Introduction

Advances in ovarian tissue cryopreservation, followed by ovarian transplantation or in vitro follicle maturation (IFM), provide options for fertility preservation in female patients with cancer. Although ovarian tissue transplantation yielded viable oocytes in monkeys (Simpson et al., 2004) and women (Downes et al., 2009, Silver et al., 2008, 2010), the IFM approach has the advantage of eliminating the possibility of reintroducing cancer cells into the patient following treatment and providing a way to harvest more mature oocytes (Woodruff, 2007). Advances in biotechnological engineering resulted in the development of an alginate-based, three-dimensional (3D) culture follicle system to maintain the cell–cell and cell–matrix connections important in regulating follicle development in vivo (West et al., 2007). This approach produced live offspring in mice (Xu et al., 2006) and studies demonstrated the potential application of IFM in human beings (Xu et al., 2010, Smale et al., 2010). Even though the micromammalian species and developmental capacity of human oocytes grown from pre-antral stages in vitro have not yet been reported, animal studies indicate that IFM is a valid approach for clinical translation to humans to overcome the destruction or damage to ovarian germ cells caused by chemotherapy and/or chemotherapeutics (Woodruff, 2007).

Recently, efforts to grow non-human primate ovarian follicles from the early stages (secondary follicles) to the antral stage during 3D culture have been successful (Xu et al., 2010) and may be valuable in identifying the optimal conditions for primate follicle culture prior to human application. Besides the clinical relevance, IFM is a powerful instrument for monitoring follicular endocrine and paracrine function, which is essential to obtain knowledge of the factors implicated in the regulation of follicular development.

Evidence suggests that FSH receptors are expressed in pre-antral follicles during in vitro development of various species, including primates (Cougoule, 1999, Findlay and Drummond, 1999). Studies indicate that FSH is essential for in vivo survival of pre-antral follicles (Wright et al., 1999, Xu et al., 2010) and promotes the growth of resident (Xu et al., 2006), non-human primate (Xu et al., 2010) and human (Xu et al., 2006) follicles during 3D culture. On the basis of rodent data reported recently, follicles cultured with a physiological (low) level of FSH tend to have oocyte and cumulus cell gene expression levels that are comparable to those in vivo (Eppig et al., 2010). In contrast, a supraphysiological level (high) of FSH alters the expression of oocyte and cumulus cell transcripts (Eppig et al., 2010). Thus, low FSH may promote oocyte-regulated cumulus cell differentiation while supporting follicle growth, whereas high FSH may inappropriately stimulate granulosa cell proliferation or differentiation.

Follicles cultured typically in the presence of atmospheric oxygen (O2) tension at 20% (v/v) (Xu et al., 2010), which is around 140 mmHg. However, the partial pressure of O2 in the peritoneal cavity where the ovaries are located is 40 mmHg (Yao et al., 1998), which approximates 5% O2 (v/v). Low O2 tension at 5% increased the viability of cumulus cells (Silver et al., 2005) and promoted developmental competence of porcine oocytes during in vitro maturation (Jamieson et al., 2005). Moreover, low O2 tension during the culture of cattle oocytes resulted in increased cumulus cell viability, maturity, and parthenogenetic activation and fertilization (Hease et al., 2009). Therefore, it is hypothesized that a low O2 environment has a positive influence on follicular development.

During spontaneous maturation of mouse oocytes in serum-free medium, the zona pellucida (ZP) becomes hardened; i.e., resistant to thyroglobulin digestion (Oo Pekki and Saravacos, 1962). The hardened ZP is resistant to sperm penetration, thus preventing fertilization. The phenomenon has also been characterized in non-human primates (VandeVoort et al., 2007) and human (Schwetz et al., 1995) oocytes. Serum, including fetal bovine serum (FBS), from various species was demonstrated to cause hardening of the zona pellucida in rodents (Eppig and Schroeder, 1986). Fetus, a major glycoprotein in serum and follicular fluid, increased the solubility of the ZP during spontaneous oocyte maturation in mice and was able to provide a serum-free culture environment (Schroeder et al., 1999). Whether fetus stimulates or is essential for follicle or oocyte maturation during macaque IFM is unknown.

