

Vascular endothelial growth factor and angiopoietin production by primate follicles during culture is a function of growth rate, gonadotrophin exposure and oxygen milieu

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STUDY QUESTION: What is the time course of production of vascular endothelial growth factor-A (VEGF-A), angiopoietin (ANGPT)-1 and ANGPT-2 by primate follicles during encapsulated three-dimensional culture, and what conditions affect their production?

SUMMARY ANSWER: Primate follicles produce VEGF-A and ANGPT-2 *in vitro*, particularly after developing to the antral stage, with VEGF production influenced by FSH concentration and O₂ tension.

WHAT IS KNOWN ALREADY: Folliculogenesis, i.e. the development of primordial follicles into mature, antral follicles, requires the creation of a vascular network in the follicle wall via a process called angiogenesis. Angiogenic factors including VEGFs and ANGPTs have documented roles in angiogenesis. However, direct studies on the production and regulation of angiogenic factors by individual, growing follicles are limited.

STUDY DESIGN, SIZE, DURATION: Ovaries ($n = 9$ pairs) were obtained from rhesus macaques during the early follicular phase of the menstrual cycle (cycle days 1–4). Secondary (125–225 μm) follicles were isolated mechanically, encapsulated into alginate (0.25% w/v) and cultured for 40 days.

MATERIALS, SETTING, METHODS: Individual follicles were cultured in a 5 or 20% O₂ environment in alpha minimum essential medium supplemented with recombinant human (h) FSH. Half of the follicles had recombinant hLH added to the media from Days 30 to 40. Follicle diameters were measured weekly. Follicles were categorized at Week 5 as no-grow (NG; <250 μm in diameter), slow-grow (SG; 251–499 μm) and fast-grow (FG; >500 μm). VEGF-A, ANGPT-1 and -2 concentrations in media were measured by ELISA.

MAIN RESULTS AND THE ROLE OF CHANCE: VEGF concentrations were low throughout the culture for NG follicles. SG and FG follicles had detectable VEGF concentrations at Week 2, which continued to rise throughout culture. VEGF concentrations were distinct ($P < 0.05$) among all three follicle categories during Weeks 4 and 5. VEGF concentrations were higher ($P < 0.05$) in SG follicles in the presence of high/mid-dose FSH at 5% O₂. In contrast, there were no dose-dependent differences in VEGF production for FG follicles based on FSH concentrations or O₂ tension. At Week 5, follicles that produced metaphase II oocytes, following exposure to an ovulatory hCG dose, secreted higher concentrations of VEGF than those containing germinal vesicle-intact oocytes. Media concentrations of ANGPT-1 were low throughout culture for all three follicle categories. ANGPT-2 concentrations were low throughout culture for NG follicles. In contrast, ANGPT-2 concentrations of SG and FG follicles continued to rise from Weeks 1 to 4. During Weeks 2–4, ANGPT-2 concentrations in FG follicles were significantly higher than those of SG and NG follicles ($P < 0.05$).

LIMITATION, REASONS FOR CAUTION: This study reports VEGF-A, ANGPT-1 and -2 production by *in vitro*-developed individual primate (macaque) follicles, that is limited to the interval from the secondary to small antral stage. After VEGF and ANGPT-1 assays, the limited remaining samples did not allow assessment of the independent effects of gonadotrophin and O₂ on the ANGPT-2 production by cultured follicles. Findings await translation to human follicles.

WIDER IMPLICATION OF THE FINDINGS: The above findings provide novel information on the process of primate follicle maturation. We hypothesize that a symbiotic relationship between elevated concentrations of ANGPT-2 and VEGF allows FG antral follicles to excel in follicle maturation, e.g. by promoting its vascularization. Elevated ANGPT-2 may also offer possible insight into future oocyte quality as early as Week 2, compared with Week 4 for VEGF and follicle size.

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Key words: VEGF / angiopoietin / follicle culture / angiogenesis / follicle development

Introduction

The ovarian vasculature is distinguished from that of many other organs by the creation and degeneration of vessels in response to physiologic conditions, notably the development of new blood vessels from pre-existing vessels to supply growing follicles. Non-growing primordial and growing early pre-antral follicles do not possess their own vascular supply, but rely on vessels within the surrounding stroma. As follicle development continues, the growing follicles form their own individualized vascular supply (Hazzard and Stouffer, 2000; Fraser, 2006; Robinson et al., 2009). This process of angiogenesis appears critical for follicular function, including steroidogenesis and oocyte maturation, and locally regulated by angiogenic factors including vascular endothelial growth factor (notably VEGF-A) and angiopoietins (ANGPTs) (Hazzard and Stouffer, 2000; Robinson et al., 2009).

VEGF-A is a potent mitogenic factor that stimulates the proliferation and migration of vascular endothelial cells for the creation and maintenance of vascular structures (Araújo et al., 2013). In contrast, ANGPTs are not mitogenic, but their actions are still fundamental in the construction of vessels (Maisonpierre et al., 1997). As an agonist to the Tie2 receptor, ANGPT-1, helps to mature and maintain blood vessels by recruiting periendothelial cells; whereas, the endogenous antagonist ANGPT-2 assists in loosening the support cell framework to allow for further vascular expansion (Hazzard and Stouffer, 2000). The angiolytic (degenerative) effects of ANGPT-2 may become pronounced in the relative absence of VEGF (Maisonpierre et al., 1997).

