Effects of inhibition of vascular endothelial growth factor at time of selection on follicular angiogenesis, expansion, development and atresia in the marmoset

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This study determined the effects of inhibiting vascular endothelial growth factor (VEGF) at follicle selection. Marmosets were given an injection of VEGF antagonist, the VEGF Trap on Day 5 of the follicular phase and ovaries were evaluated on Day 10 or 15. Ovaries from controls were assessed on Day 5 (time of selection), Day 10 (peri-ovulatory) and Day 15 (luteal phase). At Day 10, ovaries of four of the five controls contained dominant follicles, while one had ovulated. VEGF Trap-treated ovaries also contained large follicles on Day 10, but VEGF inhibition had suppressed endothelial cell proliferation, leading to reductions in the thecal vascularization and plasma estradiol relative to controls. By Day 15, ovaries of controls contained active corpora lutea whereas ovaries of four of the five treated animals still contained large antral follicles similar in size to pre-ovulatory follicles, and one had small, avascular corpora lutea. However, these follicles had a restricted vasculature, increased incidence of activated caspase-3 staining and morphological features indicating they would become degenerative non-functional cysts. These results show that after follicle selection, VEGF is essential for angiogenesis and the generation of healthy ovulatory follicles and corpora lutea, but fluid accumulation can still occur in selected follicles in the absence of VEGF.

Keywords: ovary; angiogenesis; follicle; marmoset monkey; VEGF Trap

Introduction

The ovary is distinct from other endocrine organs in that it undergoes repetitive cycles of angiogenesis within its various glandular compartments. This process is under the regulation of angiogenic growth factors, among which vascular endothelial growth factor A (VEGF-A) is pre-eminent. Pharmacological inhibition of VEGF at the beginning of the follicular phase suppresses both thecal vascularization and follicular development in the marmoset (Wulff et al., 2001a, 2002), and blockade of the VEGF receptor type 2 (VEGFR2) prevents gonadotrophin-induced follicular development in the rodent (Zimmermann et al., 2003). Furthermore, inhibition of VEGF or VEGFR2 in macaques at the early-, mid- or late follicular phase blocks the endocrine changes associated with follicular maturation and ovulation (Zimmermann et al., 2001, 2002; Fraser et al., 2005).

In addition to its pro-angiogenic role, VEGF, as a vascular permeability factor (Roberts and PaLade, 1995; Dvorak et al., 1999; Bates and Harper, 2003), is likely to play a key role in the movement of fluid into the follicular antrum during the final stages of follicle development (Koos, 1995). There is also evidence that it may act as a survival factor for granulosa cells (Greenaway et al., 2004). Thus, its inhibition at the time of follicle selection may have detrimental effects on follicles in addition to inhibition of angiogenesis.