Therefore, using the encapsulated 3D culture system, the current study tested the dose–response of FSH on follicle survival and growth, and investigated O2 tension and fetus effects on follicle follicular development in vitro. Ovarian steroids (androgens, AA, estradiol, E2, and progesterone, P4) and anti-mullerian hormone (AMH) produced by individually cultured follicles, as well as oocyte maturation, were analyzed to evaluate follicular function.

Materials and Methods

Animals and ovary collection

The general care and housing of rhesus macaque monkeys were provided by the Division of Animal Resources at the Oregon National Primate Research Center (ONPRC). Animals were pair caged in a temperature-controlled (22°C) light-regulated 12:12:0:00Z room. Diet consisted of Purina monkey Chow (Ralston-Purina, St. Louis, MO, USA) provided twice a day, supplemented with fresh fruit or vegetables once a day and water ad libitum. Animals were treated according to the Institutional Animal Care and Use Committee (IACUC). Adult female rhesus macaques (n=9; 5–10 years of age) exhibiting regular menstrual cycles of ~28 days were evaluated daily for menstruation with the first day of menses termed Day 1 of the cycle. Ovarian samples were conducted on anesthetized monkeys by laparoscopy at an early follicular phase, Day 1–4 of the cycle, as previously described (Duffy et al., 2006). Ovaries were immediately transferred into Hepes-buffered holding media (CorningSurgical, Inc., Trumbull, CT, USA) supplemented with 0.1% (v/v) human serum protein supplement (SPP, CorningSurgical, Inc.) and 10 μg/ml gentamicin (Sigma-Aldrich, St Louis, MO, USA).

Follicle isolation, encapsulation and culture

Follicles were isolated and encapsulated as previously described (Xu et al., 2006). Briefly, ovarian cortex was cut into 2 x 2 x 1 mm cortical strips and placed into 96-well plates containing 225 μl collagenase type I and 585 μl desoxyribonuclease (1 U/ml) in Wingert’s medium (Cellgro, Mediatech, Virginia, USA), at 37°C for 20 min. Follicles were mechanically isolated in the holding media using 31 gauge needles and the secondary follicles with diameters of 125–200 μm were selected for encapsulation. An intact basement membrane, two to three layers of granulosa cells and an viable, healthy oocyte that was round and centrally located within the follicle, without vacuoles or dark cytoplasm. Follicles (128 ± 12/mouse from 9 monkeys) were divided among the treatment groups with 36–60 follicles per group.

Follicles were transferred individually into 0.5 ml 25% (v/v) sterile sodium alginate (FMC, Rockland, PA) stock solution prepared in phosphate buffered saline (PBS) (137 mM NaCl, 1.0 mM phosphate, 2.7 mM KCl, Invitrogen, Carlsbad, CA, USA) and the droplets were cross-linked in 50 mM CaCl2 (Sigma-Aldrich) solution (pH 7.2) for 1 min. Each encapsulated-follicle was placed into individual wells of 48-well plates containing 300 μl alpha minimum essential medium (aMEM, Invitrogen) supplemented with 0.3% (v/v) SPP, 5 μg/ml insulin, 5 μg/ml transferrin and 5 μg/ml sodium selenite (Sigma-Aldrich).

Encapsulated follicles were cultured at 37°C in a 5 or 20% (v/v) O2 environment (in 5% CO2/95% or 5% CO2 in air, respectively) with 0.5–3.0 or 15–50 ng/ml recombinant human (rH)FSH (low or high FSH, NV Organon, Oss, Netherlands), and 0 or 1 μg/ml purified bovine follicle-stimulating factor (BFSF, Sigma-Aldrich) for 40 days. Half of the follicles in each culture condition received 0.4 μg/ml rhLH (EMD Serono, Inc., Randolph, MA, USA) in the media during Days 30–40. Follicles that reached the...
animal stage were treated with 100 ng/ml of hCG recombinant gonadotropin (CG, Medic Steroidone, INC., New Jersey) for 24 h. Oocytes were retrieved at 16 h after treatment determined for castrate mouse maturation. Half of the culture media (150 μl) was collected and replaced every other day and stored at -20°C. The media samples from each culture were assigned to ovarian steroids and AMH assays. 