These angiogenic factors have been the focus of studies evaluating their presence and specific role(s) in ovarian angiogenesis and folliculogenesis. VEGF mRNA and protein were localized to ovarian follicles/cells in rodents (Celik-Ozenci et al., 2003; Danforth et al., 2003), cattle (Greenaway et al., 2004) and primates (Christenson and Stouffer, 1997; Zimmermann et al., 2001, 2002; Wulff et al., 2002; Martinez-Chequer et al., 2003; Fraser et al., 2005), primarily through *in situ* hybridization and immunohistochemistry. Also, a number of *in vivo* studies, performed primarily by administering agents that block VEGF action (Roberts et al., 2007), generated data supporting a causal role of VEGF in promoting the growth and maturation of ovarian follicles, including primate follicles (Zimmerman et al., 2001, 2002; Wulff et al., 2001, 2002; Hazzard et al., 2002). There have been fewer studies examining the presence or role(s) of ANGPTs in the follicle (Parborell et al., 2008), especially in primate follicles (Xu and Stouffer, 2005).

Moreover, there are few reports of direct analyses of the production, regulation or action of angiogenic factors on follicles at specific stages of their development. Recently, techniques were developed for growing

individual follicles of rhesus monkeys in three-dimensional (3-D) culture (Xu et al., 2010); small pre-antral follicles grew to the antral stage, developed steroidogenic function and, in some cases, yielded meiotically mature oocytes after exposure to an ovulatory hCG bolus (Xu et al., 2010, 2011). Preliminary evidence also suggested that these follicles could produce VEGF-A *in vitro* (Xu et al., 2010). Therefore, further studies were designed to examine VEGF-A, plus ANGPT-1 and ANGPT-2, production by primate follicles *in vitro* and its regulation by gonadotrophins and O₂ tension.

Materials and Methods

Animal use and ovary collection

The general care and housing of rhesus macaques was performed by the Division of Comparative Medicine at the Oregon National Primate Research Center (ONPRC), Oregon Health & Science University. The environment was temperature controlled at 22°C in a light-regulated 12L:12D room. Animals were caged in social pairs. Diet consisted of Purina monkey chow (Ralston-Purina, Richmond, IN, USA) provided twice a day, supplemented with fresh fruit or vegetables once a day and water *ad libitum*. Animal treatment was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol for ovary collection was previously approved by the ONPRC Institutional Animal Care and Use Committee (Xu et al., 2010, 2011).

Adult, female rhesus macaques ($n = 9$) with a history of regular menstrual cycles were observed daily for menstruation. Ovariectomies were performed under anesthesia between days 1 and 4 of the cycle as described previously (Duffy and Stouffer, 2002). Ovaries were placed in HEPES-buffered holding media (Cooper Surgical, Inc., Trumbull, CT, USA) supplemented with 0.2% (v/v) human serum protein supplement (SPS; Cooper Surgical, Inc.) and 10 µg/ml gentamicin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C and immediately transferred to the laboratory (Xu et al., 2010, 2011).

Follicle isolation, encapsulation and culture

The process of follicle isolation, encapsulation and culture has been described previously (Xu et al., 2009). In short, the ovarian cortex was separated from the medulla, cut into 2 × 2 × 1 mm cortical strips and incubated in 6 ml holding media containing 275 U/ml collagenase type I and 585 U/ml deoxyribonuclease I (Worthington Biochemical Corp., Lakewood, NJ, USA) at 37°C for 20 min. Follicles were mechanically isolated using 31-gauge needles. Secondary follicles (diameter 125–225 µm) met the criteria for encapsulation if they exhibited an intact basement membrane, two to three layers of granulosa cells and a healthy, centrally located oocyte.

Follicles were individually transferred into 5 µl 0.25% (w/v) sterile sodium alginate (FMC BioPolymers, Philadelphia, PA, USA)-PBS (137 mM NaCl,

10 M phosphate, 2.7 mM KCl, Invitrogen, Carlsbad, CA, USA) and the droplets were cross linked in 50 mM CaCl₂, 140 mM NaCl, 10 mM HEPES solution for 1 min. Each encapsulated follicle was placed in individual wells containing 300 µl alpha minimum essential medium (Invitrogen), 0.3% (v/v) SPS, 1 mg/ml bovine fetuin, 5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml sodium selenite (Sigma-Aldrich) (Xu *et al.*, 2011).

