes are also more susceptible to damaging effects of reactive oxygen species (Gupta et al. 2010). Many of these parameters change after fertilization, making embryos less chilling sensitive and easier to cryopreserve (Gook et al. 1993; Fabbri et al. 2000; Ghetler et al. 2005).

Vitrification requires the presence of high concentrations of CPs. It is therefore important to minimize the damage caused to cells by the osmotic stress or chemical toxicity. No ideal cryoprotectant that meets the requirements of all different species and developmental embryonic stages has been found; vitrification studies should therefore be preceded by osmotic and cytotoxic studies. The presence of cryoprotectant in the vitrification solution decreases the probability of intracellular crystallization which is considered to cause most damage when very rapid cooling takes place, but the high concentration of the cryoprotectant required is toxic and causes osmotic injury to the oocytes even without cooling.

Different methods have been used to reduce this "solution effect": (1) short time of exposure to cryoprotectants (Fahy et al. 1984; Arav et al. 1988), (2) use of low toxicity cryoprotectants (Rall et al. 1987) or mixtures of them (Massip et al. 1987), (3) addition of nonpermeating cryoprotectants (Fahy et al. 1984), (4) reduction the cryoprotectant concentration (Rall et al. 1987), (5) exposure at low temperatures (Rall et al. 1987). Of these methods the use of nonpermeating cryoprotectants is very useful either because the shrinkage of the oocyte and consequently the amount of water inside the cell that may crystallize during rapid cooling and warming is lower (Rall et al. 1987), or because of the reduction of the amount of the cryoprotectant that penetrates the cell and thus reducing the possible toxic effect (Széll and Shelton 1987). In addition, the carbohydrates used as nonpermeating CPs have a stabilizing effect on membranes (Crowe et al. 1983). In the study reported by us (Arav et al. 1994), trehalose was less harmful than sucrose. Determination of the Boyle Van't Hoff relationship for both sucrose and trehalose produced the same regression line, so it is possible that this beneficial effect could be a consequence of its interaction with the membrane polar lipid groups (Crowe et al. 1983). Only 10 min of exposure is required for equilibration in propylene glycol and DMSO solutions or mixtures of them. The membrane is very permeable to both of them. The results of the vitrification provide evidence that propylene glycol can be used successfully. Indeed, the IVF rate of the oocytes vitrified in a solution containing 40 % (w/v) propylene glycol was 37 % and is not different from the results obtained using a slow freezing protocol (Schellander et al. 1988). The viability of vitrified mouse embryos was successfully increased by reducing the concentration of the cryoprotectant (Rall and Meyer 1989). However, concentrated solutions of permeating cryoprotectant are required for successful cryopreservation of oocytes when rapid cooling and warming rates are used. In earlier reports on immature pig oocytes, we showed that when lower concentrations of CPs are used, despite apparent vitrification, membrane destruction was unavoidable (Rubinsky et al. 1991). In 1990 M. Kasai was the first to describe the use of ethylene glycol for mouse embryo vitrification (Kasai et al. 1990). Today the most frequent solutions are based on a mixture of DMSO and ethylene glycol (Kuwayama et al. 2005a, b).

6.5 Small Volume Is the Solution for Most Problems That Occur During Vitrification

There are three major problems associated with vitrification: (1) Crystallization (during cooling), (2) Devitrification (crystallization during storage or during warming), and (3) Fractures of the glassy solution which can cause devitrification due to release of energy by the fracture. Surprisingly, at 1 μl, fractures appeared only when the concentration of the VS is high (100 % VS = 38 % ethylene glycol, 0.5 M Trehalose, and 4 % bovine serum albumin in TCM medium) but not at lower volumes (Arav et al. 2002). This means that the probability of fractures increases with the increasing of Tg or the viscosity of VS. At the low concentration of VS (50 % VS), fractures were observed only at very high cooling rate. We suggest here a simple explanation for this phenomenon, based on the following equations:

$$\text{Probability of fracturing} = \text{CR and WR} \times \mu \times V$$

$$\textit{Since probability of vitrification is} = \text{CR and WR} \times \mu \times 1/V$$

1. *Increasing the cooling rate (CR) and warming rate (WR) will increase the probability of vitrification; however it will also increase the probability of fractures.*
2. *Increasing the viscosity (μ) will also increase the probability of vitrification because Tg will increase (Yavin and Arav 2007), and therefore will increase the probability of fractions.*
3. *The only parameter that will increase the probability of vitrification and at the same time decrease the probability of fractures is reducing the volume (V) to the value of the "minimum drop size."*

