

Ovarian VEGF_{165b} expression regulates follicular development, corpus luteum function and fertility

Y Qiu¹, M Seager¹, A Osman¹, J Castle-Miller^{1,2}, H Bevan¹, D J Tortonese², D Murphy³, S J Harper¹, H M Fraser⁴, L F Donaldson⁵ and D O Bates¹

¹Microvascular Research Laboratories, School of Physiology and Pharmacology, Bristol Heart Institute, Pre-clinical Veterinary Sciences Building, Southwell Street, Bristol BS2 8EJ, UK, ²Centre for Comparative and Clinical Anatomy, University of Bristol, Pre-clinical Veterinary Sciences Building, Southwell Street, Bristol BS2 8EJ, UK,

³Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, University of Bristol, Bristol, UK,

⁴MRC Human Reproductive Sciences Unit, Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, UK and ⁵School of Physiology and Pharmacology, Medical Sciences Building, University Walk, Bristol BS8 1TD, UK

Correspondence should be addressed to D O Bates; Email: dave.bates@bristol.ac.uk

Abstract

Angiogenesis and vascular regression are critical for the female ovulatory cycle. They enable progression and regression of follicular development, and corpora lutea formation and regression. Angiogenesis in the ovary occurs under the control of the vascular endothelial growth factor-A (VEGFA) family of proteins, which are generated as both pro-(VEGF₁₆₅) and anti(VEGF_{165b})-angiogenic isoforms by alternative splicing. To determine the role of the VEGF_{165b} isoforms in the ovulatory cycle, we measured VEGF_{165b} expression in marmoset ovaries by immunohistochemistry and ELISA, and used transgenic mice over-expressing VEGF_{165b} in the ovary. VEGF_{165b} was expressed in the marmoset ovaries in granulosa cells and theca, and the balance of VEGF_{165b}:VEGF₁₆₅ was regulated during luteogenesis. Mice over-expressing VEGF_{165b} in the ovary were less fertile than wild-type littermates, had reduced secondary and tertiary follicles after mating, increased atretic follicles, fewer corpora lutea and generated fewer embryos in the oviduct after mating, and these were more likely not to retain the corona radiata. These results indicate that the balance of VEGFA isoforms controls follicle progression and luteogenesis, and that control of isoform expression may regulate fertility in mammals, including in primates.

Reproduction (2012) **143** 501–511

Introduction

Formation of new blood vessels (angiogenesis) has a critical role in the female reproductive system, by affecting, for example, the cyclic changes that occur in the ovary during the ovulatory cycle (Charnock-Jones *et al.* 1993), ovarian follicular development (Yan *et al.* 1993), corpus luteum (CL) and endometrium formation (Kamat *et al.* 1995), and remodelling of mammary gland to enable lactation (Pepper *et al.* 2000). Specifically, studies in which the principal angiogenic factor, vascular endothelial growth factor-A (VEGFA), was inhibited *in vivo* in the mouse, marmoset or macaque established that angiogenesis is required throughout follicular development, as ovarian follicles progress during the cycle (Zimmermann *et al.* 2001, 2002, 2003, Wulff *et al.* 2002), after ovulation for the formation of the CL (Wulff *et al.* 2001, 2002, Zimmermann *et al.* 2001, 2002)

and for normal endometrial development (Fan *et al.* 2008, Fraser *et al.* 2008).

VEGFA expression has been localised within the different ovarian and endometrial compartments using immunohistochemistry, PCR and *in situ* hybridisation (Charnock-Jones *et al.* 1993, Shweiki *et al.* 1993). However, VEGF is alternatively spliced to generate many isoforms that differ in the heparin-binding activity and receptor activation potential. Alternative splicing of exons 6 and 7 generates different-length isoforms (e.g. VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉) and alternative splice site selection in exon 8 generates two families of isoforms, the pro-angiogenic VEGF_{xxx} family (where xxx is the number of amino acids in the peptide monomer, e.g. VEGF₁₆₅) and the anti-angiogenic VEGF_{xxx}b family (e.g. VEGF_{121b}, VEGF_{165b}; Harper & Bates 2008). The two families differ in the choice of splice site in the terminal exon, exon 8, resulting in differing C termini,

and differing activities (Bates *et al.* 2002). Many commercial antibodies and probes do not distinguish between the VEGF_{xxx}b and VEGF_{xxx} families, and even fewer between the isoforms within each family. It has previously been shown that VEGF₁₆₅b, the most widely studied form of the VEGF_{xxx}b family, inhibits VEGF₁₆₅-mediated angiogenesis in the rabbit cornea, chick chorioallantoic membrane, rat mesentery and mouse dorsal skin chamber (Cebe Suarez *et al.* 2006), and during physiological angiogenesis in the mammary fat pad (Qiu *et al.* 2008). It also inhibits pathological angiogenesis in the retina due to ischaemia (Magnussen *et al.* 2010) or inflammation (Hua *et al.* 2010), and in cancer (Rennel *et al.* 2008a, 2008b). Switching the splice site from proximal, VEGF₁₆₅ encoding, to distal, VEGF₁₆₅b encoding, results in reduced angiogenesis in cancer (Varey *et al.* 2008) and the retina (Nowak *et al.* 2010). Analysis of VEGF₁₆₅b expression in multiple human tissues has shown that the levels of VEGF₁₆₅b vary substantially from <15% of total VEGF in placenta (Bates *et al.* 2006), to >95% in normal human colon (Varey *et al.* 2008), and that this ratio varies greatly between tissues (Woolard *et al.* 2009) in physiological and pathological conditions (Schumacher *et al.* 2007), including those where pathological angiogenesis is occurring (Pritchard-Jones *et al.* 2007).

As VEGFA has a critical role in ovarian angiogenesis, we investigated the expression pattern of VEGF_{xxx}b in the ovary of the marmoset monkey, a species in which the contribution of VEGF to ovarian function has been comprehensively studied (Fraser & Duncan 2009), and examined whether expression levels are altered during the ovulatory cycle. To investigate the effects of manipulation of VEGF₁₆₅b in the ovary, we used a transgenic (TG) mouse that over-expresses VEGF₁₆₅b. We show that VEGF_{xxx}b is expressed in primate ovaries, is downregulated during CL formation, and that over-expression of VEGF₁₆₅b in mice reduces fertility by inhibiting follicular development and CL formation.

Results

VEGF₁₆₅b is expressed in primate ovaries

Sections of marmoset ovaries were stained for VEGF_{xxx}b using an antibody raised against the nine amino acid C terminus of human VEGF₁₆₅b (Woolard *et al.* 2004; Fig. 1A). Immunohistochemical analysis showed clear VEGF₁₆₅b expression in the theca layer of follicles and in the granulosa cell layer of primary and secondary follicles (Fig. 1A), while it was absent from the granulosa cells of tertiary follicles. Localisation was also present in the CL.