Follicles (an average of 64 follicles/monkey from 9 monkeys) were randomly assigned to culture groups with 0.3, 3 or 15 ng/ml recombinant human (h) FSH (low-, mid-, high-dose; NV Organon, Oss, Netherlands), and cultured at 37°C in a 5 or 20% O₂ environment (in 6% CO₂/89% N₂ or 5% CO₂ in air atmosphere, respectively) for 40 days. The small difference in CO₂ concentration had no apparent effect on the pH. Half of the follicles from each culture condition had 0.4 ng/ml recombinant hLH (EMB Serono, Inc., Randolph, MA, USA) added to the culture media from Days 30 to 40. Each culture group contained follicles from a minimum of three monkeys. Follicles that developed an antrum were treated with 100 ng/ml recombinant human chorionic gonadotrophin (hCG; Merck Serono, Geneva, Switzerland) for 34 h before removal of the enclosed oocyte for evaluation of meiotic maturation. Every other day, half (150 µl) of the media was removed and replaced (Xu *et al.*, 2011). Media removed from individual follicles were stored at -20°C until analysis of angiogenic factors (VEGF, ANGPT-1 and ANGPT-2). Follicles and media samples were used previously to evaluate follicle survival, growth and steroid hormone (estradiol, progesterone) production (Xu *et al.*, 2011).

Follicle survival and growth

Follicle survival and maturation were assessed weekly using an Olympus CK-40 inverted microscope and an Olympus DP11 digital camera (Olympus Imaging America, Inc., Center Valley, PA, USA) as described previously (Xu *et al.*, 2009, 2010, 2011). Follicle diameter was determined by measuring the distance from the outer layer of cells at the widest diameter and then the diameter perpendicular to the first measurement. The mean of the two values was considered the follicle's overall diameter. The measurements were performed using Image J 1.42 software (National Institutes of Health, Bethesda, MD, USA). Follicles were considered atretic if the oocyte developed a dark appearance or was no longer surrounded by a layer of granulosa cells, if the granulosa cells appeared dark or fragmented, or if the follicle diameter decreased.

Macaque follicles developed in 3-D culture can be divided into three distinct cohorts based on their growth rates by Week 5 (Xu *et al.*, 2010, 2011). Follicles whose diameter increased a minimum of 3-fold (>500 µm) were considered in the fast-grow (FG) group. Those whose diameter increased a minimum of 2-fold (250–499 µm) were categorized as slow-grow (SG) follicles. And follicles whose diameter did not significantly increase (< 250 µm) were assigned to the no-grow (NG) group. As development continued, FG and SG follicles developed an antrum between 3 and 4 weeks of culture. Media from NG, SG and FG follicles were analysed for VEGF and ANGPT production.

Granulosa cell retrieval and culture

Granulosa cells were collected from large antral follicles of rhesus monkeys (*n* = 5) following controlled ovarian stimulation, as described previously (Martinez-Chequer *et al.*, 2003). Granulosa cells were obtained by follicle aspiration either before or 27 h after administration of the bolus of recombinant hCG (Merck Serono). The cells were cultured in fibronectin-coated 96-well plates, each well contained 200 µl DMEM/F12 media, 2 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite, 25 µg/ml aprotinin and 25 µg/ml low density lipoprotein (Sigma-Aldrich) at 37°C in a 20% O₂/5% CO₂/75% N₂ environment. After 24 h, the media was exchanged and the cells were cultured with the presence or absence of 100 ng/ml of hLH in

20% O₂/5% CO₂/75% N₂ or 0% O₂/5% CO₂/95% N₂. After 48 h of culture, the media was retrieved for analyses.

VEGF, ANGPT and progesterone assays

VEGF-A concentrations were determined in media collected at Weeks 2–5 of the follicle culture. Assays were performed using a Human VEGF Quantikine ELISA kit (R&D Systems, Minneapolis, MN), which was previously validated for macaque VEGF in culture media (Christenson and Stouffer, 1997).

ANGPT-1 and ANGPT-2 concentrations were determined using media from Weeks 1–4 of the follicle culture, as well as from granulosa cells after plating and 24 h of culture. Assays were performed using Human ANGPT-1 or ANGPT-2 Quantikine ELISA kits (R&D Systems), previously validated for measurement of macaque ANGPTs (Hurliman *et al.*, 2010).

Progesterone concentrations in media from granulosa cell cultures were assayed by the Endocrine Technology Support Core at the ONPRC using an Immulite 2000, a chemiluminescence-based automatic clinical platform (Siemens Healthcare Diagnostics, Deerfield, IL, USA), validated for macaque follicle culture media as reported previously (Xu *et al.*, 2009).

Statistical analysis

Statistical differences over time and between groups in the follicle culture experiments were analysed using mixed modeling of repeated measures using SAS Enterprise (9.2) with support from the ONPRC Biostatistics Unit. VEGF and ANGPT production was analysed using linear mixed models of the media concentrations over time that incorporated a random intercept for the follicle and treated measurements over time as repeated measures for the follicle. In order to meet the assumption of a normal distribution of the outcome variable and the model residuals, the analysis modeled natural log-transformed concentrations by week, group and the interaction between week and group. The reported differences between groups at each week were derived from contrasts of the interaction effect and were adjusted for multiple comparisons using Bonferroni's method. The residuals from the model did not deviate significantly from those expected from normal distribution, indicating that the use of a parametric model was justified. Because the follicles were nested within individual monkeys, an additional random effect specification for follicle source was not necessary in the models, and the model residuals did not differ significantly by the source monkey.

Statistical differences between groups in the granulosa cell experiments were analysed using one-way ANOVA using a randomized block design, followed by Duncan's multiple range test to determine differences between groups. Assay values that were below the detection limit were assigned the lowest detection value for statistical analyses. A *P* value < 0.05 was considered a significant difference between groups or time points.