The reason for the increasing probability of fractures in high concentrations of VS is thought to be related to the glass transition temperature (Tg). We know that fractures can form only at temperatures below that at which the liquid turns into glass (Tg) and above the LN temperature (−196 °C). We also know that a solution with a higher CP concentration will have a higher Tg. Therefore, if the temperature gradient increases, as in the case of higher Tg, then the probability of fracturing will also increase. Finally, the results of vitrification of bovine oocytes at the MII or GV stage, with a concentration of 75 % VS, have been reported (Arav et al. 2000). We achieved 72 and 38 % cleavage and blastocyst rates formation, respectively, for the vitrified MII oocytes and 27 and 14 % cleavage and blastocyst rates formation, respectively, for oocytes vitrified at the GV stage. We conclude that the new vitrification procedure, which features small volumes, direct contact with supercooled LN, and low concentrations of VS, reduces chilling injury and provides a high probability of vitrification in the absence of glass fractures.

6.6 Open Versus Closed Systems

Most of the methods that works very well use direct exposure to the LN, which introduces a potential risk of cross-contamination (Bielanski et al. 2003); in fact, contamination of bovine embryos by viral pathogens during storage in LN has already been reported (Bielanski et al. 2000, 2003). Volume is probably the most important factor for successful vitrification in the open systems; it was demonstrated that open systems give better results than closed systems, and these were exclusively for surface systems (Paffoni et al. 2011).

If we analyze the differences between the two systems we can see that the warming rate is not the reason for these differences as it is the same in both systems (we open the container in LN and plunge it directly into a water bath in closed system). The vitrification solution is also not very much different for both systems (actually in small tubes like Cryopette the cooling rate is very high). However, as it has been shown recently the cooling rate can be relatively slow in order to produce high survival in mouse embryos (Seki and Mazur 2012). Therefore, the only factor that is different between the two systems is the volume of the drop; while in the open system the drop volume is reduced to minimum (in the range of 0.1 μl or less), the volume in the closed system is in the range of 0.5 μl or more in order to avoid rehydration and desiccation damage during the introduction into the container and heat sealing. This strengthen the hypothesis that volume has an independant affect on vitrification.

6.7 Summary

As we can see the understanding of the vitrification process has grown over the years. In addition, using it as a preservation method has been increased and improved recently. Today, we believe that vitrification in a small drop and a relatively low concentration of CPs is the best method for oocytes preservation. The procedure can be improved, for example, by developing an automatic device that will prepare the cells for vitrification. This will help us standardize the technique of oocyte and embryo vitrification for everyone use.

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Chapter 7
Memoir of Fertility Preservation

Roger G. Gosden

Abstract Fertility preservation has been practiced for at least 50 years using semen banking, pelvic surgery, and radiation shields, but in the past 20 years it has emerged as a rapidly growing subspecialty of reproductive medicine. A dramatic rise in survivorship of young cancer patients and the widespread postponement of family building to the later years of the female reproductive lifespan have been major driving forces. Throughout the history of fertility preservation, low temperature banking has played a pivotal role, first for gametes and later for embryos and immature germ cells, while ovarian transplantation recently began to contribute and spermatogonial stem cell transfer holds future promise for men and prepubertal boys. But there are significant risks with some diseases from reimplanting residual disease, which hopefully can be eliminated by new methods for purging the tissue and germ cell culture. Since all technologies are interim, cryopreservation as a mainstay in this field will likely be swept aside eventually by a stream of progress aimed at managing fertility preservation in vivo.

Keywords Aging • Cryopreservation • Fertility-preservation • Oocyte • Ovary • Transplantation

7.1 Emerging Awareness

Everyone who started down the research road for fertility preservation has a personal story to tell. For some, it was meeting young cancer patients who had lost fertility to a life-saving treatment, for others it was the challenge of developing new

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technologies to avoid premature ovarian insufficiency or azoospermia. For me, these roads converged. This is a story of my own journey illustrated with references to projects I helped to launch or which significantly altered the course of progress. Since this is not a review but rather a brief personal account, I regret that the contributions of so many others working in this rapidly growing branch of reproductive medicine cannot all be cited here, but they are acknowledged in my reviews elsewhere.