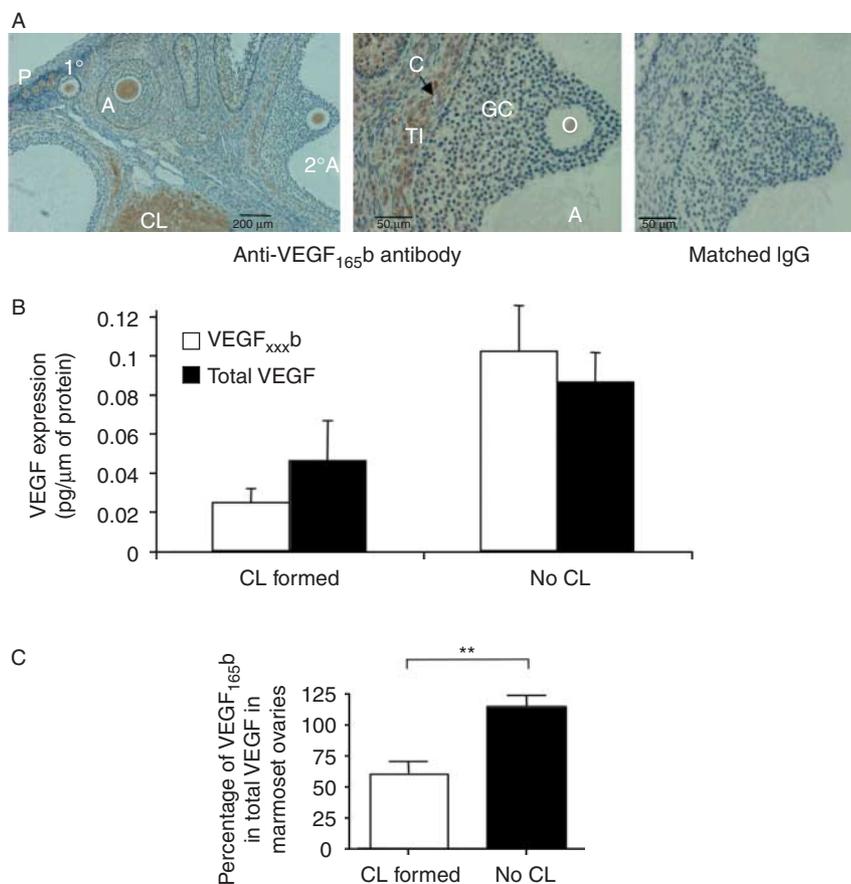


Figure 1 Expression of VEGF₁₆₅b in primate ovary. (A) Endogenous expression of VEGF₁₆₅b in marmoset ovary using an anti-VEGF₁₆₅b specific antibody. TI, theca interna; GC, granulosa cells; C, capillary; O, oocyte; CL, Corpus luteum; 2°A, secondary antral follicle; 1°, primary; A, antral; P, primordial. (B) ELISA for VEGF_{xxx}b and total VEGF on protein extracted from marmoset ovary. (C) Percentage of VEGF that is VEGF_{xxx}b in marmoset ovary. ***P*<0.01 *t*-test with Welch's correction. *n*=7 total.

Protein was extracted from whole ovaries and total VEGF and VEGF_{xxx}b levels were measured using either an ELISA for total VEGF or one specific for the VEGF_{xxx}b isoforms (Fig. 1B and C). Protein quantification showed that in ovaries that contained no corpora lutea, the measured VEGF_{xxx}b levels were similar to the total VEGF levels (Fig. 1B), suggesting that the majority of VEGF in these quiescent ovaries is VEGF_{165b} (not different from 100%). In contrast, in ovaries in which corpora lutea were found there was less VEGF_{165b} than total VEGF, and the percentage of total VEGF attributable to VEGF_{165b} was significantly smaller, $65 \pm 11\%$ ($P < 0.05$ compared to no CL, Fig. 1C, $n = 7$).

VEGF_{165b} over-expression in the ovary reduces fertility in TG mice

We generated TG mice over-expressing human VEGF_{165b} under control of the mouse mammary tumour virus (MMTV) promoter (Qiu *et al.* 2008). These mice showed strong expression of VEGF_{165b} mRNA in the ovary of virgin

animals, and weak expression in the uterus (Fig. 2A). Immunohistochemical analysis of mouse ovaries using a human specific anti-VEGF antibody showed strong expression of VEGF in the TG ovaries, but not in the wild-type (WT) ovaries (Fig. 2B). When TG females were mated with WT mice, the numbers of pups born to these mothers was significantly lower per litter (3.3 ± 0.4 compared with 5.9 ± 0.7 in the WT littermates). In contrast, WT females mated with TG males produced normal-sized litters (6.1 ± 0.7 pups per litter, Fig. 2C). To help determine whether the reduction in pup number was due to reduced ovulatory function or due to developmental defects, we determined pups birth weight and their genotype. Pups were born at a Mendelian frequency (50% of offspring were TG), and with normal birthweight (Supplementary Figure 1, see section on supplementary data given at the end of this article) indicating no offspring-dependent developmental defect. To determine whether ovulation was normal, we opened the oviducts of 13 TG and 9 WT females 0.5 days post coitus (dpc) and collected the embryos. The TG mothers had $82 \pm 6\%$ of the number of