Follicle survival and growth

Follicle survival, diameter, and armament formation were assessed weekly using an Olympus CKX51 inverted microscope and an Olympus DP11 digital camera (Olympus Imaging America Inc., Center Valley, PA, USA) as described previously (Xu et al., 2009). Follicles were measured from the outer layer of cells which included a measurement at the widest diameter of the follicle and a second measurement perpendicular to the first. The mean of the values was calculated and reported as the follicle diameter. Follicles were considered to be undergoing atresia if the oocyte was dark or not surrounded by a layer of granulosa cells, the granulosa cells became dark and fragmented, or the diameter of the follicle decreased (Xu et al., 2009b). Follicle photographs were imported into ImageJ 1.42 software (National Institutes of Health, Bethesda, MD, USA) and the diameter of each follicle was measured.

Oocyte retrieval, maturation and fertilization

Retrieved oocytes were photographed and oocyte diameters (excluding the zona pellucida) were measured using the same camera and software as described earlier. The germinal vesicle (GV)-intact and metaphase II (MI) oocytes were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for immunofluorescence imaging to identify nuclear maturation, spindle and polar body organization (Faiman and Albertini, 2007). Briefly, oocytes were incubated with primary antibody overnight at 4°C and secondary antibody for 1 h at room temperature. Spindle microtubules were labeled with a Cy3-conjugated anti-tubulin (1:100; Sigma) followed by Alexa 633-conjugated goat anti-mouse IgG (1:500; Invitrogen). F-actin was probed with Alexa 488-conjugated phalloidin (1:200; Invitrogen). Oocyte images were captured using a Leica SP5 confocal microscope equipped with DM6000 CFS microscope (Leica Microsystems Inc., Bannockburn, IL, USA). 

Serum collection and ICSI were performed for one of the MII oocytes by the Advanced Reproductive Technology (ART)/Embryonic Stem Cell Support Core at the ONPRC, as reported previously (Xu et al., 1995, 1997). The resulting embryos were transferred to a 500 μl hamster embryo culture medium 9 with 5% FBS and cultured at 37°C in 5% CO2, 5% O2, and 90% N2 (Shafter and Papicos, 2000). The ART Core (Heng and Wolf, 1997) evaluated the embryos and protocols for embryo culture were provided by the ART Core (Heng and Wolf, 1997).

Ovarian steroids and AMH assays

One media sample collected weekly during culture Weeks 1–5 was assayed for E2 and P4 concentrations by the Endocrine Technology Support Core at the ONPRC using an Immulite 2000, a chemiluminescence-based analyzer. Ovarian steroids and AMH assays were analyzed by the Endocrine Technology Support Core at the ONPRC using an Immulite 2000, a chemiluminescence-based analyzer as reported previously (Preiser et al., 2005), as previously validated for macaque follicle culture media (Xu et al., 2010). The assay has a sensitivity of 0.1 μg/ml and the intra- and inter-assay variations were <15 and 10%, respectively. Due to the cross-reaction of fetoins with the AMH antibody levels assayed in media containing fetoins, but not cultured follicles were subcategorized from AMH levels in media sample from follicle cultured with fetoins, as previously described (Xu et al., 2010).

Statistical analysis

Statistical significance was analyzed by SigmaStat 9.1 software (SPSS, Inc., Chicago, IL, USA) using a two-way analysis of variance (ANOVA) with repeated measures or one-way ANOVA followed by the Student-Newman-Keuls post-hoc test for single time points. Differences were considered significant at P<0.05 and values are presented as mean ± SEM. Follicle survival and distribution represent the percent (mean ± SEM of three individual animals in each treatment group. Follicle growth, steroid and AMH production, and oocyte maturation were analyzed for each individual follicle with total follicle numbers indicated in the figure legends, and represent follicles obtained from the three individual animals.