The development of effective inhibitors of angiogenesis may open new avenues for the treatment of reproductive disorders characterized by pathological angiogenesis, inflammation and increased vascular permeability. For example, polycystic ovarian syndrome (PCOS) is characterized by the formation of multiple follicular cysts in which the theca is hyperplastic and hyper-vascularized (Abbott et al., 2002), the granulosa cells secrete elevated levels of VEGF (Agrawal et al., 2002) and there is an increased stromal blood flow (Pan et al., 2002). In a previous study in macaques to examine the endocrine effects of inhibition of VEGF, we administered VEGF Trap at the mid-follicular phase, as it is this stage of the cycle that normal follicular size and estradiol (E₂) production are closest to that observed in the ovaries of women with PCOS (Fraser et al., 2005). This treatment regimen blocked the expected progressive rise in E₂ that ordinarily occurs in the second half of the follicular phase indicating that VEGF inhibition induces atresia of the recruited follicles at the antral stage of development (Fraser et al., 2005). We suggested that short-term administration of VEGF inhibitors in PCOS might cause...
the elaboration of the thecal vasculature, might result in failure of
fore, we sought to determine if VEGF inhibition, as well as preventing
non-human primates have been restricted to monitoring of endocrine
takes place during the mid- to late luteal phase of the preceding cycle. To syn-
Experiments were carried out in accordance with the Animals (Scientific Pro-
Adult female common marmoset monkeys (Callithrix jacchus) with a body
Materials and Methods
Animals
Adult female common marmoset monkeys (Callithrix jacchus) with a body
Expression of anti-mullerian hormone during early follicular development
was studied by (i) quantifying the number of proliferating cells stained for
Blood samples continued to be collected three times a week for the duration of the
At the end of the treatment periods, animals were injected i.v. with
20 mg bromodeoxyuridine (BrdU) (Roche Molecular Biochemicals, Essex, UK)
in saline 1 h before being sedated with 100 μl ketamine hydrochloride (Parke-
leptin-producing cells with constricted cytoplasm and atrophic nuclei.
Identification of proliferating cells
The effects of the treatment on the establishment of the thecal vascular network
was studied by (i) quantifying the number of proliferating cells stained for
BrdU, (ii) identifying endothelial cells using CD31 immunostaining and (iii)
distincting proliferating endothelial cells by co-localization of BrdU and
western 10 days follicular phase. In the main study, this dose of
VEGF Trap was given on Day 5 (mid-follicular phase), and ovaries were col-
were collected on Day 5 to assess ovarian morphology at the
treatment was initiated. In addition, controls were treated on Day 5 with
human Fc (25 mg/kg, s.c.) and ovaries were obtained on Day 10 (n = 5) or
BrdU, (rabbit anti-mouse Ig diluted 1:60 in NRS:TBS, Dako) was performed for
slides for immunohistochemistry. Sections were dewaxed in xylene, rehy-
described previously (Fraser et al., 1999). Cycles were monitored by measuring
plasma progesterone concentrations in blood samples collected three times per
Day 5 and ovulation between Day 9 and 11 (Summers et al., 1985).
The day of progesterol injection was designated follicular Day 0. This
method of synchronizing follicular recruitment is followed by follicle selection
on cycle Day 5 and ovulation between Day 9 and 11 (Summers et al., 1985).
To block VEGF, we employed the VEGF Trap, a recombinant chimeric
protein comprising portions of the extracellular domains of the human VEGF
Receptors 1 and 2 expressed in sequence with the Fc portion of human immu-
Their study on inhibition of VEGF at time of follicular selection in
Studies on inhibition of VEGF at time of follicular selection in
neutral buffered formalin. After 24 h, the ovaries were transferred to 70%
were dehydrated and embedded in paraffin according to standard procedures.

Assays
ELISA assays described previously were used to determine plasma progesterone
concentrations (Fraser et al., 2006) throughout the study, plasma E2 in terminal
samples (Taylor et al., 2004) and VEGF Trap post-treatment (Fraser et al., 2005).

Morphological characterization of follicles
All ovaries were embedded and serially sectioned, and tissue sections (5 μm)
were placed onto SuperFrost slides (BDH, Merck Co., Inc., Poole, UK). For
morphological and morphometric analyses for BrdU incorporation, every
40th section (four slides per ovary) was studied. Alternate series of sections
were used for Immunohistochemistry. Sections were dewaxed in xylene, rehy-
content of another oocyte containing a nucleus were considered to ensure proper follicular classification.
Follicles were classified as atretic when they showed signs of granulosa cell
pynosis. To determine changes in the number of dying cells in follicles, a
rabbit antibody to activated caspase-3 (Asp175) (New England Biolabs,
Hitchen, UK) was used as described previously (Fraser et al., 2006). Visualization
was achieved by DAB substrate (EnVision kit): sections were then counter-
Stages of follicular development were defined as described (Wulf et al.,
2001a, 2002), i.e. early secondary (2–4 granulosa cell layers, no antrum), late
secondary (2–4 granulosa cell layers, no antrum), tertiary (follicles containing
an antrum) and dominant (large antral follicles, >2 mm). Follicles were classi-
fied as healthy if they contained a normal-shaped oocyte surrounded by granulosa
cells that were regularly apposed on an intact basement membrane with normal
appearance of granulosa cell nuclei without signs of pynosis. For examination
of early and late secondary follicles, only those with a visible oocyte containing a

Identification of proliferating cells
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distinguishing proliferating endothelial cells by co-localization of BrdU and
CD31 (Wulf et al., 2001a, 2002).

