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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 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

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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, 2010; Ewens, 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, 2003). Human preantral follicles encapsulated in agar (Roy, 1993) or collagen (Abir, 1997, 1999, 2001) gels maintained their morphology and grew to the early antral stage. Recently, early antral follicles were obtained from ovarian cortical strip culture (Telfer, 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, 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, 2009a, 2010, 2011a, 2011b). 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, 2011, 2012), may be applied to fertility preservation in women, including cancer patients (Jeruss, 2009). Although ovarian cortex transplantation using fresh tissue in monkeys (Lee, 2004), as well as using fresh and cryopreserved tissue in women (Dittrich, 2012; Donnez, 2004; Silber, 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, 2006a), and studies demonstrated the potential application of IFM in humans (Xu, 2009b; Smitz, 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 germline 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.

3D culture of macaque follicles

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, 2009a) was replaced by human serum protein supplement (SPS; Xu, 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, 2007; Schroeder, 1990). Bovine fetuin is used currently due to unavailability of human protein. Follicles are cultured in 5% oxygen (O_2) (v/v) (Xu, 2011a), which mimics the partial pressure of O_2 in the peritoneal cavity where the ovaries are located (Tsai, 1998), in contrast to early primate follicle culture studies where 20% (v/v) atmospheric O_2 tension was used (Xu, 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, 2008). The pituitary gonadotropin, follicle stimulating hormone (FSH) promotes follicle survival and growth in a 3D matrix (Xu, 2006a, 2009a, 2009b), 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, 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, 2011a). Luteinizing hormone (LH) enables preantral follicles to respond to later LH-dependent growth during murine follicle culture (Cortvrindt, 1998; Wu, 2000). There is also an increasing body of evidence that insulin stimulates growth and improves the overall viability of human follicles *in vitro* (Wright, 1999; Louhio, 2000). In contrast, insulin can have profound detrimental effects on oocyte developmental competence during the culture of murine cumulus-oocyte complexes (Eppig, 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 needs 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, 1986). The hormonal and local milieu around follicles in follicular vs. luteal phase may have differentially effect on follicle potential.

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, 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, 2010). Survival rate is lower for follicles cultured with low- (0.3 ng/ml) compared to high-dose (15 ng/ml) FSH (Xu, 2011a). Likewise, FSH acts as a survival factor for human preantral follicles during ovarian tissue culture (Wright, 1999). These data are consistent with evidence that FSH receptors are expressed in preantral follicles of various species, including primates (Gougeon, 1996; Findlay, 1999). Folliculogenesis is blocked at the early preantral stage in FSH receptor knockout mice (Dierich, 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, 1979), in disorders of hypogonadotropic hypogonadism (Goldenberg, 1976), and selective FSH (Rabin, 1972) or FSH receptor (Aittomäki, 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, 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, 2010), which is consistent with the observation in murine follicle culture (Hirshfeld-Cytron, 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, 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, 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, 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 be 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, 2009a). More recent experiments demonstrated that 0.25% alginate promoted follicle survival (Xu, 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, 2001) are maintained in murine follicles grown in alginate (Pangas, 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, 2007).

A more physiological level of O₂ (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% O₂, relative to those without fetuin or in 20% O₂. Previous studies indicated that fetuin is present in the ovarian follicular fluid of the mouse (Høyer, 2001), horse (Dell'Aquila, 1999), and human (Kalab, 1993). A variety of cell types in culture respond to fetuin in promoting cellular attachment, growth, and differentiation (Demetriou, 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.

Follicle growth

At the beginning of the culture, diameters of collected secondary follicles range from 125-225 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, 2010, 2011a). The cohort that remains similar in size to the initial secondary follicles without significant change in diameter ($< 250 \mu\text{m}$) is termed “no-grow” follicles. Another cohort doubles their diameters (250 μm -500 μm) and is termed “slow-grow” follicles. Finally,

another group of follicles increases their diameters by a minimum of three-fold ($> 500 \mu\text{m}$, in some instances over 1 mm) and is termed “fast-grow” follicles (Figure 9.1). 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, 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, 2005) or other hormones (Xu, 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, 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, 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, 1995) and women (Schoot, 1994; Kumar, 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, 2009a).