Results

There was no difference between high- and medium-dose FSH-treated groups in any parameters analyzed (data not shown). Only data from follicles cultured with high- and low-dose FSH, except follicle survival under medium-dose FSH in Fig. 1B, are described below.

Follicle survival and growth

In the presence of high FSH, the survival rate at Day 40 was higher (P<0.05) when oocytes were cultured with fetoins at 5% O2 (data not shown; Fig. 2A). The survival rate was lower (P<0.01) for follicles cultured with low FSH and fetoins compared with low FSH cultured with high fetoins when in the presence of fetoins at 5% O2 (Fig. 2B). LH addition on Day 30 had no effect on follicle survival at Day 40 (data not shown).

At the beginning of the culture, follicle diameters did not differ among treatment groups (ANOVA, data not shown; Table 1). In culture, three distinct cohorts of surviving follicles were observed based on their growth rates as previously reported (Xu et al., 2010). The cohort that remained smaller in size to the initial follicle diameter with no significant change in diameter (<0.25 μm) through 5 weeks of culture was termed ‘no-growth’ follicles. Another cohort doubled their diameters (50–500 μm) and was termed ‘slow-grow’ follicles. Finally, another group of follicles increased their diameters by a minimum of 3-fold (>100 μm) and was termed ‘fast-grow’ follicles. An animal was considered evident in 3–4 weeks of culture for all the slow- and fast-growth follicles. During culture with high FSH, fetoins and 5% O2, 57% of the surviving follicles fell into the growing (slow- and fast-growth) follicle category (Fig. 2A). In contrast, when fetoins was absent, the majority (86%) of the surviving follicles were no-growth follicles, despite the presence of high fetoins (Fig. 2B). In the presence of low FSH, the proportions of growing follicles observed with or without fetoins were similar, 67% and 57%, respectively (Fig. 2C and D). However, fast-grow follicles were not obtained during culture with fetoins (Fig. 2C), while 22% were fast-grow follicles when cultured without fetoins (Fig. 2D). Similar patterns were observed for follicles cultured at 20% O2, except for lower (P<0.05) percentages of growing follicles compared with those cultured at 5% O2 (data not shown). The addition of LH at Day 30 had no effect on the growth distribution of surviving follicles (data not shown).

The dose of FSH and O2 tension influenced the growth rate of slow-growth (Fig. 3A and B), but not no-grow and fast-growth follicles (data not shown). In the presence of low FSH, the proportions of growing follicles observed with or without fetoins were similar, 67% and 57%, respectively (Fig. 2C and D). However, fast-grow follicles were not obtained during culture with fetoins (Fig. 2C), while 22% were fast-grow follicles when cultured without fetoins (Fig. 2D). Similar patterns were observed for follicles cultured at 20% O2, except for lower (P<0.05) percentages of growing follicles compared with those cultured at 5% O2 (data not shown). The addition of LH at Day 30 had no effect on the growth distribution of surviving follicles (data not shown). The dose of FSH and O2 tension influenced the growth rate of slow-growth (Fig. 3A and B), but not no-grow and fast-growth follicles (data not shown). In the presence of low FSH, the proportions of growing follicles observed with or without fetoins were similar, 67% and 57%, respectively (Fig. 2C and D). However, fast-grow follicles were not obtained during culture with fetoins (Fig. 2C), while 22% were fast-grow follicles when cultured without fetoins (Fig. 2D). Similar patterns were observed for follicles cultured at 20% O2, except for lower (P<0.05) percentages of growing follicles compared with those cultured at 5% O2 (data not shown).
follies during the culture interval with higher ($P < 0.05$) levels at Week 3 compared with those of slow-growth follicles (Fig. 1). Note the differences in the y-axes scales of SD and SE compared with 5A and 5B. In contrast, A4 (Fig. 5A and O), E2 (Fig. 5B) and P4 (Fig. 5C) levels of slow- and fast-growth follicles cultured without FSH stayed at baseline, except E2 (Fig. 5A) and P4 (Fig. 5F) produced by fast-growth follicles increased ($P < 0.05$) at Week 5. The steroid concentrations of both slow- and fast-growth follicles cultured with low FSH were lower ($P < 0.05$) than those of high FSH-treated follicles at Week 3.5 (Fig. 5A-D).