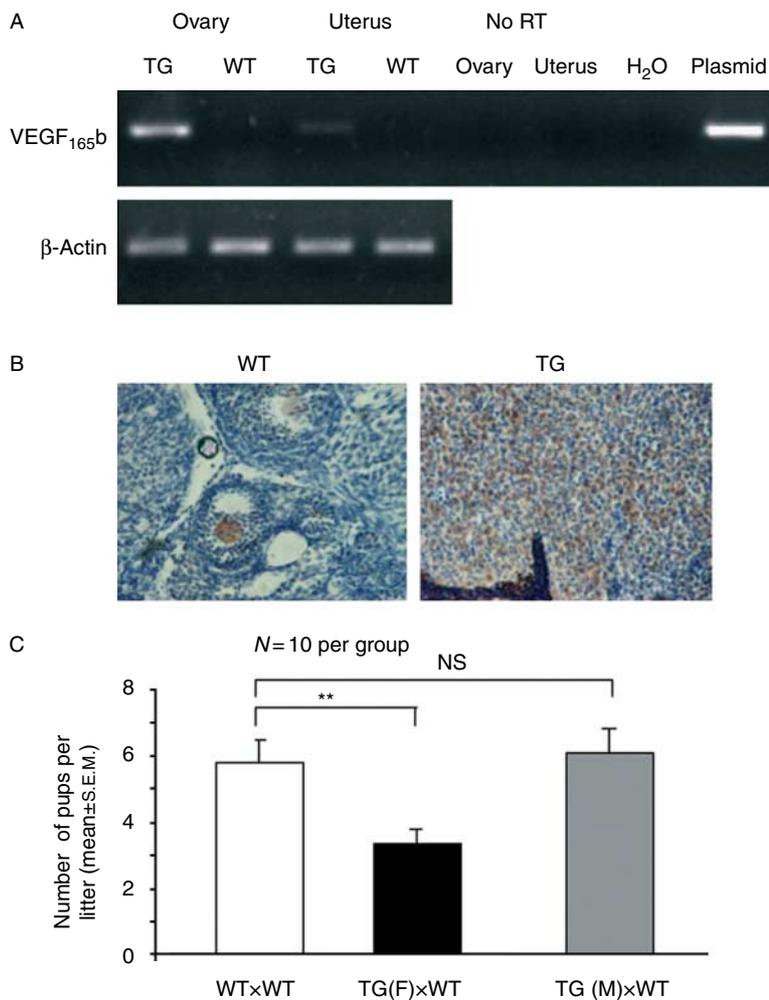


Figure 2 Over-expression of VEGF_{165b} in mouse ovary reduces litter size. (A) Mouse ovaries from wild-type or transgenic MMTV-VEGF_{165b} mice as shown were taken and RNA was extracted. RT-PCR was performed using primers specific for the human transgene. (B) Mouse tissues were fixed and stained using an anti-human VEGF antibody that detects all human isoforms but does not detect mouse VEGF. (C) Measurement of litter size in 10 transgenic (TG) or wild-type (WT) females mated to wild-type males. ** $P < 0.01$ paired *t*-test, $n = 10$ compared with littermate control. Wild-type females were also mated to TG males. NS, not significant compared with wild-type (unpaired *t*-test).

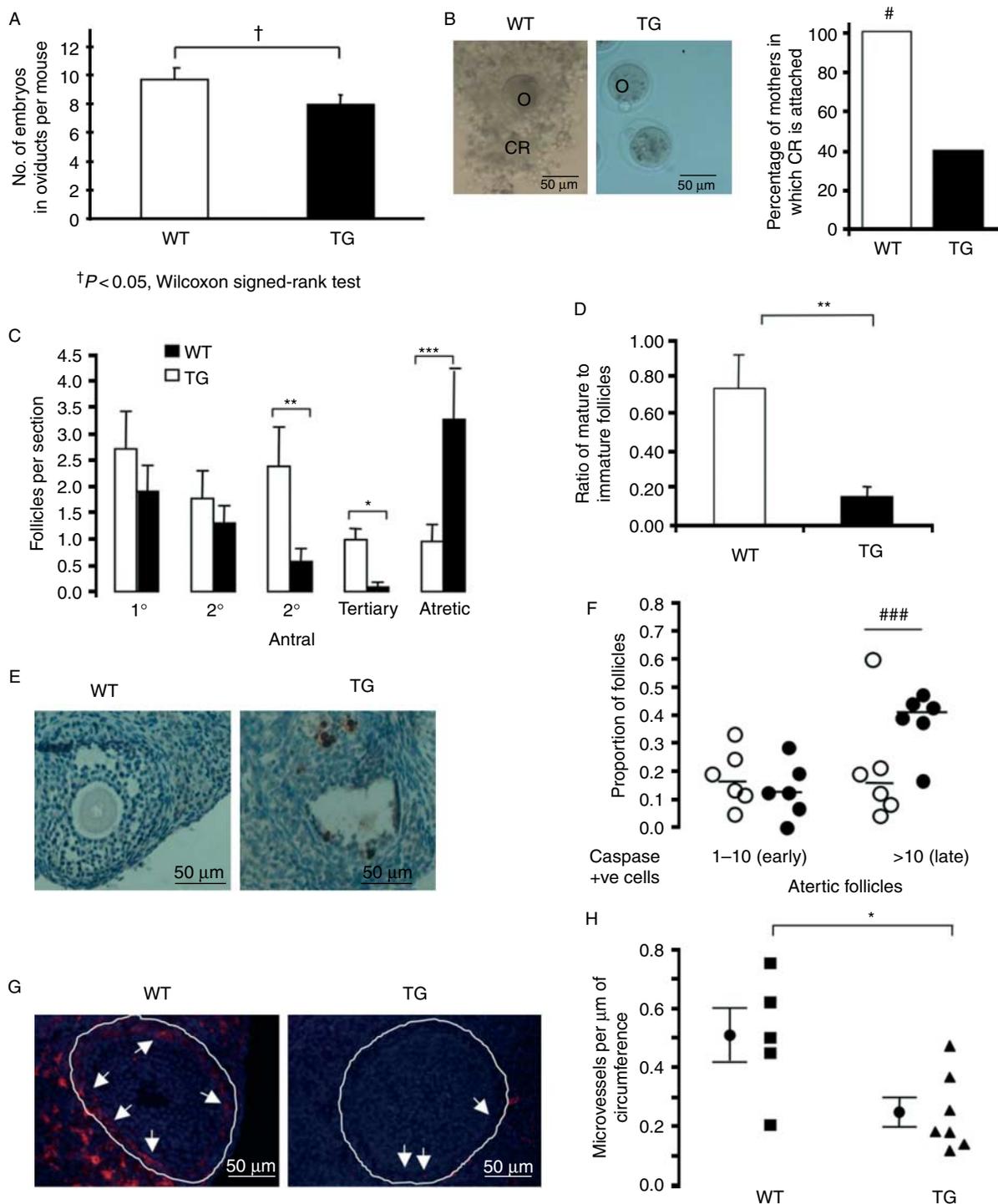


Figure 3 Changes in the embryos of the TG female mice over-expressing VEGF_{165b}. (A) Mice were culled at 0.5 dpc and embryos were collected from the oviducts. VEGF_{165b} over-expression led to a reduced number of embryos in the oviducts. $^{\dagger}P < 0.05$, Wilcoxon signed-rank test. (B) Images of embryos showing that those from TG females lack the corona radiata (CR) structure. $^{\#}P < 0.05$ Fisher's exact test. (C) Ovaries from these mice were fixed, sectioned through and stained with H&E to count follicles. $P < 0.001$ one-way ANOVA, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ Bonferroni *post-hoc* test. $n = 6$ per group. (D) The number of tertiary follicles as a percentage of secondary follicles was determined. $^{**}P < 0.01$ Mann-Whitney *t*-test. (E) Ovaries were stained with cleaved caspase-3 antibody and the number of positive (apoptotic cells) was determined. (F) The proportion of follicles with between 1 and 10 and > 10 positive cells were compared ($^{***}P < 0.001$, Fisher's exact test on follicle frequency, $n = 6$ animals per group, 274 follicles WT, 234 TG) (G). Ovaries taken at 0.5 dpc were fixed and stained using isolectin B4 to detect endothelial cells, and counterstained with Hoechst. Images of follicles from WT and TG female ovaries stained with IB4 to show MVD. (H) Microvessels were counted and expressed as microvessel covering area per unit perimeter of the follicle. $^*P < 0.05$, Mann-Whitney *t*-test.

