Focus on Vascular Function in Female Reproduction

Angiogenesis and vascular function in the ovary

R S Robinson¹, K J Woad², A J Hammond², M Laird¹, M G Hunter² and G E Mann²

¹*School of Veterinary Medicine and Science and* ²*Division of Animal Sciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, LE12 5RD, UK*

Correspondence should be addressed to R S Robinson; Email: bob.robinson@nottingham.ac.uk

Abstract

Ovarian function is dependent on the establishment and continual remodelling of a complex vascular system. This enables the follicle and/or corpus luteum (CL) to receive the required supply of nutrients, oxygen and hormonal support as well as facilitating the release of steroids. Moreover, the inhibition of angiogenesis results in the attenuation of follicular growth, disruption of ovulation and drastic effects on the development and function of the CL. It appears that the production and action of vascular endothelial growth factor A (VEGFA) is necessary at all these stages of development. However, the expression of fibroblast growth factor 2 (FGF2) in the cow is more dynamic than that of VEGFA with a dramatic upregulation during the follicular–luteal transition. This upregulation is then likely to initiate intense angiogenesis in the presence of high VEGFA levels. Recently, we have developed a novel ovarian physiological angiogenesis culture system in which highly organised and intricate endothelial cell networks are formed. This system will enable us to elucidate the complex inter-play between FGF2 and VEGFA as well as other angiogenic factors in the regulation of luteal angiogenesis. Furthermore, recent evidence indicates that pericytes might play an active role in driving angiogenesis and highlights the importance of pericyte–endothelial interactions in this process. Finally, the targeted promotion of angiogenesis may lead to the development of novel strategies to alleviate luteal inadequacy and infertility.

Reproduction (2009) **138** 869–881

Introduction

Angiogenesis, the formation of new blood vessels from existing ones, involves a complex series of cellular processes and molecular changes. In adults, it is largely limited to pathological situations such as tumour growth and wound healing. However, the ovary undergoes continual cyclical changes and so requires continual angiogenesis (Reynolds & Redmer 1999, Fraser & Lunn 2001). An established vasculature consists of an inner lining of endothelial cells, associated mural cells such as pericytes and vascular smooth muscle cells (vSMC). These vessels remain quiescent until there is an angiogenic stimulus such as hypoxia or wounding, which then upregulates proangiogenic factors, such as vascular endothelial growth factor A (VEGFA; Gerhardt & Betsholtz 2003). After this stimulus, the existing vessels start to destabilise through the disruption of endothelial and mural cellular contacts. At the same time, numerous proteases are activated and the extracellular matrix (ECM) is degraded. Endothelial cells, then, migrate towards the angiogenic stimuli and

proliferate under the influence of proangiogenic factors. Once connected and aligned, the endothelial cells form a lumen and the newly formed vessel is then stabilised by the recruitment of pericytes (Gerhardt & Betsholtz 2003). Thus, angiogenesis is a highly regulated process involving a balance between a plethora of pro- and anti-angiogenic factors.

Key angiogenic regulators

The principal proangiogenic factors include fibroblast growth factor 2 (FGF2), VEGFA, platelet-derived growth factor (PDGF) family and the angiopoietin (ANGPT) system. They have many overlapping functions but there are some important differences. These factors and associated properties are summarised in Table 1. Blockade of VEGFA/PDGF signalling has highlighted the critical roles that these factors play in controlling not only angiogenesis but also ovarian function. For example, inhibition of VEGFA signalling by various methods disrupted ovulation, completely blocked the vascularisation of the subsequent corpus luteum (CL) and prevented the post-ovulatory rise in progesterone (Fraser & Lunn 2001). Conversely, much less is generally known about the anti-angiogenic factors. They generally associate with the ECM and suppress

This paper is one of four papers that form part of a special Focus Issue section on Vascular Function in Female Reproduction. The Guest Editor for this section was H N Jabbour, Edinburgh, UK.

Table 1 The principal angiogenic factors and their associated properties.