For slow-growth follicles cultured at 5% O$_2$ with and without fetuin, media levels of A4 (Fig. 5A), E2 (Fig. 5B) and P4 (Fig. 5C) did not increase between Week 4 and 5 in the presence of high FSH. However, for these follicles, the addition of LH at Day 30 increased ($P < 0.05$) estradiol production between pre-LH (Week 4) and post-LH (Week 5) exposure (data not shown). Moreover, LH treatment increased ($P < 0.05$) A4 (Fig. 6A) and P4 (Fig. 6C), but not E2 (Fig. 6B), levels in media for the slow-growth follicles cultured with low FSH and the levels were higher ($P < 0.05$) at Week 5 than those without LH administration. In contrast to the slow-growth follicles, LH supplementation at Day 30 had no effect on the patterns or levels of steroids during culture 5 for fast-growth follicles regardless of culture conditions (data not shown), as previously noted (Xu et al., 2010).

**Anti-müllerian hormone**

Since fetuin had no effect on AMH production by cultured follicles (data not shown), media AMH concentrations from follicles cultured with and without fetuin were combined for data analysis. When follicles were cultured with high FSH at 5% O$_2$, AMH levels produced by the no-growth follicles did not change over the culture (Fig. 7A). Although diameters of no-, slow- and fast-growth follicles were not different at the onset of culture, levels of AMH produced by slow- and fast-growth follicles at Week 1 were higher ($P < 0.05$) than those of no-growth follicles. Moreover, AMH levels of fast-growth follicles increased ($P < 0.05$) at Week 2 and remained at high levels until Week 5 ($P < 0.05$) (Fig. 7A). AMH levels during Weeks 3 and 4 were distinct ($P < 0.05$) among all three follicle categories. When cultured with low FSH at 5% O$_2$, the slow- and fast-growth follicles produced higher ($P < 0.05$) levels of AMH during Week 2–4 compared with slow-growth follicles cultured with 20% O$_2$. While AMH levels declined ($P < 0.05$) at Week 5, the no-growth follicles had basal levels of AMH (Fig. 7A). Furthermore, the levels were lower ($P < 0.05$) than those of no-growth follicles. Although diameters of no-, slow- and fast-growth follicles were not different at the onset of culture, levels of AMH produced by slow- and fast-growth follicles at Week 1 were higher ($P < 0.05$) than those of no-growth follicles. Moreover, AMH levels of fast-growth follicles increased ($P < 0.05$) at Week 2 and remained at high levels until Week 5 ($P < 0.05$) (Fig. 7A). AMH levels during Weeks 3 and 4 were distinct ($P < 0.05$) among all three follicle categories. However, the levels were lower ($P < 0.05$) than those of no-growth follicles. Although diameters of no-, slow- and fast-growth follicles were not different at the onset of culture, levels of AMH produced by slow- and fast-growth follicles at Week 1 were higher ($P < 0.05$) than those of no-growth follicles. Moreover, AMH levels of fast-growth follicles increased ($P < 0.05$) at Week 2 and remained at high levels until Week 5 ($P < 0.05$) (Fig. 7A). AMH levels during Weeks 3 and 4 were distinct ($P < 0.05$) among all three follicle categories. When cultured with low FSH at 5% O$_2$, the slow- and fast-growth follicles produced higher ($P < 0.05$) levels of AMH during Week 2–4 compared with slow-growth follicles cultured with 20% O$_2$. While AMH levels declined ($P < 0.05$) at Week 5, the no-growth follicles had basal levels of AMH (Fig. 7A). Moreover, LH treatment increased ($P < 0.05$) A4 (Fig. 6A) and P4 (Fig. 6C), but not E2 (Fig. 6B), levels in media for the slow-growth follicles cultured with low FSH and the levels were higher ($P < 0.05$) at Week 5 than those without LH administration. In contrast to the slow-growth follicles, LH supplementation at Day 30 had no effect on the patterns or levels of steroids during culture 5 for fast-growth follicles regardless of culture conditions (data not shown), as previously noted (Xu et al., 2010).