embryos found in the oviduct of the WT mothers ($P < 0.05$; Fig. 3A). Moreover, strikingly, the embryos in the oviducts from the TG lacked adherent corona radiata in 60% of the mothers (Fig. 3B). Embryos were cultured for 4 days, and survival was measured by determining the rate of progression to blastocyst stage. There was no difference in the percentage of embryos reaching blastocyst stage when comparing embryos from TG and WT mothers (Supplementary Figure 2, see section on supplementary data given at the end of this article). This suggested a defect in folliculogenesis rather than developmental defects. We, therefore, collected ovaries from six TG and six WT female mice at 0.5 dpc and sectioned through the ovaries to determine follicle number. There was no statistical difference in the total number of follicular elements (follicles and corpora lutea) between WT (12 ± 2.6 per section) and TG (8.6 ± 1.3 per section, $P > 0.1$ *t*-test) mice sampled per section. However, as shown in Fig. 3C, there was a significant reduction in secondary, tertiary and mature follicle numbers in the TG compared with the WT mice. There was no statistical difference in primordial or primary follicles per section between WT and TG animals. Furthermore, there was a significant reduction in the percentage of tertiary to secondary follicles (Fig. 3D), suggesting not only a reduction in follicular development, but also a further defect in follicular maturation to the tertiary stage. Interestingly, there were more atretic follicles in the TG mice than those in the WT. The mechanisms for increased atresia were investigated by staining for apoptotic cells in the ovaries (Fig. 3E). Activated caspase-3-positive cells have been used as an indicator of early and late atresia (Utsunomiya *et al.* 2008). Atretic follicles with fewer than 10 cleaved caspase-3-positive cells have previously been regarded as early atresia and those with more than 10 as late atresia. There was no increase in the proportion of follicles in early atresia in TG mice (11%) compared with WT (13%), but a statistically significant increase in the proportion of late atretic follicles in TG (40%, $n = 6$ mice; $P < 0.01$, Fisher's exact test) compared with WT (24%, $n = 6$, Fig. 3F). To determine whether this reduction was associated with reduced vascular density around follicles, we stained the ovaries with isolectin B4, and determined the number of vessels covering the area (Fig. 3G). There was a significant reduction in blood vessels surrounding follicles from TG mothers (Fig. 3H, $P < 0.05$).

Angiogenesis is also required for corpora lutea formation. As VEGF_{165b} levels are reduced in marmoset ovaries containing CL, it is possible that a down-regulation occurs, to allow the intense angiogenesis that takes place in this organ. We, therefore, measured the size, number and vascularity of the CL in the largest cross section of ovaries from seven TG and five WT mice, to determine whether VEGF_{165b} over-expression inhibits CL formation. The number of CL per ovary was reduced in TG animals, consistent with reduced follicular development (Fig. 4A). In addition, the size of the CL was also reduced (Fig. 4B) from 0.82 ± 0.19 mm² in the

WT to 0.23 ± 0.08 mm² in the TG ($P < 0.01$, *t*-test). The proportion of CL with both between 1 and 10 cleaved caspase-3-positive cells (early CL regression) was also increased in TG (31% compared with 18% in WT) and with more than 10 cleaved caspase-3-positive cells (26% compared with 1%, Fig. 4C). These results indicate that regression of the CL is more rapid in the TG mice. Figure 4D shows microvascular (red) and pericyte (green) staining in the CL. Both the microvascular density (MVD; Fig. 4E) and the mean pericyte coverage (Fig. 4F) were reduced in the TG mice (both P values < 0.05 , *t*-test with Welch's correction).

To determine whether the reduced follicular development resulted in, or was associated with, altered ovarian cyclicity, we staged mice by daily vaginal smearing (Fig. 5A). Figure 5B shows that the TG mice had a 23% increase in ovulatory cycle length (6.0 ± 0.25 days, $n = 6$) compared with WT littermates (4.85 ± 0.26 days, $n = 7$, $P = 0.022$, Mann-Whitney *U*-test). Closer examination showed that the increase in the length of the estrous cycle was due to extension of estrus (Fig. 5C) from 1.6 ± 0.24 to 2.5 ± 0.19 days ($P < 0.05$, Mann-Whitney *U*-test).

Discussion

Follicular development in the ovary has been shown to be dependent on angiogenesis, in particular on blood vessel growth driven by the angiogenic isoforms of VEGF. VEGF₁₆₅ is upregulated in the follicle during development (Ravindranath *et al.* 1992, Shweiki *et al.* 1993, Taylor & Mueller 2004) and inhibition of VEGF by administration of VEGF-TRAP (Wulff *et al.* 2002), antibodies to VEGF (Zimmermann *et al.* 2001) and VEGFR tyrosine kinase inhibitors (McFee *et al.* 2009) results in inhibition of follicular development. However, all these inhibitors target both the pro-angiogenic and the anti-angiogenic isoforms of VEGF (Varey *et al.* 2008). VEGF_{165b} has been shown to be anti-angiogenic in both pathological circumstances, such as ischemic retinopathy (Konopatskaya *et al.* 2006), models of age-related macular degeneration (Hua *et al.* 2010), prostate (Rennel *et al.* 2008a), lung (Merdzhanova *et al.* 2010), renal (Rennel *et al.* 2008a), skin (Pritchard-Jones *et al.* 2007) and colon (Varey *et al.* 2008) cancers, and in physiological angiogenesis including gonadogenesis (Artac *et al.* 2009) and mammary gland formation (Qiu *et al.* 2008). The results shown here indicate that the regulation of follicular development and hence fertility are under control of differential splicing of VEGF. We show that VEGF_{165b} is localised within different cell types in the marmoset ovary and levels decrease in ovaries containing corpora lutea. Crucially, the over-expression of VEGF_{165b} in the mouse ovary, driven by the MMTV promoter, demonstrated a functional role for this isoform, causing delayed follicular and corpus luteal development that contributed to a fertility defect in these mice. The MMTV promoter drives expression in tissues