Insulin affects fast-, 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, 2010). Insulin receptor mRNA and protein are located in theca, granulosa, and stromal cells of antral follicles in women (el-Roeiy, 1993; Samoto, 1993). Insulin may mimic local insulin-like growth factor (IGF) activity, which is suggested to improve viability of cultured follicles in primates (Louhio, 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, 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, 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, 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 preant 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, 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, but not no- and fast-grow follicles. Follicles cultured at 20% O₂ are less likely to grow compared to those cultured at 5% O₂. Follicles maintain similar growth rates during the first 3 weeks in 5% and 20% O₂. However, diameters increase and become larger at week 5 for follicles cultured at 5% O₂ than those of 20% O₂. This observation is consistent with follicle culture studies using domestic animals, where low O₂ concentration (5%) stimulated follicle growth with a high proportion of them developing an antral cavity (Cecconi, 1999; Silva, 2010). It is hypothesized that the ovarian follicle, in a low-oxygen environment, is often challenged by hypoxia (Redding, 2008), and antrum formation provides a way to support further growth by avoiding hypoxia in the follicle wall (Redding, 2007). The effects of O₂ tensions less than 5% (hypoxic, i.e., 1-3% O₂) on macaque follicles during 3D culture have not been tested.

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.

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, 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,

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, 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, 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, 1984; Erickson, 1990; Langhout, 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 (Figure 9.2). 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, 1980). This LH responsiveness suggests the presence of theca cells in *in vitro* developed primate follicles, although this has not yet been conclusively demonstrated. 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, 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, 2009a). This may result from the desensitizing of LH receptors by continuous LH exposure or not distinguishing slow- 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 adult monkeys at week 5. Follicles from prepubertal animals produce less E2 and more P4 compared to follicles from older adults (Xu, 2010). This may be due to less FSH sensitivity of the follicles as described above (Davoren, 1984). Follicle function may be suboptimal in prepubertal and older adults if insulin enhanced FSH-stimulated steroidogenesis is lacking.

Fetuin exposure and O₂ tension (5 or 20%) do not alter steroid levels in culture media of *in vitro*-developed macaque follicles (Xu, 2011a).

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, 2010, 2011a).

AMH production by *in vitro*-developed follicles correlates positively with growth rate. When follicles are cultured with high-dose FSH at 5% O₂, 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, 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, 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, 2002; Weenen, 2004; Thomas, 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 system. The addition of AMH into cultures of human or rat ovarian cortical strips reportedly improved the recruitment, survival and growth of primordial follicles (Schmidt, 2005; McGee, 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, 2002; La Marca, 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, 2011a). Based on data from human (Weenen, 2004), marmoset (Thomas, 2007), and rodent (Salmon, 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 co-culture experiments determined that *AMH* mRNA expression within granulosa cells of mouse preantral follicles is regulated by signals from the oocyte (Salmon, 2004).

Also, there is the evidence that the production of AMH, like other potential growth factors (e.g. VEGF; Shweiki, 1992), is regulated by O₂ tension *in vitro*. A physiological level of O₂ (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% O₂, except that AMH declines to basal levels earlier at week 3 (Xu, 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, 1992) and protein (Yamamoto, 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, 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, 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, 2004). VEGF may also promote nuclear and cytoplasmic maturation of bovine oocytes *in vitro* (Luo, 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, 2010, 2011a; unpublished data).

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 hrs to examine reinitiation of meiotic maturation of the oocyte within the follicle (Xu, 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 (Figure 9.3A), 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 2 cells within 24 hrs after ICSI (Figure 9.3B). Subsequent cleavage is observed, but embryonic development arrests by day 3 post-ICSI (Figure 9.3C; Xu, 2011a). In rhesus monkeys, the transition from maternal to embryonic genome occurs at the 6- to 8-cell stage (Schramm, 1999). Therefore, the oocyte must contain the appropriate instructions, involving expression of new proteins from maternal mRNAs/genes (Kocabas, 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 ($> 110 \mu\text{m}$) than GV oocytes ($\sim 100 \mu\text{m}$) (Xu, 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, 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, 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, 2005). Conversely, a decrease in FSH dose seemed to limit inappropriate gene expression (Sánchez, 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 O₂ 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, 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% O₂. It is well established that high O₂ tension is associated with higher levels of reactive oxygen species, and oxidative stress plays a role in cytotoxic activity (Evans, 2004; Devine, 2011). As such, low O₂ tension (5%), compared to 20%, reduced cumulus cell apoptosis in canine cumulus-oocyte complexes during culture (Silva, 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, 1998) and

mouse oocyte competence for embryonic development (Acevedo, 2007), the lower insulin concentration in encapsulated 3D culture system does not improve oocyte quality in macaques (Xu, 2010, 2011a).