**Discussion**

Advances in the encapsulated 3D system allow primates secondary follicles to grow to the small antral stage and to produce local (AMH) and endocrine (steroids) factors. Compared with our previous study (Xu et al., 2010), the current culture conditions improved follicle growth (to >100 μm in diameter) as well as the health and growth of the oocytes to reach the size of those that mature in vivo (>100 μm in macaques; Busto et al., 2008). For the first time, oocytes retrieved from in vitro developed primate follicles displayed the competence to reinitiate meiosis for fertilization, and hence early embryonic cleavage. Further indices of oocyte quality need to be examined, such as cumulus–oocyte communication (Kimura et al., 2007), to monitor the competence to undergo meiotic maturation, as well as maternal to zygotic transition. In the present study, fetuin, in combination with high FSH and 5% O$_2$, increased follicle survival and promoted growth of follicles. A variety of cell types in culture respond to fetuin in promoting cellular attachment, growth and differentiation (Niko, 1992; Demers et al., 1996). Previous studies found that fetuin is present in the ovarian follicular fluid of the mouse (Heyer et al., 2001), horse (Dell’Aquila et al., 1999) and human (Kalab et al., 1993). Our current data suggest that fetuin cultured with low FSH without added fetuin grew faster after antrum formation. 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 FSH. Fetuin may also stimulate the action of macrophages (Kimura et al., 1998), and healthy granulosa cells may behave in a macrophage-like manner during follicular atresia in the mouse (Inoue et al., 2000; Heyer et al., 2001), guinea pig (Kanaya, 1997), and cow (Van Wezel et al., 1999). The specific effects of fetuin on follicle cell proliferation and differentiation remain to be determined. It has been proposed that the protein inhibitory activity of fetuin plays an important role in preventing ZP hardening (Schwarz et al., 1993). Since one MB oocyte, which originated from a follicle grown in the absence of fetuin, was fertilized by ICSI, ZP ‘hardening’ that prevented sperm penetration was not evident. One unexpected observation was that alFa beads became non-transparent, brittle and fragmentary after 2 weeks of culture without fetuin. This phenomenon is not evident in murine follicle culture during the shorter culture period (<2 weeks; Xu et al., 2006). The mechanism whereby fetuin maintains alFa integrity is unknown. Since fetuin is currently not a recombinant protein, additional complexity caused by fetuin impurity cannot be ruled out. Compared with the typical culture condition of 20% O$_2$, the lower O$_2$ tension at 5% and fetuin supported higher follicle survival in the presence of high FSH, increased the rate of antrum formation and promoted the growth of slow-growth follicles after antrum formation when cultured with low FSH. Investigators using caprine pre-antral follicles cultured with 5% O$_2$ and LH supplementation at Day 30 achieved significant differences over time (low and high O$_2$), or between conditions (apparent) are indicated by different letters ($P < 0.05$). Data are presented as the mean ± SEM with three animals per treatment group. Parentheses indicate the total number of follicles from each category surviving follicles per treatment group.
FSH, O2 and fetuin effects on follicle culture

Table I Characteristics of oocytes retrieved from antral follicles on Day 40 of encapsulated 3D culture with 5% O2 (4 h after addition of rhCG).

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Follicles harvested</th>
<th>Oocytes retrieved</th>
<th>Degenerate oocytes</th>
<th>Healthy oocytes</th>
<th>Diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GV-intact</td>
</tr>
<tr>
<td>High FSH + fetuin</td>
<td>25</td>
<td>20</td>
<td>8</td>
<td>11</td>
<td>I</td>
</tr>
<tr>
<td>High FSH</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Low FSH</td>
<td>9</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Low FSH + fetuin</td>
<td>8</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
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</table>

*Values are the mean ± SEM with each oocyte diameter as an individual data point.