My recollections begin in the late 1980s. It would be misleading to imply that fertility preservation was first emerging at that time because semen banking and surgical translocation of the ovaries, to mention only two important procedures, had already been practiced for a long time. But it was a time when more children and young adults were surviving cancer as a result of advances in disease management which were causing side-effects from ever more potent medications. The personal costs of more successful treatment could be profound, as often as not iatrogenic sterility as well other late effects which have been well documented by Dror Meirow and others (Meirow et al. 2010).

As a laboratory scientist I rarely encountered patients, but my first introduction to someone needing fertility preservation at the Christie Hospital in Manchester, England, had a lasting impact. Joanne was a young policewoman who had recently become engaged to be married. She had been diagnosed with non-Hodgkin's lymphoma. I remember very clearly when she told me that it was a bigger blow to hear that her treatment would make her sterile than when she was told she had cancer. A clinical oncology colleague said that some young patients even asked for less effective treatment if it was less likely to prevent them from having children one day. These were stunning revelations, made all the more poignant when Joanne died.

In those days, although not so long ago, few options were available for such women, and none of them were rosy. A woman about to undergo high-dose chemotherapy could be offered embryo banking in advance, with all its emotional and ethical baggage. Men were somewhat luckier as they could opt for semen cryopreservation before sterilizing chemotherapy, radiation, or orchidectomy, although there are still few options for prepubertal boys. Gamete or embryo donation was offered as a backup, but was not always available or even acceptable. However, I was well aware of relevant progress being made in reproductive technology, and some projects underway in my own laboratory seemed to hold promise of addressing problems faced by patients. This dawning revelation steered my own research goals in a fresh and more practical direction for the years ahead.

7.2 Ovarian Transplantation and Cryopreservation

My first foray into fertility preservation came in a roundabout way. I had wondered whether we might be able to rescue the few primordial follicles in the ovaries of young women with a mosaic XO/XX karyotype or, more radically, if we could help those with non-mosaic Turner's syndrome to become fertile by transplanting donor germ cells. A potential solution to the first group came later with the development

of ovarian banking, and for the second I wondered about the prospect of using germ cells from fetal ovaries to populate streak gonads with follicles. Healthy fetal tissue from pregnancy terminations sometimes became available for research, although I realized this was obviously going to be a controversial agenda. Yet it seemed better to find some use for the tissue than simply discard it.

After disaggregating ovaries from 12 to 16 week-old fetuses, we studied premeiotic and meiotic germ cells in vitro, and afterwards tested their viability by transplantation into immunodeficient SCID mice (Gosden 1992). I had recently reported that ovarian xenografts from several (potentially all) animal species thrive in that environment (Gosden et al. 1994a). Human germ cells could be grown in SCID mice too (Newton et al. 1996), but what would happen if the tissue was transferred to a human recipient? At one time, fetal tissue was widely assumed to be "antigenically immature," but there was no assurance and I left the question to other investigators. Anyway, my studies did not progress very far, although similar projects were starting elsewhere (Abir et al. 2009), because of a lack of grant funding and a mostly hostile response to the very idea of using donor fetal ovaries for fertility.

I wondered whether I could turn the project around from that cul-de-sac to find a practical application for the techniques that we had developed. I hoped to find a strategy and tissue that were less ethically charged. It was then that the idea of fertility preservation for cancer patients with their *own* germ cells dawned on me. By storing these cells in a protected environment they would be available for patients until needed, perhaps many years later, when they could be thawed for natural conception after autotransplantation or cultured to generate mature gametes for an IVF procedure.

We began harvesting primordial follicles from immature mouse ovaries disaggregated with collagenase, most of which remained viable. After disappointment with injecting the follicles into host ovaries sterilized by X-irradiation or aging, we tried recombining the follicles in a fibrin clot or collagen gel, inspired by an old paper from the Rockefeller developmental biologist, Paul Weiss. Sticky grafts proved to be highly successful: follicles grew and the ovary, a plastic organ, was quickly restored to morphology that was indistinguishable from fertile controls. Most important, the animals were fertile again (Gosden 1990). This progress was extended to cryopreservation of the follicles using protocols that John Carroll had successfully adapted for mature oocytes: again we were successful (Carroll and Gosden 1993).

In a relatively underexplored field, there seemed to be so many experimental opportunities, and we made the most of them. Studying human ovarian tissue with Kutluk Oktay who joined the laboratory soon afterwards, we found small follicles isolated enzymatically generally survived despite the fibrous stroma, and even after cryopreservation (Oktay et al. 1997). Today, viability rates are even higher with more specific enzymes (Dolmans et al. 2006), but since it is a slow process disaggregating human ovarian tissues and they yield few follicles the future seemed to lie with intact tissue slices.