Results

VEGF-A concentrations

Since preliminary studies did not detect VEGF-A in media during Week 1 of culture (Xu *et al.*, 2010), VEGF concentrations were measured from Weeks 2 to 5. Values from follicles exposed to high- and mid-doses of FSH at 5% O₂ were combined (Fig. 1) since there was no significant difference in VEGF concentrations between these treatment groups (data not shown). Media VEGF concentrations for NG follicles remained low and unchanged throughout culture. In contrast, SG and FG follicles produced detectable VEGF concentrations at Week 2, which increased (*P* < 0.05) continuously during the subsequent culture. VEGF concentrations were distinct (*P* < 0.05) among all three follicle categories at Weeks 4 and 5 (Fig. 1).

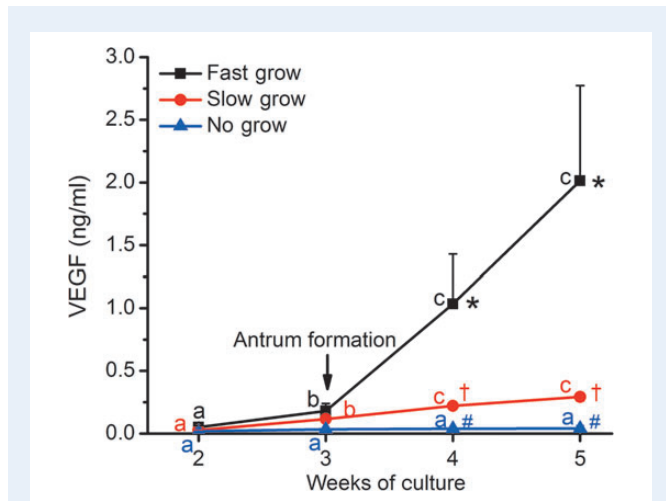


Figure 1 VEGF-A concentrations in the media of no-grow (NG) ($n = 27$), slow-grow (SG) ($n = 49$) and fast-grow (FG) ($n = 15$) follicles (from 6 monkeys) as a function of weeks in culture in the presence of 5% O_2 and high/mid FSH levels. Data are presented as non-transformed—mean \pm SEM. ^{a,b,c}Significant differences between Weeks 2, 3, 4 and 5 for NG (blue), SG (red) and FG (black) follicles, respectively ($P < 0.05$). ^{*†#}Significant differences between NG, SG and FG follicles at Weeks 4 and 5 ($P < 0.05$). Growing follicles reached the antral stage (arrow) at 3–4 weeks of culture (Xu et al., 2011).

At Week 5 of culture, the high/mid-dose of FSH did not promote an increase in VEGF concentrations by NG follicles, compared with low-dose FSH, at 5% O_2 (Fig. 2A). In contrast, the high/mid-dose of FSH increased ($P < 0.05$) concentrations of VEGF produced by SG follicles. For FG follicles, both high/mid- and low-dose of FSH induced a higher ($P < 0.05$) level of VEGF production compared with SG and NG groups, but there was no dose-dependent difference despite the ~ 2 -fold greater VEGF concentrations with high/mid-FSH treatment (Fig. 2A).

In the presence of high/mid-dose of FSH, there was no difference in the VEGF concentrations for NG follicles at Week 5 with the different O_2 tensions (5 versus 20%) (Fig. 2B). In contrast, higher ($P < 0.05$) VEGF concentrations were produced by SG follicles in the 5% O_2 , compared with 20% O_2 , milieu. For FG follicles, VEGF concentrations were higher ($P < 0.05$) than those of SG and NG follicles, but there was no observed change between different O_2 tensions (5 versus 20%) (Fig. 2B).

There was no significant effect of adding LH after Day 30 on VEGF concentrations in any treatment group (data not shown).

Oocyte quality related to VEGF-A production

Following 5 weeks of culture, FG and SG follicles were exposed to hCG with oocytes retrieved subsequently. Oocytes retrieved from follicles cultured in different conditions (FSH doses and O_2 concentrations) were combined for the following analysis. The majority of follicles contained oocytes that degenerated ($n = 45$), and those with healthy oocytes typically remained at germinal vesicle (GV) stage ($n = 19$). However, three follicles produced oocytes that progressed to metaphase II (MII) stage. Follicles containing healthy immature (GV intact) oocytes produced higher ($P < 0.05$) concentrations of VEGF than

those with degenerated oocytes, which were lower ($P < 0.05$) than those producing meiotically matured (MII) oocytes (Fig. 3).

ANGPT-1 and -2 concentrations

In the presence of high/mid-FSH and 5% O_2 , media from all three follicle categories contained ANGPT-1 concentrations near the limit of detection (64 pg/ml) throughout culture (Fig. 4A). No change in ANGPT-1 concentrations was noted for follicles cultured in different FSH concentrations (high/mid- versus low-dose) or O_2 tensions (5 versus 20%) (data not shown).