Ligand	Isoforms	Biochemical properties	Receptor/cellular target	Functions	References
<i>Pro-angiogenic growth factors</i>					
VEGFA	VEGFA ₁₂₁ VEGFA ₁₄₅ VEGFA ₁₆₅ VEGFA ₁₈₉ VEGFA ₂₀₆	Binds to heparin and ECM (except VEGFA ₁₂₁) VEGFA ₁₂₁ , ₁₄₅ and ₁₆₅ are secreted and soluble while VEGFA ₁₈₉ , ₂₀₆ are cell associated	VEGFR1 (FLT) (signalling capability is unresolved) VEGFR2 (KDR) (tyrosine kinase activity)	Stimulates endothelial proliferation, migration and tube formation Vascular permeability factor	Ferrara <i>et al.</i> (2003)
FGF2	18 kDa (cytoplasmic) 22–34 kDa (nuclear)	Heparin binding	FGFR1–4 (tyrosine kinase activity)	Stimulates endothelial proliferation and migration Mitogen for fibroblasts Cell differentiation	Presta <i>et al.</i> (2005)
PDGF	PDGFA PDGFB PDGFC PDGFD	Dimerisation required for activity PDGFAA, BB (homodimers) PDGFAB (heterodimer)	PDGFRA (PDGFAA, AB, BB) PDGFRB (PDGFBB) Both tyrosine kinase receptors	Activation of PDGFRB by PDGFBB stimulates recruitment of pericytes	Fredriksson <i>et al.</i> (2004)
ANGPT	ANGPT1 ANGPT2		Tie2 (tyrosine kinase receptor) ANGPT1 activates Tie2 ANGPT2 is an endogenous antagonist	ANGPT1 stimulates vessel maturation while ANGPT2 destabilises endothelial–pericyte contacts	Maisonpierre <i>et al.</i> (1997)
<i>Anti-angiogenic factors</i>					
Thrombospondin	TSP1 and TSP2	High molecular weight glycoprotein Heparin binding ECM associated	CD36 Integrin associated protein (IAP, CD47)	TSP1: induces endothelial apoptosis and destabilises endothelial cell contacts TSP2: inhibits endothelial cell migration	Armstrong & Bornstein (2003)
Angiostatin	–	Produced by cleavage of plasminogen by protease Contain kringle domains	α v β 3 integrin	Inhibits endothelial cell proliferation and migration	Wahl <i>et al.</i> (2005)

angiogenesis by inhibiting endothelial migration or stimulating apoptosis in endothelial cells (Armstrong & Bornstein 2003).

This review will focus on angiogenesis and its regulation during the key stages of the follicular–luteal timeline in farm animals, but also incorporates data from other species where appropriate. This timeline includes the recruitment of the theca layer, antral follicle development and dominance, ovulation and subsequent luteal development.

Initial recruitment of thecal vasculature

Both primordial and primary follicles receive sufficient nutrients and oxygen by passive diffusion from stromal blood vessels. However, the formation of an individual capillary network around each follicle is required for follicles to grow beyond these stages. This network is initially thin, roughly structured and has a single layer. It is confined to the theca layer with the granulosa layer remaining avascular throughout folliculogenesis (Tamanini & De Ambrogi 2004).

There is remarkably little information regarding how the follicle initially recruits its vascular network. The likely candidate is VEGFA, which is first detected in the granulosa and theca layers of secondary follicles in cows (Yang & Fortune 2007) while ANGPT and FGF2 do not appear in these cells until the antral stages (van Wezel *et al.* 1995, Hayashi *et al.* 2004). Furthermore, administration of VEGFA stimulated the development of secondary follicles in cows (Yang & Fortune 2007). While VEGF trap administration in primates reduced the endothelial cell area of secondary follicles and inhibited the formation of antral follicles (Wulff *et al.* 2002).

However, it is unclear what stimulates VEGFA expression because hypoxia-induced factor 1 α (HIF1A) (a transcription factor induced by hypoxia and a potent inducer of VEGFA) was absent from pre-antral follicles (Duncan *et al.* 2008). It is also unlikely to be gonadotrophins because pre-antral follicle growth is gonadotrophin independent. It could alternatively be an oocyte-derived factor. Both PDGF and FGF2 are present in the oocyte of primordial and primary

follicles (van Wezel *et al.* 1995, Nilsson *et al.* 2006). Additionally, both factors promote the primordial to primary transition, pre-antral follicular growth and recruitment of theca cells (Nilsson *et al.* 2006, Matos *et al.* 2007). However, their effects on theca vascularity are currently unknown.