Working with David Baird a few years earlier, we had shown that thin cortical slices of cryopreserved sheep ovaries were restored to cyclical function by retransplantation to the same animal (Gosden et al. 1994b; Baird et al. 1999). This was an encouraging advance because the sheep ovary is so much larger and more fibrous

than the mouse model. The tissue became revascularized within only 2–3 days because ovaries are rich in angiogenic factors. Subsequently, with Oktay, Tony Rutherford, Helen Newton, Yves Aubard and others in Leeds we found that slices of human cortex could be successfully engrafted into SCID mice with a majority of follicles surviving the double insult of cryopreservation and ischemia (Newton et al. 1996). With Samuel Kim, the hu-SCID ovarian model was adapted for the vital study of testing whether ovarian tissue from patients with lymphomas carry residual disease, which could potentially cause recrudescence of the disease from transplanted tissue (Kim et al. 2001). The results were especially reassuring for Hodgkin's disease, which became the first condition requiring fertility preservation to benefit clinically from this strategy after a baby was born to a former patient who had undergone ovarian transplantation in Brussels (Donnez et al. 2004).

There were arguments whether that child had indeed been conceived from the transplanted tissue or from a fragment that had remained in situ: a definitive molecular test was obviously impossible because the tissue was autografted. Nevertheless, there is no longer any doubt that ovarian banking-transplantation can be successful since more than 30 babies have been born at independent centers. Transplantation always appeared to be more challenging than cryopreservation because we had to rely on timely revascularization of a tissue in jeopardy of necrosis from ischemia. Most of our experience with vascular ovarian transplants has been in rats in which follicular survival rates were much improved by reducing ischemia to only a few minutes (Wang et al. 2002), but novel techniques were needed to bank the intact ovaries of larger species before retransplantation (Arav et al. 2010). In a remarkable series of monozygotic twin sisters discordant for ovarian function, Sherman Silber showed that when ovarian slices were carefully transplanted (most were fresh) the technique is remarkably robust. All nine patients returned to menstrual cycles in about 4 months, and most of them conceiving healthy pregnancies, sometimes more than once (Silber and Gosden 2007).

7.3 Cryopreservation of Oocytes and Follicles

There have been plenty of historical precedents for ovarian transplants in experimental animal science, and even claims for clinical cases more than a century ago, but transplantation combined with cryopreservation was a novelty in the 1990s (Gosden 2008). Various primary cryoprotective agents have been used over the years, but the protocol has otherwise remained much the same, based on very slow-freezing and rapid rewarming. Unfortunately, attempts to optimize protocols were hindered by a scarcity of clinical material and because the number of follicles contained in a tissue slice can only be determined by destroying it for histological analysis. Hopes for even better results now hang on vitrification which is gradually replacing the formerly conventional method of freezing for tissue, and especially now for oocytes (Gosden and Gosden 2012).

Ovarian banking was gaining more attention when freezing mature oocytes was still relatively ineffective and possibly risky. While the first cases of egg banking

were reported in the mid-1980s, soon after the breakthrough with embryo banking, subsequent experience was discouraging. Oocyte survival rates were often <30 %, and animal data showed that the chill-sensitive cells were at risk of aneuploidy after the spindle depolymerized. Since primordial follicles were more tolerant of cooling and didn't have a spindle apparatus, ovarian banking and transplantation seemed a safer strategy. Over time, and particularly at large Italian centers, the slow freeze–rapid rewarming technique has produced progressively better results with egg banking, and the children born were healthy (Borini et al. 2008).

The cryopreservation laboratory for ARTs has subsequently been transformed by vitrification, in which the protocols developed by Kuwayama's group have been most influential (Kuwayama 2007). Ultrarapid cooling enabled the cells to safely cross a critical thermal range at which they are at risk of chill-injury, and the highly concentrated cryoprotective agents employed avoid the hazard of nucleating ice crystals. As a result, egg banking is now a common procedure in ART worldwide, replacing embryo banking in many cases to find a key role in fertility preservation. Moreover, since the oocyte is enclosed by a zona pellucida the risk of transferring metastatic cancer cells should be close to zero. Such progress is unreservedly welcome, and yet I suspect that ovarian banking will continue to fulfill needs that cannot be met by egg banking. Sometimes there is not enough time to prime the ovaries for oocyte harvesting for IVF, children are unsuitable for ovarian stimulation, and IVF success rates are so poor by the fifth decade of age (and probably further compromised by cancer therapy) that natural conception after a transplant could be a better option. Culture of cryopreserved follicles to produce mature oocytes may be an even better choice in future.