**Different letters indicate significant differences within the column (P < 0.05).

Figure 4 GV-intact (A, bottom left) and MII (B, bottom left) oocytes at retrieval with corresponding confocal microscopy images (A, B) and pictures of the cumulus–oocyte complex (arrows) in a fast-grow follicle (C) and retrieved MII oocytes prior to (D) and after (E, F) in vitro maturation using ICSI. The GV oocyte contains a perinucleolar ring (A) and the MII oocyte shows the polar body adjacent to the spindle (B). Green tubulin (A and B, top right) and red F-actin (A and B, top right) staining were overlapped on bottom right of (A) and (B). The fertilized MII oocyte cleaved to two cells (B) and arrested with uneven cleavage (F). Scale bar = 50 μm for oocytes and 200 μm for follicle.

Figure 5 Dose–response of FSH on steroid production by slow- (A–C) and fast- (D–F) growing follicles in vitro. Androstenedione (A and D), estradiol (B and E) and progesterone (C and F) levels in media containing either low- or high-dose FSH at 5% O2, without LH supplementation at Day 30. Significant differences over time (lowercase) or between the FSH dosage groups (uppercase) are indicated by different letters (P < 0.05). Data are presented as the mean ± SEM, n, number of follicles.

formation. This is the first evidence that AMH, like other potential growth factors (e.g., vascular endothelial growth factor; Svedsjo et al., 1993), is regulated by O2. On the basis of evidence from human (Wiesner et al., 2004), non-human primate (Thomas et al., 2007) and rodents (Salmon et al., 2009) ovaries, AMH production by follicles cultured at 5% O2 after antrum formation may originate from mural or cumulus cells. Thus, a physiological level of O2 (5%) is beneficial for macaque follicle survival, growth and function, as well as oocyte quality, during encapsulated 3D culture.

The addition of FSH produced dose-dependent effects that are different for follicle survival and growth. In the presence of fetuin at 5% O2, follicles cultured with high FSH had a higher survival rate than those of low FSH. In contrast, most of the follicles cul-

cells of mouse pre-antenal follicles is regulated by signals from the ovary (Salmon et al., 2004). Thus, high levels of AMH may be induced by oocyte-derived factors to reduce the negative effects caused by high FSH.

For the first time, MII-stage oocytes were retrieved from primate antral follicles, following growth from pre-antenal follicles under chemi-
cally defined conditions. Nevertheless, most of the healthy oocytes obtained after the rhCG stimulus remained at the GV stage, and the MII oocyte that was fertilized only reached the 2-cell stage before embryonic development arrested. While insulin had deleterious effects on oocyte development in mouse follicle culture (Figure et al., 1996), lowering insulin concentration in our 3D culture system did not improve oocyte quality in macaques (Xu et al., 2010). In rhesus monkeys, the transition from maternal to embryonic genome occurs at the 6- to 8-cell stage (Schramm and Bavister, 1999). Therefore, the oocyte must contain the appropriate instructions, involving the expression of new protein-coding genes (Kocabas et al., 2004), to drive the first few divisions and the awakening of the embryonic genome. Further studies are warranted to improve cytoplasmic and nuclear maturation, as well as the developmental competence of embryos produced from oocytes retrieved from primate follicles after encapsulated 3D culture.
LH-receptor signaling promotes A4 production with high FSH (Xu et al., 2010). LH addition was reported previously during primate follicle culture. A moderate increase in steroid production by slow-grow follicles followed but not on E2, production in slow-grow follicles cultured with low FSH, with a significant difference over time (low FSH) or among the follicle categories (apoptosis) are indicated by different letters (P < 0.05). Data are presented as the mean ± SEM, n, number of follicles.