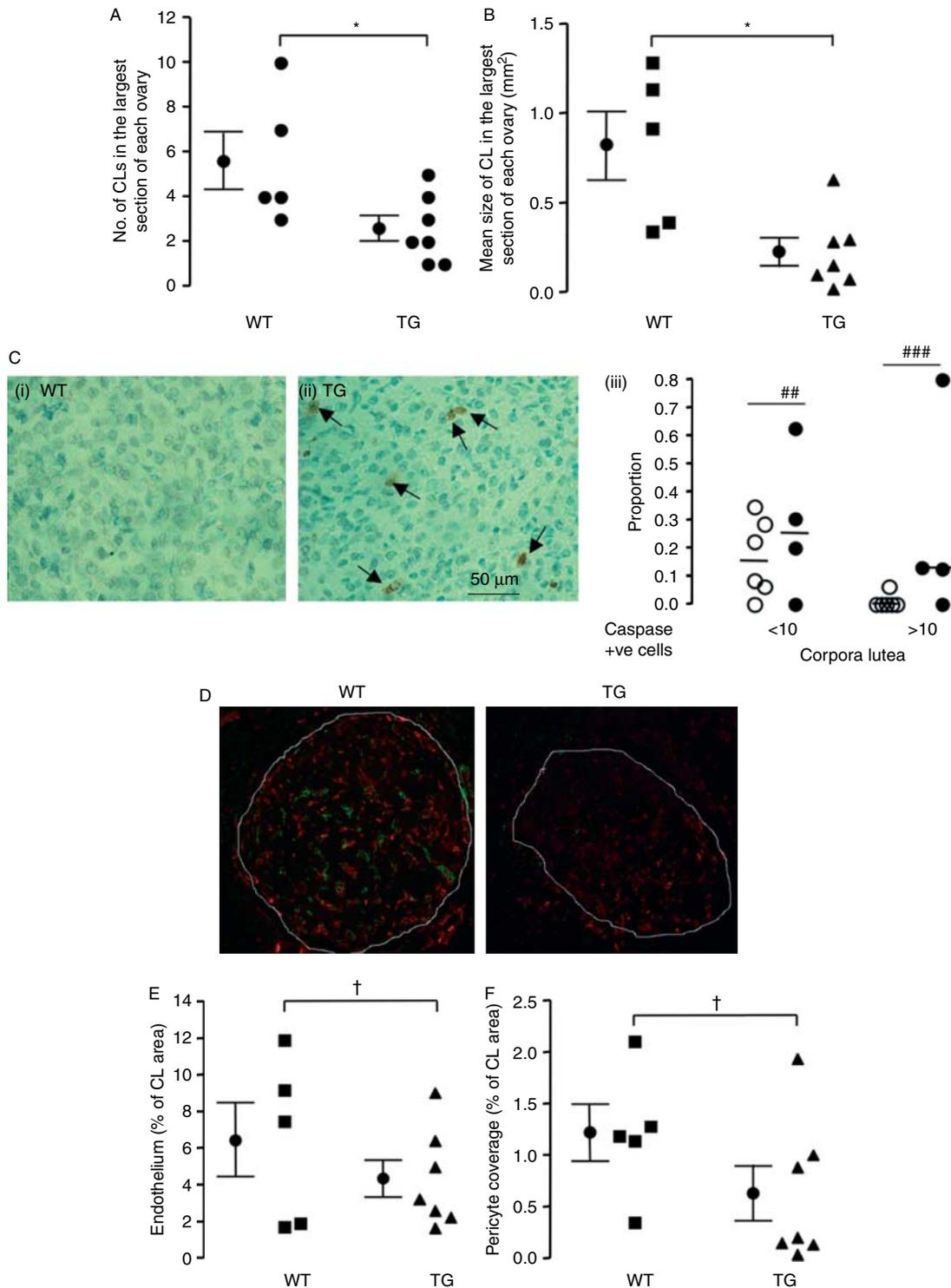


Figure 4 Over-expression of VEGF_{165b} in the mouse ovary results in changes in the corpus luteum (CL). Ovaries collected from mice at 0.5 dpc were fixed and stained by H&E to investigate CL structure. (A) The number of CL in the largest cross section of each ovary was counted. **P* < 0.05, *t*-test. (B) The total size of the CL in the largest cross section of each ovary was measured with ImageJ. **P* < 0.05, *t*-test. (C) Ovaries stained for cleaved caspase-3 were analysed for corpus luteum staining in WT (i) and TG (ii). Numbers of CL were counted (iii) according to the proportion with 1–10 (early regression) and > 10 cleaved caspase-3-positive cells (late regression; ##*P* < 0.01, ###*P* < 0.001, Fisher's exact test on CL frequency). *n* = 6 mice per group, 90 CL in WT, 45 in TG. (D) Ovaries collected from mice at 0.5 dpc were fixed and stained for microvessels using isolectin B4 (red) and pericytes with anti-NG2 antibody (green). (E) Blood vessel density was counted and expressed as microvascular density (microvessel covering area per mm² of CL). (F) Pericytes covering area per unit area of CL were counted, †*P* < 0.05, *t*-test with Welch's correction.