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, 2004). Only a few reports have applied the baboon model to understand early events of folliculogenesis (Fortune, 1998; Wandji, 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, 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, 1997; Roy, 1993). Initial studies on macaque (Xu, 2009b) and baboon (Xu, 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, 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 non-permissive 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, 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 (Figure 9.4).

A necessity for FSH in mouse (Abir, 1997), macaque (Xu, 2011a), and human (Adriaens, 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, 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 $288 \pm 9 \mu\text{m}$ to $439 \pm 24 \mu\text{m}$ 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.

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- α -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 9.1). 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, 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 9.1), 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, 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 9.1). Exogenous FSH in the culture media may promote the *GCLC* and *EGFR* mRNA expression, as reported in rat SAF and granulosa cell culture (Hoang, 2009; Fujinaga, 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.

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 O₂ 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 AMH level decreases (Figure 9.5). 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 of 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, 1997) or human (Telfer, 2008) ovarian cortex can survive and develop to the secondary stage in serum-free culture. Human (Vanacker, 2011) and macaque (Hornick, 2012) primordial or primary follicles can be isolated and maintain their viability when cultured in groups. To date, efforts to grow and mature individual primordial and primary follicles *in vitro* have not been reported in primates, especially under chemically-defined conditions. Preliminary experiments conducted in rhesus macaques indicate that it is possible to grow individual primary follicles (80-120 μ m in diameter) *in vitro* to the small antral stage, which function in steroidogenesis, local factor production, and oocyte maturation (Xu, 2011c). 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 0.5-1.5 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, 2010) and baboons (Xu, 2011b), with demonstration of fertilization and early embryonic development *in vitro* to the expanded blastocyst stage (Peluffo, 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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Figure 9.1.

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.

Figure 9.2.

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 $\pm$ SEM. N, number of follicles. (This figure was originally published in and reproduced with permission from Xu et al., Hum Reprod 2011a)

Figure 9.3.

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 μ m. (This figure was originally published in and reproduced with permission from Xu et al., Hum Reprod 2011a)

Figure 9.4.

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 was recovered from the follicle for in vitro maturation (IVM; panel c).

Cumulus cells expanded after 24 hrs of IVM (panel d). The oocyte resumed meiosis, reached the metaphase II (MII) stage within 48 hrs (panel e), and displayed a normal spindle structure (panel f). Arrow, polar body (PFB). Scale bar = 100 μ m (panel a-d), 50 μ m (panel e), 10 μ m (panel f). (This figure was originally published in and reproduced with permission from Xu et al., Biol Reprod 2011b)

Figure 9.5.

Conceptual graph of macaque follicle development during encapsulated 3-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.

Table 9.1
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 (<i>in vitro</i> / <i>in vivo</i>) [*]
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
Transcription regulation	Cytochrome P450 family 19 subfamily A polypeptide 1	Cytochrome P450 aromatase	CYP19A1	NM_000103	NC
	Anti-Müllerian hormone	Müllerian-inhibiting factor	AMH	NM_000479	NC
	Anti-Müllerian hormone receptor, type II	Anti-Müllerian hormone type-2 receptor	AMHR2	NM_020547	NC
Angiogenesis	Vascular endothelial growth factor A	Vascular endothelial growth factor	VEGFA	NM_001025366	NC
	Vascular endothelial growth factor receptor 1	Fms-related tyrosine kinase 1	VEGFR1/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
	Becclin 1, autophagy related	Becclin-1	BECN1	NM_003766	NC

* Fold changes in mRNA levels, assayed by Affymetrix macaque gene arrays; follicles from n = 3 animals.

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