Because of limited sample size and/or media volume after measuring VEGF and ANGPT-1 (plus steroids and anti-Müllerian hormone in other studies, Xu et al., 2011), data from different culture conditions (FSH doses and O_2 concentrations) were combined for analysis of ANGPT-2 (Fig. 4B). ANGPT-2 concentrations remained low in NG follicles throughout culture. For SG follicles, there were detectable ANGPT-2 concentrations at Week 1, which increased ($P < 0.05$) by Week 4. ANGPT-2 concentrations produced by FG follicles increased ($P < 0.05$) at Weeks 2–4, and were greater ($P < 0.05$) than those produced by SG and NG follicles. Limited sample sizes also did not allow the assessment of LH effects on ANGPT production.

To initially consider the cell source(s) of ANGPTs in the macaque antral follicle, media from short-term cultures of granulosa cells collected from large antral follicles of controlled ovarian stimulation protocols were analysed (Table 1). Non-luteinized and luteinizing granulosa cells collected before and after the hCG bolus, respectively, produced > 10 -fold more ($P < 0.05$) ANGPT-2 than ANGPT-1 at 20% O_2 . Granulosa cells responded to hCG *in vivo* (luteinized versus non-luteinized controls) and LH *in vitro* (non-luteinized controls versus LH) with enhanced progesterone concentrations. However, there were no significant differences in ANGPT concentrations produced by luteinized or non-luteinized granulosa cells when cultured in different O_2 tensions (20 versus 0% O_2 ; data not shown) or exposed to LH *in vitro*, or following hCG *in vivo* (Table 1).

Discussion

Using 3-D culture technology, this study directly evaluated the production of angiogenic factors, notably VEGF-A, ANGPT-1 and ANGPT-2, by individual primate follicles at discrete (secondary to small antral) stages of development *in vitro*, as well as its regulation by gonadotrophins and O_2 tension. The data suggest that growing (SG and FG) follicles produce significant quantities of VEGF-A and ANGPT-2, but not ANGPT-1, especially after antrum formation. VEGF production by SG follicles was influenced by FSH concentration and O_2 tension.

VEGF-A accumulation in media was a function of follicle growth rate *in vitro*. Prior to antrum formation, and if follicles failed to form an antrum (NG follicles), VEGF concentrations did not increase. However, following antrum formation of growing (SG and FG) follicles, VEGF increased and reached appreciable concentrations (ng/ml) in FG follicles. Our findings are consistent with previous reports of absent VEGF mRNA expression, as judged from *in situ* hybridization on ovarian sections, in primordial, primary and early secondary follicles in non-human primates (Wulff et al., 2002; Taylor et al., 2004), and VEGF mRNA expression increased after antrum formation in rodent and monkey follicles (Taylor et al., 2004; Abramovich et al., 2009). Whereas the onset of