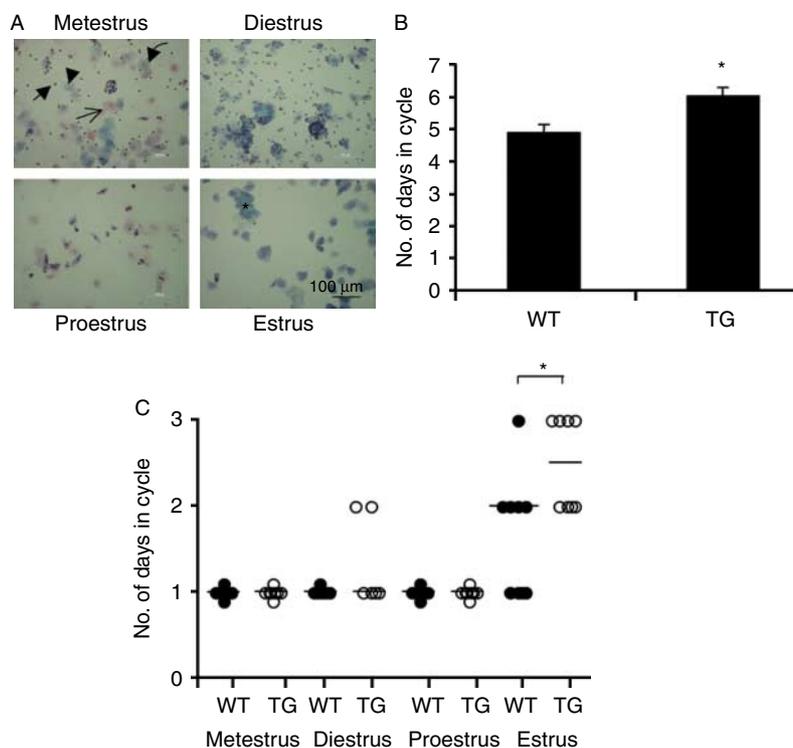


Figure 5 VEGF_{165b} over-expression alters estrous cycling. Estrus cycle was determined in MMTV-VEGF_{165b} transgenic mice, expressing VEGF_{165b} in the ovary, and littermate sisters, by composition of cells in vaginal smears. Arrow, neutrophils; arrow head, parabasal cells; curved arrow, intermediate cells and *cornified superficial cells. (B) The TG mice had a significantly longer cycle time than the littermate controls. (C) Determination of the lengths of the cycles of each mice showed that estrus was longer by a day in the TG compared with WT mice. * $P < 0.05$, Mann-Whitney U -test.

in response to glucocorticoid hormones, and specifically progesterone (Otten *et al.* 1988). It is therefore expressed in a variety of tissues during pregnancy and the ovarian cycle, but the strongest expression is in the mammary gland and the ovary in healthy mice (Wang & Greenwald 1993, Wagner *et al.* 2001).

From the results described above it appears that VEGF-mediated angiogenesis, inhibited by VEGF_{165b} expression, is important for multiple components of the ovulatory cycle. However, we did not discern an alteration in the total number of follicles in these mice, indicating that any effect of VEGF_{165b} over-expression on oogenesis was not detectable. It is the maturation of the follicle from primary to secondary and to mature follicles that was inhibited by the anti-angiogenic VEGF_{165b} isoform over-expression, as it is by VEGF inhibition (Zimmermann *et al.* 2003). Importantly, we show here that the quality of the progression is also inhibited, as the relationships between the cumulus cells and the ovum appear to be disrupted, resulting in release of an ovum lacking the cumulus cell-oocyte complex. The percent reduction in embryos in the oviduct was not as great as the reduction in pup number, suggesting that there was a reduction in the ability of the embryos to implant in the uterus, in addition to the reduction in embryos released. However, culture of the embryos did not show any evidence of developmental defects in the embryos themselves, which together with the normal birth weight indicate that the reduction in litter size is dependent on ovarian function.

The increase in the length of the estrous cycle, due to an increase in the length of estrus, could be a result of, or lead

to, the delay in follicular development and defective CL formation as has been shown when VEGFA is inhibited (Fraser & Duncan 2009). Normally, the breakdown of the basement membrane in the mature follicle results from intense invasive angiogenesis across the theca interna that is stimulated by an increase in VEGF₁₆₅ expression by the granulosa cells (Shweiki *et al.* 1993). The over-expression of VEGF_{165b} in the granulosa cells driven by the MMTV promoter appears to be able to delay this vessel growth with resulting reduction in the size of the CL. This is likely to affect steroid production by the CL (Fraser *et al.* 2000), but the impact of VEGF_{165b} on progesterone secretion has yet to be determined.

Generation of the different variants of VEGF results from a process of alternative splicing that is tightly regulated in normal conditions. Splice site selection is normally under the control of a co-ordinated and regulated set of splice factors, including SRSF6 (SRp55; Nowak *et al.* 2008), ASF/SF2 (Nowak *et al.* 2010) and SC35 (Merdzhanova *et al.* 2010), which themselves are regulated by kinases such as SRPK1 (Nowak *et al.* 2010). Very little is known about the regulation of splice factors in the ovary, but the results of the present study add to findings of other mRNAs that are alternatively spliced in the ovary (e.g. LH receptor, Mandai *et al.* 1997; FGFR2, Parrott & Skinner 1998; FSHR, Kraaij *et al.* 1998 and oestrogen receptor, Chu *et al.* 2000) to suggest that regulation of ovarian function may be controlled not only by regulation of gene transcription, but also by regulation of alternative splicing.

In conclusion, we show that alternative splicing of VEGF occurs in the primate ovary, and can regulate ovarian

function and fertility in mice. These results raise the intriguing possibility that, in humans, the balance of pro- vs anti-angiogenic isoforms may regulate follicular development, menstrual cycle length, and ultimately, fertility.

Materials and Methods

Animal maintenance: mice

The TG MMTV-VEGF_{165b} (TG) line was generated on C57BL6×CBA/CA background and back-crossed with C57BL6 mice as described (Qiu *et al.* 2008). All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986, and associated guidelines and with approval of the University of Bristol Ethical Review Panel. These animals have three copies of the MMTV-VEGF_{165b} construct (Qiu *et al.* 2008). For ovary experiments, 2- to 4-month-old F₅₋₇ generation female TG mice and WT littermate controls were used. For experiments in the oviduct and ovary, TG or WT females were mated with WT male mice. Once plugged, female mice were culled and oviducts and ovary were dissected.

Marmoset tissue

Immunohistochemical localisation of VEGF_{165b} was carried out on ovaries obtained from normal adult marmoset monkeys (*Callithrix jacchus*), which had been fixed in neutral buffered formalin and stored in paraffin blocks, having been generated from experiments described previously (Duncan *et al.* 2008). Two pairs of ovaries from the mid-follicular and mid-luteal phase of the ovulatory cycle were used. For measurement of levels of VEGF and VEGF_{xxx}b within the marmoset ovary, ovaries were obtained from adult marmosets being killed as a result of health problems not related to the reproductive system. Four marmosets were sedated and killed as described previously (Wulff *et al.* 2002). Their ovaries were removed, weighed and rapidly frozen in dry ice before being stored at -70 °C until required. Four of the ovaries contained follicles, while four consisted predominantly luteal tissue.

RT-PCR

Briefly, total RNA was isolated with TRIzol (Invitrogen) extraction and DNase I (Invitrogen) was digested as per the manufacturer's instructions to prevent gDNA contamination. DNase-treated RNA (1 µg) was reverse transcribed into cDNA with avian myeloblastosis virus (AMV) reverse transcriptase using method described by the manufacturer (Promega). Both cDNA and RNA treated with DNase I were subjected to PCR with forward primer 5'-ACA AGA TCC GCA GAC GTG TA-3' and reverse primer 5'-ACA GAT GGC TGG CAA CTA GA-3' for transgene detection. PCR amplification was initiated at 94 °C for 4 min, 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s, followed by final extension at 72 °C for 10 min. A band at 199 bp indicated VEGF_{165b} transgene expression.