Pre-antral follicular vasculature

There is a large increase in not only the total vasculature but also the vascular density during pre-antral follicular growth in pigs. Not surprisingly, ~40% of all proliferating cells in the theca are of endothelial origin (Martelli *et al.* 2009). There were parallel increases in the expression of VEGFA mRNA in both granulosa and theca. Intriguingly, during early pre-antral follicle growth, there was a positive correlation between the degree of proliferation and vascular area (Martelli *et al.* 2009). From this, it is tempting to speculate that pre-antral follicular selection is based on vascular supply.

Antral follicle and dominance

By the antral follicle stage, the vascular sheath consists of two concentric capillary networks, with one layer directly below the basement membrane and the other in the theca externa (Martelli *et al.* 2006). The use of vascular corrosion casts have suggested that the nature of angiogenesis in the theca layer changes during follicular development. Initially, there is budding which is followed by pre-dominantly sprouting during early antral follicle stages and then capillary elongation in the later stages (Jiang *et al.* 2003).

Neovascularisation is crucial for antral follicle growth, dominance and pre-ovulatory development since numerous studies have shown that anti-angiogenic compounds (e.g. VEGFA trap) reduced the thecal vascularity and consequently severely comprised follicular development (Wulff *et al.* 2002, Fraser & Duncan 2009). However, whether dominance is achieved by a follicle having a more extensive vasculature and thus receiving greater hormonal support (Zelevnik *et al.* 1981) remains to be elucidated. This hypothesis is supported by the observation that, during dominant follicle selection, those follicles that were oestrogen-active had vastly greater vascularisation and VEGFA concentrations than their oestrogen-inactive counterparts. This was despite the oestrogen-inactive follicle being larger in diameter (Grazul-Bilska *et al.* 2007). There is also strong evidence that, shortly after selection, there is a rapid degeneration of the thecal vasculature, once atresia has been initiated in the subordinate follicles (Jiang *et al.* 2003, Macchiarelli *et al.* 2006). However, any vascularisation differences are likely to be subtle and its temporal aspect makes it very difficult to prove the original hypothesis definitively. The recent advances in measuring ovarian blood flow

have begun to shed more light on this issue. In mares, the follicles that became dominant had an increased blood flow prior to deviation when compared to their subsequent subordinates (Acosta *et al.* 2004). While a similar study in the cow was less conclusive, there was a rapid reduction in blood flow in subordinate follicles after deviation (Acosta *et al.* 2005). However, these technologies will enable us to increase our understanding of the regulation of follicular blood flow. It is possible that this will lead to the development of strategies to promote follicular function by manipulating blood flow.

Regulation of follicular angiogenesis

The regulation of follicular angiogenesis and in particular the crucial role of VEGFA has been extensively and expertly reviewed (Fraser & Duncan 2009). In this review, we will only focus on a few of these aspects. Since the nature of angiogenesis changes as the follicle grows (Jiang *et al.* 2003), it is highly likely that additional influencing factors are involved in controlling all these different processes. For example, there are five VEGFA isoforms, which have varying biochemical and biological properties (Ferrara *et al.* 2003). It is possible that these isoforms are differentially expressed during folliculogenesis. This is supported by observations that VEGFA₁₆₄ mRNA expression in bovine granulosa cells was upregulated by oestradiol (E₂) but downregulated by progesterone, while these steroids had the opposite effect on VEGFA₁₂₀ mRNA (Shimizu & Miyamoto 2007). It is equally possible that the VEGF co-receptors, neuropilin-1 and -2, might influence the effects of VEGFA. While these receptors have no signal transduction, they enhance the binding efficiency of VEGFA to VEGFR2. Interestingly, VEGFA₁₂₀ is unable to bind to neuropilin-1 and this might explain its lower mitogenic activity (Karamysheva 2008). There is limited information on neuropilins in the ovary, but Shimizu *et al.* (2006) showed that neuropilin-1 mRNA was present in granulosa and theca cells, while neuropilin-2 mRNA was only expressed in the theca layer. Moreover, neuropilin-1 was regulated by progesterone and E₂ in bovine granulosa cells (Shimizu *et al.* 2006). It is also intriguing that VEGFA is principally located in the avascular granulosa cells (Berisha *et al.* 2000a, Greenaway *et al.* 2005). This is likely to create an angiogenic gradient to stimulate the vascularisation towards the basement membrane thereby maximising the supply of oxygen, nutrients and hormones to the granulosa cells.