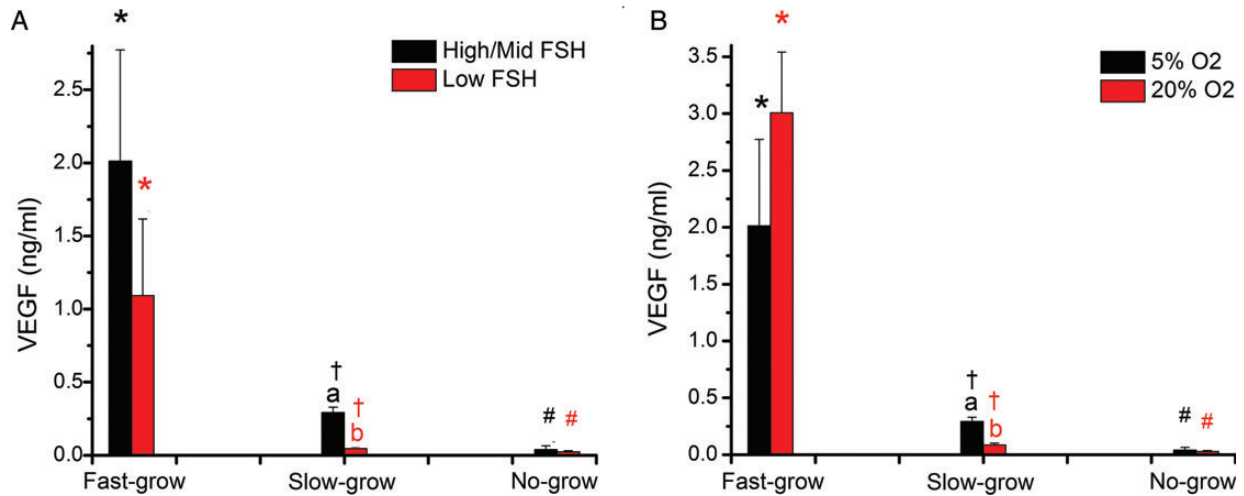


Figure 2 VEGF-A concentrations in media from no-grow (NG), slow-grow (SG) and fast-grow (FG) follicles following 5 weeks of culture in (A) low or high/mid FSH (NG, $n = 11$ and 27 ; SG, $n = 13$ and 49 ; FG, $n = 6$ and 15 from 9 monkeys) at 5% O_2 and (B) 5 or 20% O_2 (NG, $n = 27$ and 20 ; SG, $n = 49$ and 8 ; FG, $n = 15$ and 2 from 6 monkeys) in the presence of high/mid FSH. Data are presented as mean \pm SEM. Panel A: “a”s on FG data points mean VEGF concentrations in high/mid FSH culture is not different from low FSH culture for fast-grow follicles; a and b on SG data points mean VEGF concentrations in high/mid FSH culture is different from low FSH culture for slow-grow follicles; “a”s on NG data points mean VEGF concentrations in high/mid FSH culture is not different from low FSH culture for no-grow follicles. *† and # on high/mid FSH data points mean VEGF concentrations in FG is different from SG and NG, and SG is different from NG at high/mid dose of FSH; *† and # on low FSH data points mean VEGF concentrations in FG is different from SG and NG, and SG is different from NG at low dose of FSH. ^{a,b}Significant differences between low (red) and high/mid (black) FSH doses for SG follicles ($P < 0.05$). *†#Significant differences between NG, SG and FG follicles in the presence of low and high/mid FSH ($P < 0.05$). Panel B: “a”s on FG data points mean VEGF concentrations in 5% O_2 culture is not different from 20% O_2 culture for fast-grow follicles; a and b on SG data points mean VEGF concentrations in 5% O_2 culture is different from 20% O_2 culture for slow-grow follicles; “a”s on NG data points mean VEGF concentrations in 5% O_2 culture is not different from 20% O_2 culture for no-grow follicles. ^{a,b}Significant differences between 20% (red) and 5% (black) O_2 concentrations for SG follicles ($P < 0.05$). *†#Significant differences between NG, SG and FG follicles in the presence of 20% and 5% O_2 ($P < 0.05$). *† and # on 5% O_2 data points mean VEGF concentrations in FG is different from SG and NG, and SG is different from NG at 5% O_2 ; *† and # on 20% O_2 data points mean VEGF concentrations in FG is different from SG and NG, and SG is different from NG at 20% O_2 .

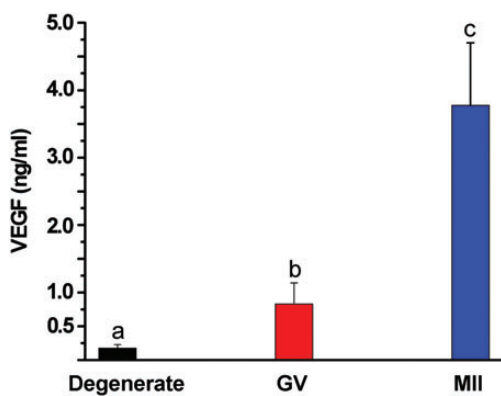


Figure 3 VEGF-A concentrations at Week 5 from cultures of growing (slow- or fast-grow) follicles as a function of oocyte quality/maturation following exposure to 100 ng/ml hCG. Values are the mean \pm SEM, $n = 45$, 19 and 3 for follicles from 9 monkeys providing degenerate (black), germinal-vesicle (GV, red) or metaphase II (MII, blue) oocytes, respectively, at retrieval. Significant differences between groups are denoted by different letters.

VEGF production appears to be related to the stage of follicle growth (antrum formation), the magnitude of VEGF production may be influenced by follicle size and activity. VEGF secretion correlates with its purported angiogenic actions as the antrum forms to create an extensive surrounding vasculature for increasing transport of nutrients and hormones to/from the growing follicle (Taylor *et al.*, 2004; Abramovich *et al.*, 2009). As follicles grow larger and mature, their need for vasculature increases. In the current study, FG follicles produced higher concentrations of VEGF, which supports previous data that larger or faster growing follicles from various species produced more VEGF (Danforth *et al.*, 2003; Greenaway *et al.*, 2004; Chowdhury *et al.*, 2010).

VEGF-A production by *in vitro*-developed follicles can be dependent on FSH and O_2 milieu. Increasing FSH concentrations promoted VEGF production by SG follicles at 5% O_2 . The dose-dependent, stimulatory effect of FSH in small antral follicles extends the previous evidence that gonadotrophins promote VEGF production by granulosa cells in the periovulatory follicle. Since survival and growth of macaque follicles in 3-D culture requires FSH (Xu *et al.*, 2011), FSH may have, at least in part, a ‘permissive’ action that allows other factors to regulate VEGF production. Notably, decreasing O_2 concentration, from the typical tissue culture milieu (20%) to concentrations commonly found in vascularized tissues (5%), increased VEGF production by SG follicles in the presence