For BrdU and CD31 staining, antigen retrieval was performed by pressure-
cooking sections in 0.01 M citrate buffer (pH 6), for 6 min at high-pressure
setting 2 (Tefal Clypso pressure cooker, Tefal, Essex, UK). Slides were then
left for 20 min in hot buffer and washed in Tris-buffered saline (TBS)
(0.05 mol/l Tris and 9 g/l NaCl). To reduce non-specific binding, sections
were blocked in normal rabbit serum (NRS) (1:5 diluted in TBS containing
20 mg/protein. To separate follicular recruitment, cortical egg cells were
removed with cold PBS containing 5% bovine serum albumin for 30 min.
Primary antibodies to CD31 (mouse anti-human CD31, Dako Corporation,
Copenhagen, Denmark) or BrdU (mouse anti-BrdU, Roche Molecular Biochemicals)
were diluted 1:20 and 1:30 in TBS, respectively. Incubation was carried out overnight
at 4°C. Slides were washed three times in TBS. Incubation with the secondary antibody
(rabbit anti-mouse Ig diluted 1:60 in NRS:TBS, Dako) was performed for
40 min at room temperature, followed after two washes in TBS by incubation
in alkaline phosphatase-anti-alkaline phosphatase complex (1:100 dilution in
TBS, Dako) for 40 min at room temperature. Visualization was performed
using 500 μl/ slide nitro blue tetrazolium (NBT) solution containing 45 μl
NBT substrate (Roche Molecular Biochemicals), 10 ml NBT buffer, 35 μl
5-bromo-3-chloro-3-indolyl-phosphate and 10 μl levamisole. Sections for
BrdU were counterstained with haematoxylin, whereas sections for CD31

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were not counterstained, so that quantitative image analysis could be performed. For dual labelling, slides were incubated first with CD31 and visualized with Fast Red (Sigma, Poole, UK; 1 mg Fast Red in 1 ml Fast Red buffer (20 mg napthol AS-MX phosphate, 2 ml dimethyl formamide and 98 ml 0.1 M Tris, pH 8.2)]. After staining for CD31, incubation with BrdU was performed. BrdU-stained cells were visualized with NBT as described above.

Quantification of BrdU and CD31 immunohistochemistry

Follicular angiogenesis is not initiated in the marmoset until follicles contain more than four granulosa cell layers (Wulf et al., 2001a). Thus, only follicles with four or more layers of granulosa were analysed. All follicles of this size or greater were analysed in the Day 10 VEGF Trap-treated group and Day 10 controls, while quantification was confined to the large antral follicles in the Day 15 VEGF Trap-treated group. Captured images were thresholded, and the thecal and granulosa cell compartments were outlined and analysed separately. Four sections per ovary were analysed under x 200 magnification. Cellular proliferation was measured using an image analysis system set up to measure the number of black-stained nuclei (BrdU positive), and the number of black and light blue (haematoxylin)-stained nuclei (total number of cells) in the outlined compartment of interest. A proliferation index (BrdU-positive cells expressed as a percentage of the total number of cells) was calculated for the thecal and granulosa compartments for each follicle and expressed as a mean value for the number of follicles assessed within each follicular stage per animal.

The automated image analysis of BrdU in the granulosa of secondary follicles could not reliably distinguish between single cells because of small cytoplasmic volume and close proximity of nuclei. Thus, the granulosa cell proliferation index in these follicles was obtained by manual counting of the number of granulosa cells in 50 secondary follicles to determine a mean cell number per unit area, measuring the total area occupied by the granulosa in each follicle and converting the area to cell number. Proliferating endothelial cells (dual stained cells with red stained cytoplasm BrdU positive black stained nucleus) were also counted manually at x 200.

The endothelial cell area (CD31-positive cells) was measured by thresholding a captured grey-scale image and converting it to a binary image. The whole area of the thecal compartment and the CD31-positive area within the compartment were measured. The CD31-positive area was then calculated per unit area of the thecal compartment and expressed as a mean value for the number of follicles assessed within each follicular stage per animal.

Quantitative analysis was performed using an image analysis system linked to an Olympus Corporation camera, and the data were processed using Imaging-Pro Plus version 3.0 for Windows (Microsoft Corporation, USA).