For the first time, a significant LH effect was observed on A4 and P4, but not on E2, production in slow-grow follicles cultured with low FSH. A moderate increase in steroid production by slow-grow follicles following LH addition was reported previously during primate follicle culture with high FSH (Xu et al., 2010). According to the 2-cell, 2-gonadotrophin theory, LH-receptor signaling promotes A4 production from P4, which allows steroidogenic maturation of the follicles by providing substrate for E2 biosynthesis in granulosa cells (McNatty et al., 1980). The LH responsiveness suggests the presence of these cells in in vitro developed primordial follicles, although this has not yet been conclusively demonstrated in our system. In low-FSH cultured follicles, well-developed theca cells may be stimulated by LH to produce A4 and P4, while granulosa cells undergoing appropriate proliferation may utilize A4 and P4 efficiently to synthesize high levels of E2. It is also possible that healthy theca cells produce insulin-like growth factors (Bongio et al., 2010) or other paracrine factors that can support steroidogenesis in granulosa cells independent of FSH stimulation. Steroid production by fast-grow follicles was not altered by LH addition, which extends our previous findings on cultured conditions with high-FSH (Xu et al., 2010). High steroid production prior to the LH addition may prevent further stimulation. While there is a dose-dependent effect of FSH, LH addition does not appear to impact AMH production by in vitro developed primordial follicles.

Three groups of surviving follicles with different growth rates were observed in the current, as well as our previous, studies in macaques (Xu et al., 2010). Also, AMH production in vitro by early pre-antral follicles correlated positively with growth rate as we reported previously (Xu et al., 2010). AMH acts as a paracrine factor to modulate folliculogenesis in the early stages of mouse follicular development (Durlinger et al., 2002). Thus, early AMH production may be a potential marker for predicting further development of pre-antral follicles with different growth rates during culture. Notably, some of the follicles were removed from fast-grow follicles. However, it is not known whether AMH production by early pre-antral follicles predicts the quality of the oocyte enclosed within a small atretic follicle that develops during the encapsulated 3D culture.

In summary, under improved conditions, individual pre-antral follicles are able to grow in vitro and produce mature oocytes for fertilization. On the basis of the current data, the following conditions for follicle culture are being investigated: (i) medium (2 ng/ml) FSH for the first 3 weeks to support follicular survival, followed by low (0.3 ng/ml) FSH after antrum formation to promote follicle growth; (ii) low concentration (0.5 mg/ml) of fetuin to maintain the perifollicular gel integrity without causing oocyte overmaturation and apoptosis; and (iii) low LH concentration at 5% O₂ may be a marker of follicle growth potential to screen for no-growth follicles at the early stage of their development. The encapsulated 3D culture system provides a way to understand the process and regulation of folliculogenesis in primates, including gene and protein expression, as well as metabolic pathways during follicular development. This information can be used to discover biomarkers that predict or report follicle and/or oocyte condition during FSH stimulation. The ultimate goal is to translate the experimental results to patients, thereby developing new diagnostic and therapeutic approaches for ovarian preservation, and hence female fertility.

Authors’ roles

J.Y. contributed to (i) experimental design, (ii) follicle collection and culture, and (iii) critical manuscript reviewing for important intellectual content and final approval of the version to be submitted for publication. R.R.Y. contributed to (i) experimental design, (iii) follicle collection and culture, (iv) critical reviewing on the manuscript and (iv) final approval of the version to be submitted for publication. K.Y.P. contributed to (i) experimental design, (ii) statistics and AMH assays, (iii) critical reviewing on the manuscript and (iv) final approval of the version to be submitted for publication. M.B.Z. provided contributions to (i) conception and design of the experiments, (ii) follicle collection and oocyte retrieval, (iii) data interpretation on follicle development, oocyte maturation, starch and AMH production, (iv) critical manuscript reviewing for important intellectual content and (iv) final approval of the version to be submitted for publication. R.L.S. contributed to (i) conception and design of the experiments, (ii) immunofluorescence imaging and data interpretation, (iii) critical reviewing on the manuscript and conceptions of the experiments, (iv) follicle collection and oocyte retrieval, (v) data interpretation on follicle development, oocyte maturation, starch and AMH production, (vi) critical manuscript reviewing for important intellectual content and (v) final approval of the version to be submitted for publication.

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Freed JR, Drummond AE. Regulation of the FSH receptor in the ovary. Trends Endocrinol Metab 1999; 10: 183–188.