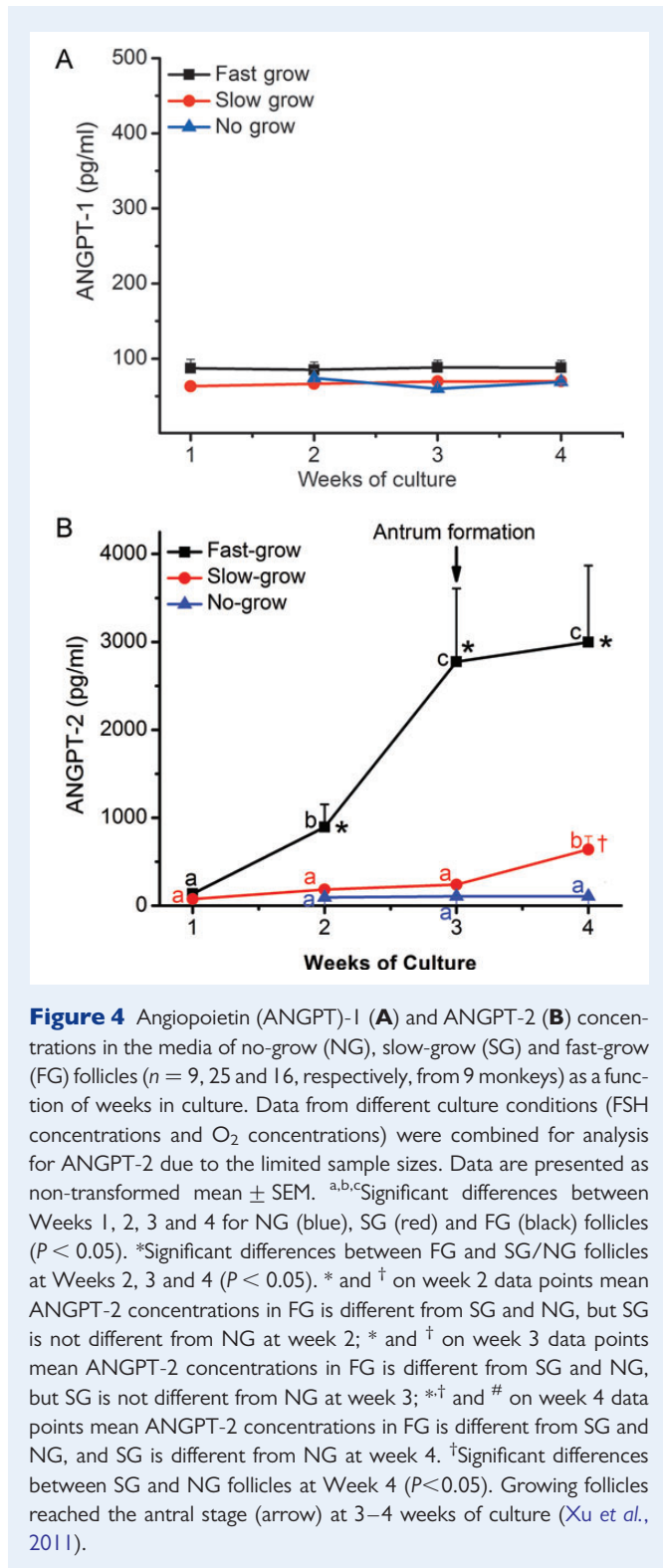


Figure 4 Angiopoietin (ANGPT)-1 (**A**) and ANGPT-2 (**B**) concentrations in the media of no-grow (NG), slow-grow (SG) and fast-grow (FG) follicles ($n = 9, 25$ and 16 , respectively, from 9 monkeys) as a function of weeks in culture. Data from different culture conditions (FSH concentrations and O_2 concentrations) were combined for analysis for ANGPT-2 due to the limited sample sizes. Data are presented as non-transformed mean \pm SEM. ^{a,b,c}Significant differences between Weeks 1, 2, 3 and 4 for NG (blue), SG (red) and FG (black) follicles ($P < 0.05$). *Significant differences between FG and SG/NG follicles at Weeks 2, 3 and 4 ($P < 0.05$). * and † on week 2 data points mean ANGPT-2 concentrations in FG is different from SG and NG, but SG is not different from NG at week 2; * and † on week 3 data points mean ANGPT-2 concentrations in FG is different from SG and NG, but SG is not different from NG at week 3; *† and # on week 4 data points mean ANGPT-2 concentrations in FG is different from SG and NG, and SG is different from NG at week 4. †Significant differences between SG and NG follicles at Week 4 ($P < 0.05$). Growing follicles reached the antral stage (arrow) at 3–4 weeks of culture (Xu et al., 2011).

of high/mid-dose of FSH. Hypoxia is a primary stimulus for VEGF production in many tissues (Lee et al., 1997; Neeman et al., 1997; Tesone et al., 2005). The current data extend the evidence that hypoxic-to-normoxic (0–5%) O_2 concentrations promote VEGF production by luteinizing granulosa cells collected from women during controlled

Table 1 Angiopoietin (ANGPT) and progesterone levels in media from acute (24 h) culture at 20% O_2 of macaque granulosa cells collected before (non-luteinized) or after (luteinized) administration of an hCG bolus in controlled ovarian stimulation cycles.

Granulosa cells	ANGPT-1 (pg/ml)	ANGPT-2 (pg/ml)	Progesterone (ng/ml)
Luteinized			
Control	39 ± 2	944 ± 438	$923 \pm 385^{b,d}$
LH	39 ± 2	1480 ± 1675	$1380 \pm 397^{b,d}$
Non-luteinized			
Control	36 ± 2^a	906 ± 780	$68 \pm 50^{b,e}$
LH	39 ± 2	527 ± 991	$482 \pm 276^{c,e}$

^aLimit of assay detection.

^{b,d}Different letters indicate significant differences ($P < 0.05$) in progesterone levels between treatments with the same cell type.