Statistical analysis

Data obtained for different cycle and follicular stages were tested for significant differences using ANOVA followed by Bonferroni’s multi-test. Effects of the treatment compared with late follicular controls were determined using a two-tailed, unpaired t-test. Differences were considered significant at P < 0.05. The tests were performed using SPSS version 11 for Macintosh (SPSS, Inc., Chicago, IL, USA). All values are given as the mean ± SEM.

Results

VEGF Trap concentrations in plasma

All treated marmosets exhibited plasma concentrations of VEGF Trap between 100 and 200 mg/l one day post-treatment. VEGF Trap levels then fell progressively to 48–74 mg/l by Day 5 and 5–30 mg/l by Day 10. The detection limit of the assay was 0.5 mg/l, and it has been estimated that effective pharmacological blockade of VEGF in the ovary is retained until VEGF Trap levels falls below 1–2 mg/l (Fraser et al., 2006). Thus, levels of unbound VEGF Trap in all treated marmosets remained within the anticipated pharmacologically effective range for the duration of the study.

Hormonal changes

All marmosets responded to the administration of prostaglandin analogue during the luteal phase with a rapid fall in plasma progesterone concentration, confirming the induction of luteolysis. VEGF Trap treatment on cycle Day 0 or 5 resulted in a significant suppression (P < 0.05) of E2 concentrations in blood samples collected on Day 10, being 253 ± 21 pmol/l (treatment starting Day 0) and 691 ± 140 pmol/l (treatment starting Day 5), compared with 1965 ± 466 pmol/l in Day 10 controls (P < 0.05). On Day 15, E2 in treated animals was 444 ± 102 pmol/l, which was also significantly lower (P < 0.05) than Day 10 controls. Plasma progesterone concentrations remained at follicular phase values (<30 nM/l) in treated animals, while by Day 15 in controls they were elevated to 206 ± 14 nM/l.

Size of selected follicles

The predominant structures in control ovaries on Day 5 were medium-sized antral follicles (Fig. 1A) and by Day 10, two or three large pre-ovulatory follicles (11 in all) (Fig. 1B) were present in the ovaries.
from four animals, while the ovaries of the remaining marmoset contained two recently ovulated follicles. By Day 15, all controls had ovulated and large healthy corpora lutea predominated (Fig. 1C). In contrast, treatment with VEGF Trap at Day 0 markedly suppressed follicular development, such that at Day 10 the ovaries contained only pre-antral and small antral follicles (Fig. 1D). Paired ovarian weights was also significantly reduced (84.3 ± 3 mg) compared with Day 10 controls (165.0 ± 22 mg) (P < 0.05). These changes were associated with a marked suppression of endothelial cell proliferation and attenuation of the thecal vasculature (data not shown), comparable to that seen in studies where VEGF Trap was administered every other day over the equivalent period (Wulff et al., 2002). Thus, when administered on cycle Day 0, a single 25 mg/kg injection of VEGF Trap was fully effective in suppressing follicular angiogenesis and development for at least 10 days.

When VEGF Trap treatment was initiated on cycle Day 5, after follicle selection had occurred, paired ovarian weights at Day 10 (125 ± 19 mg) were not significantly different from controls, and the ovaries of all five treated marmosets contained 1–3 large antral follicles (11 in all) (Fig. 1E). The mean volume of these follicles was significantly greater than that of the largest tertiary follicles present at the time treatment was initiated (Day 5 untreated group, P < 0.05) (Fig. 2), indicating that expansion had occurred despite VEGF inhibition. However, these follicles were significantly smaller than the pre-ovulatory follicles of their respective (Day 10) controls (P < 0.01) (Fig. 2).

For animals evaluated on cycle Day 15, the ovaries of all controls contained new corpora lutea. In contrast, the ovaries of four of five VEGF Trap-treated animals were dominated by large antral follicles (8 in all) (Fig. 1F); these were of significantly greater volume than those found at Day 10 in treated marmosets, and now of similar volume to that of the pre-ovulatory follicles in Day 10 control ovaries (Fig. 2). Paired ovary weight (104 ± 6 mg) was significantly less than that of Day 15 control ovaries that contained well-developed corpora lutea (225 ± 26 mg) (P < 0.001). The remaining Day 15 treated animal had apparently ovulated, but the two corpora lutea were exceptionally small (Fig. 1G) and combined ovarian weight was only 58 mg.