The effects of VEGFA will be further modulated by the co-ordinated action with other angiogenic factors (e.g. ANGPT, PDGF and FGF2). Recently, Greenberg *et al.* (2008) demonstrated that VEGFA's action was markedly modulated if FGF2 and/or PDGF were present. It has been shown that FGF2 is present in the theca interna

layer of antral follicles and in the granulosa, albeit at lower levels. Additionally, FGF2 concentrations increased during the final stages of follicular maturation in cows (van Wezel *et al.* 1995, Berisha *et al.* 2000a) and were increased by eCG in gilts (Shimizu *et al.* 2002). However, no studies have investigated the effects of inhibiting FGF2 at any stage of follicular development and much remains to be elucidated about its role in regulating follicular angiogenesis. While the majority of the studies have focused on pro-angiogenic factors, one anti-angiogenic factor, namely, thrombospondin (TSP) has received some attention. Greenaway *et al.* (2005) found that TSP1 and its receptor CD36 were present at maximal levels in small antral follicles in the cow. Thereafter, TSP1 concentrations decreased as the antral follicles developed, but were found to be upregulated during atresia in the marmoset (Thomas *et al.* 2008). Thus, the upregulation of TSP1 might play a key role in follicular atresia by inhibiting angiogenesis. Intriguingly, TSP1 expression was increased by LH in the rat granulosa cells and were present in the early CL (Petrik *et al.* 2002). This indicates that there is still much to learn about the role of TSP in controlling ovarian angiogenesis.

Follicle–luteal transition: a period of intense angiogenesis

The transition from pre-ovulatory follicle to CL is a dynamic process involving a series of biochemical and morphological changes following the LH surge that includes angiogenesis (Reynolds & Redmer 1999). Indeed, the rates of luteal growth and angiogenesis are such that they are only rivalled by the fastest growing tumours and in the mature CL the majority of luteal cells are adjacent to one or more capillaries (Reynolds & Redmer 1999). While luteal and follicular angiogenesis are likely to be broadly similar, there are several important differences. Firstly, following ovulation, the breakdown of the basement membrane enables endothelial cells and pericytes to invade and vascularise the

luteinising granulosa cells. Secondly, the timescale of luteal development is much shorter and thus angiogenesis is more intense. Indeed, it has been estimated that up to 85% of the proliferating cells in the developing CL are of vascular origin (Reynolds & Redmer 1999). Finally, the process in ruminants is likely to involve extensive vascular remodelling since there is extensive intermingling of all cell types (Stocco *et al.* 2007). These processes are under the influence of numerous growth factors and the temporal regulation of the key factors during this period is shown in Fig. 1.

Control of luteal vascularisation

Pre-ovulatory follicle

It is likely that the degree of luteal vascularisation is, at least in part, programmed within the pre-ovulatory follicle. Firstly, the follicular vascular bed provides the basis on which the luteal vasculature is formed and secondly, there is an accumulation of pro-angiogenic growth factors (e.g. VEGFA and FGF2) during the latter stages of pre-ovulatory follicle development (Berisha *et al.* 2000a). Presumably, this accumulation enables the intense angiogenesis after ovulation to occur. It is curious that the accumulation of VEGFA in the granulosa is unable to stimulate protease activity and enable the migration of endothelial cells into the granulosa layer. This is presumably due to the more complex composition of the follicular basement membrane (mainly collagen type IV, laminin $\alpha 1$, $\beta 1$ and $\beta 2$ as well as nidogen, perlecan and focimatrix), while the ECM associated with the vasculature is principally composed of collagen IV and laminin $\beta 2$ (Irving-Rodgers *et al.* 2006).