^{d,e}Different letters indicate significant differences ($P < 0.05$) in progesterone levels between cell types with the same treatment.

ovarian stimulation cycles (Friedman et al., 1997) or dispersed luteal cells from monkeys or women (Tesone et al., 2005). VEGF production by FG follicles was not influenced by FSH or O_2 concentrations, which is consistent with the conclusions that cells in the tertiary or large pre-ovulatory follicle produce VEGF independent from gonadotrophins (Taylor et al., 2004) and O_2 (Martinez-Chequer et al., 2003). Due to limited sample sizes, the interaction between FSH dose and O_2 concentration on VEGF production was not evaluated.

Notably, follicles that contained an oocyte capable of reinitiating meiosis in response to hCG produced higher concentrations of VEGF at Week 5 *in vitro*. *In vivo* studies support an angiogenic or angiotropic action of VEGF that promotes follicular development and steroidogenic function (Zimmerman et al., 2001, 2002; Wulff et al., 2002; Celik-Ozenci et al., 2003; Roberts et al., 2007). There is also increasing evidence that VEGF has extra-vascular effects in the ovary (Luo et al., 2002; Araújo et al., 2013; Greenaway et al., 2004). Further studies are warranted to discern vascular versus extra-vascular actions of VEGF in the growing follicle, and whether VEGF is a possible marker for high-quality follicles destined to provide a mature oocyte capable of fertilization.

In the present study, ANGPT-1 production was low for *in vitro*-developed follicles regardless of culture conditions and growth rates. Previous evidence in rodent (Abramovich et al., 2009), bovine (Hayashi et al., 2004) and monkey (Wulff et al., 2001) follicles suggests that ANGPT-1 mRNA and protein expression increases in antral follicles. The limited ANGPT-1 production in SG and FG follicles after antrum formation may be due to the immaturity of growing follicles. In contrast to ANGPT-1, *in vitro*-developed follicles produced ANGPT-2 with increasing concentrations after antrum formation, especially in FG follicles which display considerable estrogenic activity (Xu et al., 2011) and produce mature oocytes. The high ANGPT-2:ANGPT-1 ratio in developing follicles *in vitro* is consistent with the current granulosa cell study, where non-luteinized cells produced more ANGPT-2 than ANGPT-1 during short-term culture. There is also evidence of similar characteristics in the follicular fluid of 'lead' follicles from controlled ovarian stimulation protocols in women (Hurliman et al., 2010; Nishigaki et al., 2011). These data suggest

that a high ANGPT-2:ANGPT-1 ratio is associated with final maturation of the periovulatory follicle in primates. Due to limited sample sizes, gonadotrophin and O₂ effects on ANGPT-2 production were not assessed for cultured follicles. But, effects of gonadotrophins and O₂ on ANGPT production by cultured granulosa cells were also not detected in the current study. Further studies are needed to examine the regulation of ANGPT-2 expression in the developing follicle and clarify its vascular and extravascular actions (Hayashi *et al.*, 2004; Parborell *et al.*, 2008). We hypothesize that a symbiotic relationship between elevated concentrations of ANGPT-2 and VEGF allows FG follicles to excel in follicle maturation. Also, elevated ANGPT-2 production may offer possible insight into future oocyte quality as early as Week 2, compared with Week 4 for VEGF and follicular size.

The current data add to our understanding of the dynamic activity of primate follicles as they develop from the early secondary stage to the small antral (~1 mm in diameter) stage during 3-D culture. The production of angiogenic factors, VEGF-A and ANGPT-2, as well as other local factors (e.g. anti-Müllerian hormone) and steroid hormones (progesterone, androstenedione and estradiol) (Xu *et al.*, 2010, 2011, 2013), is a function of the follicle growth rate, with FG follicles producing higher concentrations than SG and NG follicles. Likewise, their production is a function of the stage of follicular development, e.g. the production of VEGF and ANGPT-2 correlates with steroid hormone production (Xu *et al.*, 2011); concentrations are low prior to onset of antrum formation and continue to rise after antrum formation. The 3-D culture system provides a unique opportunity to explore possible local actions of follicular factors that promote the follicular development, both from a steroidogenic and gametogenic perspective, including VEGF and ANGPTs independent of angiogenesis.

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Authors' roles

As lead author, T.E.F. performed VEGF and ANGPT assays of media from follicle cultures, analysed the data, led the discussion of data and its relevance to the literature, and provided the first draft of the manuscript. T.A.M. and A.V. performed the granulosa cell experiments, including ANGPT assays, analysed the data and participated in preparation of the manuscript. M.B.Z. assisted with follicle isolation. M.B.Z. and R.L.S., as collaborators and leaders of their respective laboratories, provided oversight of the research trainees (T.E.F. and A.V.) during experimental design and performance, data analyses, literature evaluation and manuscript preparation. J.X. performed the follicle culture, analyzed follicle growth and oocyte parameters, as well as contributed to data interpretation, critical manuscript revising for important intellectual content and preparation of the final manuscript to be submitted for

publication. All authors approved the final version to be submitted for publication.

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Conflict of interest

The authors have no conflict of interests to disclose.

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