For mouse β-actin control, forward primer 5'-AGC CAT GTA CGT AGC CAT CC-3' and reverse primer 5'-CTC TCA GCT GTG GTG GTG AA-3' give a 228 bp PCR product, after amplification under the same cycle conditions as for transgene.

ELISA of VEGF_{xxx}b

Tissue protein lysate was prepared from seven marmoset ovaries in RIPA buffer. Protein concentration was determined by Bio-Rad assay (Bio-Rad) and the amount of VEGF_{165b} was determined by ELISA as previously described (Varey *et al.* 2008) with a specific detection antibody against VEGF_{xxx}b isoforms with the operator masked to luteal content.

Briefly, 0.08 µg goat anti-VEGF polyclonal IgG (AF293-NA; R&D Systems, Abingdon, UK) diluted in 1×PBS (pH 7.4) was adsorbed onto each well of a 96-well plate (Immulon 2HB; Thermo Life Sciences, Basingstoke, UK) overnight at room temperature. The plate was washed three times between each step with 1×PBS-Tween (0.05%). After blocking with 100 µl of 5% BSA in PBS for 1 h at 37 °C, 100 µl recombinant human VEGF_{165b} (R&D Systems) diluted in 1% BSA in PBS (ranging from 62.5 pg/ml to 4 ng/ml) or protein samples was added to each well. After incubation for 1 h at 37 °C with shaking and three washes, 100 µl mouse monoclonal anti-VEGF_{xxx}b biotinylated IgG (clone 264610/1; R&D Systems) at 0.4 µg/ml was added to each well, and the plate was left for 1 h at 37 °C with shaking. Streptavidin-HRP (100 µl; R&D Systems) at 1:200 dilution in 1% BSA in PBS was added, the plate was left at room temperature for 20 min and 100 µl/well O-phenylenediamine dihydrochloride solution (substrate reagent pack DY-999; R&D Systems) was added, protected from light and incubated for 20 min at room temperature. The reaction was stopped with 50 µl/well 1 M H₂SO₄, and absorbance was read immediately in the Ophys MR 96 well plate reader (Dynex Technologies, Chantilly, VA, USA) at 492 nm, with control reading at 460 nm.

ELISA of pan-VEGF

Pan-VEGF detection was followed with the manufacturer's suggestion (Duoset human VEGF from R&D, cat. no. DY293). Capture antibody (0.08 µg) diluted in 1×PBS (pH 7.4) was adsorbed onto each well of the 96-well plate (Immulon 2HB; Thermo Life Sciences) overnight at room temperature. The plate was washed three times between each step with 1×PBS-Tween (0.05%). After blocking with 100 µl of 1% BSA in PBS for 1 h at 37 °C, 100 µl recombinant human VEGF₁₆₅ diluted in 1% BSA in PBS (ranging from 62.5 pg/ml to 4 ng/ml) or protein samples was added to each well. After incubation for 1 h at 37 °C with shaking and three washes, 100 µl detection antibody at 0.05 µg/ml was added to each well, and the plate was left for 1 h at 37 °C with shaking. Streptavidin-HRP (100 µl; R&D Systems) at 1:200 dilution in 1% BSA in PBS was added, the plate was left at room temperature for 20 min and 100 µl/well O-phenylenediamine dihydrochloride solution (substrate reagent pack DY-999; R&D Systems) was added, protected from light and incubated for 20 min at room temperature. The reaction was stopped with 50 µl/well 1 M H₂SO₄, and absorbance was read immediately in the Ophys MR 96 well plate reader at 492 nm, with control reading at 460 nm.

H&E staining and follicle counting

Mouse ovaries were fixed in Bouin solution and embedded in paraffin wax, and 5 µm sections were subjected to H&E staining

using standard procedure. Every fifth section for each ovary was selected for follicle counting, and for atretic follicle number, sections were sampled. Primary, secondary and tertiary follicles were categorised by using a follicle classification developed by Pedersen & Peters (1968) and Myers *et al.* (2004).

Immunohistochemistry

Ovary samples from WT, TG mice were fixed with Bouin solution, while marmoset ovaries were fixed in neutral buffered formalin, and embedded in paraffin. Sections (5 µm) were mounted onto gelatin/poly-L-lysine-coated glass slides. The sections were dried onto the slides in a 37 °C incubator overnight. Sections were dewaxed in xylene for 15 min and rehydrated through graded ethanol solutions (100, 90 and 70%, v/v). Microwave antigen retrieval was performed in 0.01 M sodium citric buffer (pH 6.0) for 7 min at 95 °C at 800 W followed by 9 min at 120 W. Sections were cooled to room temperature before being washed twice in deionised water for 5 min each time. Sections were incubated with freshly prepared 3% (v/v) hydrogen peroxide (BDH, Poole, UK) diluted in 1×PBS for 5 min, then washed twice for 5 min with 1×PBS and blocked with 5% (w/v) BSA (Sigma) followed by 1.5% (w/v) normal goat serum (Vector Laboratories, Peterborough, UK) in 5% (w/v) BSA for 30 min. The sections were washed twice with 0.05% (v/v) PBS–Tween at room temperature for 5 min, then incubated with the primary antibody diluted in 1.5% w/v normal goat serum in 1×PBS. A polyclonal rabbit VEGF antibody (A20 sc152; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used. Tissue sections were treated with a matched concentration of normal, affinity-purified rabbit IgG (Sigma), used as a negative control. The sections were washed twice in 0.05% (v/v) PBS–Tween, for 5 min each time. The blocking step was repeated as before, followed by two 5-min washes in 0.05% (v/v) PBS–Tween. All sections, including the controls, were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted in 1.5% (w/v) normal goat serum for 1 h in a humid chamber at room temperature. Sections were washed twice with 0.05% (v/v) PBS–Tween, 5 min/wash, then incubated with a pre-prepared avidin–biotinylated enzyme complete kit (Vector Laboratories) for 45 min in a humid chamber at room temperature. Again, the sections were washed twice with 0.05% (v/v) PBS–Tween, 5 min each time, followed by incubation with 3,3'-diaminobenzidine substrate (Vector Laboratories) to yield a brown-coloured product. The reaction was stopped by washing twice with deionised water for 5 min. Sections were counterstained with Mayer's haematoxylin (BDH) for 5 min, then differentiated in water. Sections were dehydrated by passing through increasing concentrations of ethanol (70, 90 and 100%, v/v) for at least 2 min each, cleared in xylene for at least 10 min, and permanently mounted in DPX Mountant for histology. Staining was examined with a Nikon Eclipse E-400 microscope; images were captured using a DCN-100 digital imaging system (Nikon Instruments).