In the pre-ovulatory follicle, there is likely to be a shift away from vascular expansion to vessel maturation and this notion is supported by increases in the ANGPT1:ANGPT2 ratio at this time in cows (Hayashi *et al.* 2004). Moreover, the injection of ANGPT2 into pre-ovulatory follicles of rhesus monkeys attenuated

Figure 1 Schematic representation of the temporal changes in the levels of angiogenic factors during the bovine follicular–luteal transition. The data are based on observations from Goede *et al.* (1998), Berisha *et al.* (2000b, 2006), Hayashi *et al.* (2004), Rosiansky-Sultan *et al.* (2006), Robinson *et al.* (2007) and Hunigen *et al.* (2008). There is also a spatial change in FGF2 from the theca vasculature to luteinising granulosa cells after the LH surge and then back to the vasculature on day 3 (Berisha *et al.* 2006). Since there are no data available from the cow, the ¹HIF1A and ²PDGFB data are based on observations in the marmoset (Duncan *et al.* 2008) and mouse (Sleer & Taylor 2007) respectively and the bars are drawn in grey.

follicular maturation and prevented ovulation presumably by disrupting pericyte–endothelial cell interactions (Xu & Stouffer 2005). This highlighted the importance of the recruitment of pericytes and/or vSMC during the latter stages of follicular development. These cells, through their contractile properties, are likely to influence the follicular blood flow as well as stabilising the vasculature. Further investigation into how and when these mural cells are recruited during folliculogenesis and in particular the role of PDGF and transforming growth factor β is essential.

LH surge: the initiation of angiogenesis

The LH surge upregulates numerous genes (e.g. cyclo-oxygenase and progesterone receptor) that induce a series of cellular and biochemical processes that culminate in ovulation (Reynolds & Redmer 1999). A number of these events (e.g. breakdown of the basement membrane, immune-like response) play a fundamental role in initiating angiogenesis. LH might also have some direct effects on angiogenesis. For example, follicular FGF2 mRNA and protein concentrations dramatically increase following the LH surge in cows (Berisha *et al.* 2006, Robinson *et al.* 2007). At the same time, FGF2 also spatially translocates from thecal endothelial cells to the nucleolus of granulosa cells (Berisha *et al.* 2006). However, little is known about FGF2 during this time in other species. The limited information that is available, indicates that FGF2 is unaffected by the LH surge in women (Seli *et al.* 1998) and FGF2 production by human luteinising granulosa cells *in vitro* remains constant (Phan *et al.* 2006). The ANGPT2:ANGPT1 ratio in follicles also increases after the LH surge in cows (Shimizu *et al.* 2007) and macaques (Hazzard *et al.* 1999) and this may induce the destabilisation of existing vessels. Whether LH can upregulate follicular VEGFA remains unresolved. In most *in vitro* studies, LH or hCG stimulated VEGFA production by granulosa cells in primates (Martinez-Chequer *et al.* 2003, van den Driesche *et al.* 2008) and cows (Schams *et al.* 2001). However, *ex vivo* studies have been less conclusive with some showing LH stimulation in primates (Stouffer *et al.* 2001) and mice (Kim *et al.* 2009), while others, in cows, showed only small and transient increases (Berisha *et al.* 2006) or no effect (Robinson *et al.* 2007). It appears that this effect is similar for the different VEGFA isoforms 121, 165 and 189 (Berisha *et al.* 2008). Conversely, in pigs, VEGFA concentrations initially decreased in the granulosa layer in response to LH, but increased in the theca layer (Martelli *et al.* 2006). While the exact regulation of VEGFA by the LH surge remains to be elucidated, it is clear that VEGFA is in abundance in the periovulatory follicle in preparation for the intense angiogenesis that occurs after ovulation.