7.4 In Vitro Growth (IVG) and In Vitro Maturation (IVM) of Oocytes

Growth and maturation of oocytes in culture offers an alternative strategy to transplantation which could be applied at any premenopausal age and should avoid transmitting residual disease to the patient. And banking the immature oocytes as follicles or in ovarian tissue is a natural companion technology.

Since the large majority of follicles in the ovary are dormant at the primordial or "intermediary" stages, there is an enormous reserve of gamete potential (Faddy et al. 1992). The culture strategy is also attractive because oocyte–granulosa complexes (GOCs) are avascular developmental units and, provided the integrity of the units is preserved and necessary nutrients and growth factors are supplied, production of healthy, competent oocytes should be possible. That the culture period would have to be as protracted as the normal span of follicular growth in vivo was seen as a problem, but is no longer regarded as quite the drawback that we once feared.

Our interest in IVG and IVM began in the late 1980s with Evelyn Telfer, Nicola Boland, Alison Murray, and others in the Edinburgh laboratory, and around the same time progress was underway at the Jackson Laboratory (Bar Harbor, ME) and in Kansas City. In each center, complementary approaches were adopted independently, although all worked on rodent ovaries. In two of the centers, agar or collagen

gels were used for 3D support to either GOCs or intact follicles from early stages of development (Roy and Greenwald 1989; Torrance et al. 1989), whereas in the third center GOCs were cultured on hydrophobic membranes (Eppig and Schroeder 1989). It was in the Jackson Laboratory that oocytes were first grown successfully to competent gametes.

Innovative modifications soon emerged in these centers and elsewhere. Small follicles were grown in V-wells of microtiter plates (Spears et al. 1994), on conventional culture plastic surfaces (Cortvrindt et al. 1996), and more recently in alginate which offers greater scope to engineer the 3D scaffold than the original gel matrices (Xu et al. 2006). All these systems have generated fertile oocytes from early growing follicles, although a multi-stage culture system starting with an intact explant was needed to make the breakthrough with primordial follicles because they rapidly break down after incubation in proteolytic enzymes and are too small for large scale microdissection (Eppig and O'Brien 1996).

Progress with animal models (mainly mice) has enabled a few centers with access to human and subhuman primate tissue to begin translational studies. Evelyn Telfer's Edinburgh team has adapted a multistage system to grow small follicles in wafers of human cortical tissue to a stage at which they can be isolated in microwells for a further period in vitro until oocytes become competent for IVM (Telfer et al. 2008; Telfer and McLaughlin 2011). One of the more remarkable findings was a marked acceleration of follicle growth in vitro compared with in vivo, which implies an advantage of a truncated culture period provided the oocytes still attain their development milestones. Teresa Woodruff's laboratory at Northwestern University working with Oregon primatologists has used alginate encapsulation for preantral human and macaque follicles, which grew to antral stages in a month. The oocytes were apparently normal, being physiologically coupled to cumulus cells via trans-zonal projections, and some even attained metaphase II after IVM. The results after cryopreservation were almost equivalent (Ting et al. 2011).

These studies bode well for clinical applications. Around the world, there are thousands of ovarian biopsies stored in liquid nitrogen for patients pending decisions when or whether to use the tissue for reproductive care. Although reimplantation was assumed to be the most practicable technique until recently, by the time that many women are ready to start a family a better option for them might be follicle culture. In the meantime, much work is needed to improve the efficiently of growing oocytes to maturity, as well as such safety studies as are feasible with human material and as many molecular and cytogenetic tests in animal models as are necessary.

7.5 Fertility Preservation in Males

Sperm banking and ICSI have brought revolutionary benefits for men facing cytotoxic treatment or orchidectomy, and also for those seeking a "backup" after vasectomy. Although the author's research has been focused mainly on female

fertility, this memoir would be incomplete if it ignored males, and particularly the vexed problem of managing the reproductive potential of prepubertal boys. With survival rates now exceeding 80 % for some malignancies of childhood, 1 in 5,000 males in the population are cancer survivors, and the number needing fertility preservation continues to grow.