When the viability of the granulosa cells in the largest follicles was assessed by incidence of staining for activated caspase-3 it was found that in dominant follicles from control animals, staining for activated caspase-3 was either entirely absent or present in a few granulosa cells per follicle (Fig. 1H). In contrast, activated caspase-3 was present in all largest antral follicles from the VEGF Trap-treated marmosets on Day 10, albeit to a variable degree (in 2–9 cells per follicle in 6 follicles from three marmosets, and in 30–>100 cells per follicle in 5 follicles of the remaining two animals (Fig. 1I). Staining for activated caspase-3 was generally more extensive in the largest antral follicles in the animals receiving VEGF Trap evaluated on Day 15; one dominant follicle contained 11 stained cells, while the remaining seven follicles contained 30–>100 positive cells (Fig. 1J). These results indicated that atresia of these large follicles was beginning at Day 10 and imminent or established by Day 15.

Theca and granulosa cell proliferation
Detailed histological evaluation revealed that in selected follicles, VEGF inhibition produced alterations in follicular morphology, characterized by decreased cellular proliferation in both the theca and granulosa, attenuation of the thecal vasculature and thinning of the theca.

The effect of VEGF Trap treatment on BrdU incorporation into cells in the thecal layer and granulosa cells is illustrated in Fig. 3. In Day 10 control ovaries, the proliferation rate in the theca layer rose significantly from the early secondary to the late secondary (P < 0.001) and again between late secondary and tertiary stage (P < 0.05). The rate of proliferation in the theca of dominant follicles was decreased relative to other tertiary follicles (P < 0.01) (Fig. 3A, C and E). On Day 10, treated ovaries exhibited a markedly reduced rate of proliferation in the thecal layer of late secondary, tertiary and large antral follicles (P < 0.001) (Fig. 3B, D and E). Proliferation rates in the theca of treated follicles were similarly inhibited on Day 15, although only changes in the largest antral follicles were quantified (Fig. 3E and F).

In control marmosets, granulosa cell proliferation was also significantly greater in late secondary, compared with early secondary follicles (P < 0.01) (Fig. 3F). This high rate of proliferation was maintained in tertiary follicles (Fig. 3F), but in dominant follicles granulosa proliferation was significantly less (P < 0.05, Fig. 3F). Injection of VEGF Trap had no significant effect on granulosa cell proliferation in early or late secondary follicles (Fig. 3F). However, tertiary follicles of VEGF Trap-treated animals displayed markedly lower rates of granulosa cell proliferation relative to controls (P < 0.001) (Fig. 3D and F). In dominant follicles in controls, the granulosa cell proliferation rate was reduced. Proliferation in the largest follicles in VEGF Trap-treated animals was rather variable (Figs. 3D and F), being significantly lower (P < 0.05) only in the Day 15 treated group. These effects on proliferation rate resulted in a reduction in granulosa cell number in dominant follicles of treated animals (Fig. 3G) and this was statistically significant (P < 0.001) in the VEGF Trap ovaries on Day 10.

Thecal endothelial cell proliferation and vascularization
Examination of follicles in sections dual stained for BrdU and CD31 revealed that endothelial cell proliferation in the theca was almost completely abrogated by VEGF inhibition (Fig. 4A and B). In control ovaries, the proportion of proliferating cells that were endothelial in the theca was 19.9 ± 3% in late secondary, 18.7 ± 2% in tertiary follicles, rising significantly (P < 0.001) to 56.0 ± 6% in dominant follicles. Total numbers of proliferating endothelial cells in follicles were markedly suppressed by VEGF Trap treatment in vascularized follicles at all stages of development including the largest antral follicles (P < 0.001 Fig. 4B and C).

These changes were associated with a reduction in the thickness of the thecal layer in all classes of vascularized follicles in treated
ovaries, compared with follicles of the same class in control ovaries. In control ovaries, the thickness of the thecal layer increased progressively with follicle size: from late secondary (27.0 ± 2.3 μm) to tertiary stages (57.0 ± 3.5 μm) (P < 0.001), and from tertiary to dominant stages (74.9 ± 4.1 μm) (P < 0.01). Thecal thickness was significantly reduced in each class of follicle by VEGF Trap treatment in Day 10 marmosets: late secondary (18.6 ± 0.9 μm) (P < 0.01), tertiary (29.6 ± 3.1 μm) (P < 0.001) and large antral (44.7 ± 5.8 μm) (P < 0.01). Similarly, in the Day 15 treated group, thecal thickness of the largest follicles was 49 ± 6.1 μm, significantly lower (P < 0.05) than in Day 10 controls.