For staining of microvessels, 5 µm ovary sections were dried thoroughly on SuperFrost slides (Fisher Scientific, Loughborough, UK). Non-specific binding was blocked with 5% normal goat serum in 0.5% PBS–Triton 100 for 1 h and avidin/biotin blocking reagent (Vector Laboratories) was applied for 15 min each on sections. Vasculature staining was performed with

incubation with 5 µg/ml biotinylated isolectin B4 (Life Technologies, Paisley, UK) at 4 °C overnight and washing in 0.5% PBS–Triton 100 four times each for 5 min followed by 1 h incubation with 2 µg/ml AlexaFluor 555-conjugated streptavidin (Vector Laboratories). After washing, sections were counterstained with 5 µg/ml Hoechst for 30 min and coverslipped with Vectashield (Vector Laboratories). Images were analysed with Leica confocal microscope using a ×40 oil objective. Microvessels stained in red were selected in Photoshop and MVD (microvessel covering area per unit perimeter of the follicle or microvascular coverage area/total area % for corpus lutea) was calculated in ImageJ.

For pericyte staining, antigen retrieval was performed in 0.01 M sodium citrate buffer (pH 6.0), for 7 min at 95 °C at 800 W followed by 9 min at 120 W. Avidin/biotin blocking reagent (Vector Laboratories) was applied for 15 min each on sections and non-specific binding was blocked with 1% BSA, 2% normal horse serum in 0.05% PBS–Tween 20 (pH 7.4) for 1 h. Pericyte staining was performed with incubation with 1:200 anti-NG2 antibody in blocking solution (Molecular Probe) at 4 °C overnight and washing in 0.05% PBS–Tween 20 four times each for 5 min followed by 2 h incubation with 2 µg/ml AlexaFluor 488-conjugated goat anti-rabbit IgG (Vector Laboratories) in blocking solution. After washing, sections were counterstained with 5 µg/ml Hoechst for 30 min and coverslipped with Vectashield (Vector Laboratories). Images were analysed with Leica confocal microscope using a ×40 oil objective. Pericytes stained in green were selected in Photoshop and pericyte density (pericyte coverage area/total area %) was calculated in ImageJ (Abramoff *et al.* 2004).

For cleaved caspase-3 staining, antigen retrieval was performed by bringing slides to a boil in 10 mM sodium citrate buffer (pH 6.0) followed by maintaining at a sub-boiling temperature for 10 min. Sections were incubated with freshly prepared 3% (v/v) hydrogen peroxide in 1×PBS for 5 min. Non-specific blocking was achieved by incubation of sections for 1 h with 5% normal goat serum diluted in TBS–0.1% Tween 20 and with avidin D and biotin solution each for 15 min. A polyclonal rabbit cleaved caspase-3 antibody (1:400; (New England Biolabs, Hitchin, UK)) diluted in blocking solution was applied on sections and incubated overnight at 4 °C. Biotinylated goat anti-rabbit IgG diluted in blocking solution was then applied for 30 min in a humid chamber at room temperature. After washing, sections were incubated with a pre-prepared avidin–biotinylated enzyme complete kit followed by incubation with 3,3'-diaminobenzidine substrate (Vector Laboratories) to yield a brown-coloured product. The reaction was stopped by washing with deionised water for 5 min. Sections were counterstained with Mayer's haematoxylin (BDH) for 2 min and then differentiated in water. Sections were dehydrated by passing through increasing concentrations of ethanol (70, 90 and 100%, v/v) for at least 2 min each, cleared in xylene for at least 10 min and permanently mounted in DPX Mountant for histology. Staining was examined with a Nikon Eclipse E-400 microscope; images were captured using a DCN-100 digital imaging system (Nikon Instruments).

Collection of embryos

Both oviducts from each female mouse at 0.5 dpc were dissected and transferred into M2 medium in Petri dish.

The ampulla was torn with fine forceps and embryos were gently squeezed out. Embryos were collected with a mouth pipette and released into M2 medium with hyaluronidase (10 mg/ml). The number of embryos was counted. Embryos were washed four times in M2 medium and transferred into well-equilibrated M16 medium in an incubator. Cells were maintained in M16 medium in the incubator for 4 days to blastocyst stage, checked each day and the number of alive and dead cells was recorded.

Cycle determination

Ten microlitre of 0.9% NaCl was injected into a female mouse vagina with a pipette. After pipetting, the solutions were collected, pipetted onto a clean slide and pulled to form a smear. After drying at room temperature, a smear was stained with 10% Giemsa for 5 min, rinsed with tap water, and dried and viewed under a light microscope. Female cyclic stage was determined, as shown in Fig. 5. Cycle determinations were repeated every day for at least two cycles and the number of days for each cycle was calculated. Four mice were excluded (two WT and two TG) as cycle time in diestrus was >3 days, indicating a missed cycle.

Statistical analysis

Figures are given as mean \pm S.E.M. unless stated; $P < 0.05$ was regarded as significant. Methods of statistical analysis are included in relevant figure legend. For single comparisons t -tests were used with Welch's correction where variances were significantly different, and data normally distributed, or paired t -test when samples were matched to littermates. Integral data (e.g. number of embryos), or non-normally distributed data (determined by Kolmogorov–Smirnov test) were tested by non-parametric statistics (Wilcoxon paired or Mann–Whitney unpaired U -test). For comparison purposes, VEGF expression, litter size, number of embryos, follicle number and proportions, vessel densities and luteal sizes, and days in cycles were considered as independent variables. The experimental units were numbers of mice in TG or WT groups. Frequencies were compared by a χ^2 -test (Fisher's exact test for 2×2 tables). Power analysis for non-significant values was carried out with G.Power using 80% power as a minimum cut-off.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-11-0091>.

Declaration of interest

D O Bates and S J Harper are inventors on the VEGF_{165b} patent.

Funding

This work was funded by a BHF grant (PG/08/054/25272) to D O Bates and LFD and BS/06/005 (to D O Bates), and Wellcome Trust grant 69029 (to D O Bates and S J Harper). H M Fraser was funded by a core grant (U.1276.00.002.00003.01) to the Medical Research Council Reproductive Sciences Unit.

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Received 23 March 2011

First decision 5 May 2011

Revised manuscript received 7 December 2011

Accepted 9 January 2012