Following our success with primordial follicle transfer (Gosden 1990), we considered a parallel strategy for males. We reasoned that spermatogonial stem cells (SSCs) could be recovered for low temperature banking and returned to their seminiferous tubules after they had been sterilized. But before we had made any significant progress, Ralph Brinster's group in Philadelphia had made a breakthrough with mice (Brinster and Avarbock 1994). When they injected SSCs into the lumens of busulfan-sterilized tubules the cells crossed the junctional apparatus separating Sertoli cells to reach their physiological niche in the basal compartment (Oatley and Brinster 2012). Their findings were conclusive because reporter genes proved that the repopulating cells had indeed originated from a donor source and not from residual SSCs in the testes. Most importantly, fertility was restored and the technique worked almost equally well after cryopreservation, even after storing for as long as 14 years (Wu et al. 2012).

Since considerable skill is needed to inject murine tubules only ~170 μm in diameter, it might be assumed that the task would be easier in the larger seminiferous tubules of humans. However, human tubules are more difficult to impale with pipettes. Using orchidectomy specimens, we found the rete testis is an effective route for injection, from which cells can disperse through the testicular plumbing to colonize virtually the entire organ (Brook et al. 2001). Provided Sertoli cell function is not compromised by cancer treatment, this technique holds clinical promise.

One of the chief attractions of SSC transfer, like ovarian transplants, is the restoration of natural fertility so that a patient (or his spouse) need not undergo expensive medicalization with ARTs after having already endured a stressful course of cancer therapy. Another attraction is that SSC transfer is as equally applicable for prepubertal children as to adults, since testicular biopsies can be banked until needed. There is however another potential option in which the tubules are grafted intact into the subject from which they were harvested for cryopreservation. The principle has been validated with immature mice in which spermatogenesis was initiated after grafting to the scrotum or even subcutaneously, and from such sites enough mature sperm can be harvested to fertilize oocytes using ICSI (Honaramooz et al. 2002; Schlatt et al. 2002).

There is again the drawback that tissue might return the original disease to the patient if he had a systemic disease like leukemia. This problem might be avoided using immunodeficient animals as hosts for patient's xenografts, although there could be a much more acceptable and satisfactory solution over the horizon, a dream technology analogous to follicle culture. For a long time it has been hoped that spermatogenesis might be completed entirely in vitro. This is a challenging goal that will require maintaining the physiological integrity of the germ cell–Sertoli cell relationship, but perhaps within reach using 3D culture systems (Mahmoud 2012).

7.6 Prospect

The most exciting advances in fertility preservation technologies still lie ahead, and will have to address new clinical challenges. We have seen astonishing progress over the past 25 years, most notably new hope for women where few options were available before, and ongoing developments that could at last enable most child patients of both sexes to avoid iatrogenic sterility. While emphasis has been on avoiding irreplaceable loss of reproductive potential during cancer treatment or sometimes from the disease itself, attention will likely switch focus as oncology advances. Less ovotoxic treatment regimens for cancer are emerging and, for example, the tyrosine kinase inhibitor, imatinib (Gleevec), for chronic myelogenous leukemia may not affect ovarian function or spermatogenesis (Schultheis et al. 2012). Even as iatrogenic causes of sterility recede, in future genetic causes will continue to be presented for attention, and we may see new concerns emerging, such as genetic risks from other medications or occupational hazards, or for space travelers from cosmic rays and coronal mass ejections. But keeping our feet firmly on the ground, the looming elephant in fertility preservation will be the demography of fertility. As more women postpone a family, we may witness a mushrooming demand for egg banking from healthy women seeking to preserve their oocytes. Whether many will choose or afford this expensive fertility insurance or if demography changes again is in the realm of speculation, but we can be certain that current technologies will eventually be passed over in favor of better ones.

Cryopreservation has occupied center-stage in my career for fertility preservation of both females and males, having proved to be versatile, safe, and effective. Perhaps evaporative drying in trehalose solutions may supersede sperm cryopreservation, although I doubt its application to the voluminous cytoplasm of the egg (Li et al. 2007). More likely, we will see new pharmaceutical strategies that can be applied in vivo, avoiding the need to remove germ cells to a protected environment outside the body. The molecular toolkits of the future may enable pathways leading to the apoptosis of germ cells to be blocked or control the prodigious wastage of the follicle reserve, perhaps through the phosphoinositol-3-kinase pathway. Such strategies could, in theory, preserve follicles and even postpone the menopause. If these failed, there is the dawning possibility that stem cell technology, although not strictly fertility preservation, could create patient-specific germ cells de novo for either sex (Hayashi et al. 2012). I therefore think there are plenty of reasons to be optimistic about helping people who need fertility preservation, and if new technologies reduce the need for donor gametes they should be welcomed wholeheartedly.

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