Quantitative analysis of the vascular density in control ovaries showed a progressive increase from late secondary (3.8 ± 0.4%), to tertiary (6.1 ± 0.2%) (P < 0.01), to large antral (dominant) follicles (12.9 ± 0.7%) (P < 0.001). Treatment with VEGF Trap significantly decreased thecal vascular density relative to controls in Day 10 marmosets in both late secondary (1.9 ± 0.3%) (P < 0.05) and tertiary follicles (2.0 ± 0.3%) (P < 0.001). In tertiary follicles of control ovaries, the thecal vascular network extended to the membrana propria, and VEGF Trap treatment largely prevented the development of this feature. In contrast, vascular density was not significantly different between dominant follicles of control and treated animals (10.3 ± 0.8%) on Day 10. However, as noted above, thecal thickness
was significantly reduced by VEGF inhibition, and this effect was most apparent in dominant follicles. Therefore, to obtain an estimate of the total vascular investment of follicles at various stages of develop-
ment, the area vascular density was multiplied by thecal thickness. This analysis confirmed that in control ovaries, relative thecal vascularization increased progressively as follicles developed from late secondary to tertiary ($P < 0.001$) and from tertiary to dominant stages ($P < 0.01$) (Fig. 5C). It also revealed a significant reduction ($P < 0.001$) in the relative vascular investment of every class of follicle in the ovaries of VEGF Trap-treated marmosets, relative to controls (Fig. 5C).

In the one VEGF Trap treated Day 15 animal that had ovulated, cellular proliferation was virtually abolished in the corpora lutea and the vascular density also was severely reduced compared with control corpora lutea (not shown).

**Discussion**

In marmosets, when treatment with a single dose of VEGF Trap is initiated in the early follicular phase (Day 0), inhibition of VEGF blocks thecal endothelial cell proliferation, vascularization and antrum formation, resulting in the absence of large antral follicles. Here, we have focussed on the role of VEGF on the latter stages of follicle development, specifically the process of angiogenesis, granulosa cell proliferation and follicle expansion that occurs after follicle selection. The results show that VEGF inhibition blocks angiogenesis, impairs normal development and initiates atresia in the selected follicles. Follicle expansion was delayed but not prevented in these follicles, indicating that fluid can accumulate in selected follicles independently of VEGF and can take place during the onset of atresia.

Thus, although selected follicles had enlarged during the course of treatment, particularly when the anovulatory follicles were examined on Day 15, this masked important morphological and functional differences. It may be predicted that the predominant fate of the follicles, as judged by their status on Day 15 of the cycle is to become degenerative non-functional cysts. Only one of the five treated marmosets ovulated, and the resultant corpora lutea were very small and characterized by a near absence of endothelial cell proliferation, failure to elaborate a microvasculature and low progesterone output. Perhaps follicle development had progressed further at the start of the cycle.
VEGF Trap treatment was accompanied by specific changes in cellular proliferation within growing follicles. Endothelial cell proliferation within the theca was markedly inhibited, and the proliferation of parenchymal cells in the theca also was reduced. As a consequence, the theca layer was thinner in the follicles of treated animals than in follicles at the same developmental stage in control ovaries. VEGF inhibition also was associated with a suppression of granulosa cell proliferation in tertiary follicles as described previously (Wulff et al., 2002). In the large antral follicles of controls, granulosa cell proliferation was declining by the pre-ovulatory period, but was further suppressed in treated marmosets, being statistically significant by Day 15. The observed reductions in the proliferation of non-vascular components of the theca and the granulosa are probably secondary to the arrested development of the thecal vasculature, and the potential reduction in VEGF-mediated vascular permeability to macromolecules that could further limit the diffusion of gonadotrophins and other proteins into the developing follicles. The reduction in granulosa cell proliferation could also be the result of inhibition of an autocrine survival effect of VEGF that has been demonstrated on bovine granulosa cells (Greenaway et al., 2004). The result of all of these inhibitory effects was to suppress plasma E2 levels compared with controls and prevent ovulation. By Day 15, in treated marmosets, the incidence of activated caspase-3 in granulosa cells increased, showing that the large follicles were becoming atretic. GnRH antagonist treatment following follicle selection has also been associated with follicular atresia accompanied by continued expansion (Taylor et al., 2004). These observations indicate that the increase in follicle volume can occur during the process of atresia and that this passive increase in volume does not require sustained proliferation of blood vessels, theca or granulosa cells or additional VEGF or gonadotrophin signalling.

The mechanisms that regulate follicle expansion are unclear. It is a very rapid process, such that follicular diameter increases at a ~50-fold greater rate than during the pre-antral phase of follicle growth (Gosden et al., 1988). VEGF acts as a potent pro-permeability factor in a variety of tissues in vitro and in vivo (Roberts and PaLade, 1995; Dvorak et al., 1999; Bates and Harper, 2003). In addition, VEGF is thought to be a prime mediator of ovarian hyperpermeability (Wang et al., 2002; Gomez et al., 2003); specific inhibition of VEGFR2 inhibits vascular permeability in a rat model of ovarian hyperstimulation syndrome (Gomez et al., 2002) and administration of VEGF Trap at the ‘post-angiogenic’ period of the luteal phase in marmosets suppresses the secretion of plasma progesterone, suggesting an inhibition of ovarian vascular permeability (Fraser et al., 2006). Based upon this information, we had expected to find that, in addition to mediating follicular angiogenesis, VEGF would also play a critical role in follicular expansion following follicle selection. While it is clear that VEGF is required for expansion of small follicles, once vascularity has been established by VEGF in selected follicles fluid accumulation can occur in the absence of additional VEGF signalling. However, given the perceived importance of VEGF in mediating vascular permeability, it may be that the macromolecular component of follicular fluid of the treated ovaries is deficient.

The possibility remains that VEGF-A is ordinarily involved in this process, but that additional mechanisms come into play that allow a degree of follicular expansion in its absence. For example, other members of the VEGF family not inhibited by VEGF Trap may be involved. VEGF-C, is best characterized as a lymphangiogenic factor acting through VEGFR3 (Jussila and Aliitalo, 2002). However, VEGF-C can also bind to VEGFR2 and developing blood vessels can express VEGFR3. Currently, little is known regarding the expression of VEGF-C or VEGFR3 in the developing follicle, or their ability to modulate vascular permeability. While it may be that

Figure 5: CD31 staining in theca layer of large antral follicles from (A) Day 10 control ovary and (B) VEGF Trap treated ovary (Day 10). Note the thinner thecal layer in the treated ovary. Quantification of relative vascularization increases progressively with follicle size in control ovaries. VEGF inhibition results in a relative reduction in thecal vascularization at all stages. Different capital letters denote statistical differences within a class of follicle associated with treatment. G, granulosa; t, theca. Bar = 50 μm.
follicular expansion is mediated in part by other classes of angiogenic or vasoactive factors, entirely distinct mechanisms may play a pivotal role. For example, aquaporins are expressed in the ovary where they are believed to mediate transcellular water movement in granulosa cells, which would contribute to follicle expansion (McConnell et al., 2002). The available data do not indicate that aquaporin expression in the ovary is regulated by VEGF, so it is possible that aquaporins act to mediate fluid accumulation in its absence. Further studies are required to elucidate the potential role of the above molecules in follicle expansion.

In conclusion, delaying VEGF antagonist treatment until after follicle selection profoundly suppresses endothelial cell proliferation and further elaboration of the thecal vasculature leading to either a failure of ovulation or formation of non-functional corpora lutea. Although follicles continue to expand they are likely to become atretic. The current results provide support for the idea that VEGF Trap may induce atresia of accumulated follicles in the PCOS ovary and justify the extension of this approach to models of this condition.

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Conflict of interest statement

S.W. is employed by and holds stock in Regeneron Pharmaceuticals Inc., maker of the proprietary VEGF inhibitor, VEGF Trap.

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