During the periovulatory period, there is also hyperaemia and increased ovarian blood flow (Acosta *et al.* 2003). This is probably due to increased nitric oxide production (Mitsube *et al.* 2002) following the upregulation of endothelial nitric oxide synthase (eNOS) and inducible NOS in the thecal vasculature (Zackrisson *et al.* 1996). However, this is more likely to be an E_2 mediated upregulation rather than the effect of LH since E_2 is a potent, rapid stimulator of eNOS in endothelial cells (Kim *et al.* 2008). VEGFA also plays a role since it stimulates vascular permeability. Increases in blood flow would normally result in increased supply of oxygen to the tissue, however, HIF1A is upregulated in the periovulatory follicle of marmosets (Duncan *et al.* 2008) and in the collapsed follicle of pigs (Boonyaparakob *et al.* 2005) which suggests that the tissue is hypoxic. Since hCG was a more potent stimulator of HIF1A than hypoxia itself in luteinising granulosa cells (van den Driesche *et al.* 2008), it is possible that the LH surge induces HIF1A expression directly. Thus, it is possible that any increases in VEGFA following the LH surge are mediated through the induction of HIF1A mRNA (Duncan *et al.* 2008). To date no studies have investigated HIF1A expression in ruminants during the follicular–luteal transition.

Periovulatory events: the breakdown of the basement membrane

The breakdown of the basement membrane involves a plethora of proteases that includes members of the matrix metalloprotease (MMP) family such as collagenases, gelatinases and membrane type (MT) MMP. Serine proteases such as plasmin, which is generated from plasminogen, are also involved by degrading fibrinogen and fibrin (Curry & Smith 2006). Several of these proteases are upregulated by the LH surge (e.g. MMP1, MMP9, MMP13, MT-MMP1 as well tissue and urokinase plasminogen activators), while others such as MMP2 are not (Bakke *et al.* 2002, 2004, Dow *et al.* 2002, Kliem *et al.* 2007, Berisha *et al.* 2008). These proteinases are nevertheless integral components in the ovulatory process. Furthermore, the administration of an anti-MMP2 antibody to sheep pre-ovulatory follicles not only disrupted ovulation but also the luteal tissue that was formed was vascular deficient (Gottsch *et al.* 2002). This suggests that protease activity and/or breakdown of the basement membrane is important for the initiation of luteal angiogenesis and is likely to have numerous effects: firstly, it removes the physical block to the vascularisation of the granulosa layer. Secondly, it could fragment and spread ECM components as well as creating a more spacious environment. This would generate conditions that are more conducive to endothelial (and other cells) motility and migration. Thirdly, any angiogenic factors sequestered in the basement membrane would be released. Finally, it could stimulate

the differentiation of the follicular cells (e.g. granulosa cells exposed to fibronectin undergo luteinisation). The increased proteolytic activity would also stimulate the degradation of the ECM surrounding the existing vasculature, which is a pre-requisite for angiogenesis. This is supported by the observation that there is a decline in the vascular area in the periovulatory follicle (Cavender & Murdoch 1988, Martelli *et al.* 2006). However, the injection of galardin (a broad spectrum MMP inhibitor) to either normal or plasminogen-deficient mice had no effect on either ovulation rates or subsequent luteal vasculature (Wahlberg *et al.* 2007). Furthermore, there are no apparent reproductive defects in single MMP gene knockout mice (Wahlberg *et al.* 2007). These contrasting findings may reflect differences between species. Alternatively, there is considerable redundancy and overlapping of activities in the different proteases such that one protease can overcome the loss of another making it difficult to pinpoint the precise roles of each factor.

One protease that is critical for follicular development and ovulation in mice is a disintegrin and metalloproteinase with a TSP type 1 motif (ADAMTS1; Shozu *et al.* 2005). ADAMTS1 cleaves the matrix proteoglycans versican and aggrecan as well as pro-collagen, and is expressed in the periovulatory follicle. In addition, it is increased by gonadotrophin stimulation (Madan *et al.* 2003) and this may occur through the HIF1A pathway (Kim *et al.* 2009). ADAMTS1 might play a role in regulating endothelial cell invasion since it is transiently upregulated when these cells invade into collagen matrix following VEGFA/FGF2 stimulation. Moreover, small interfering RNA directed against ADAMTS1 attenuated the ability of endothelial cells to invade (Su *et al.* 2008). Conversely, the overproduction of ADAMTS1 enhanced infiltration of myofibroblasts and ECM deposition as well as accelerating tumour development (Su *et al.* 2008). Collectively, these studies suggest that ADAMTS1 might play a key role in the initial stages of angiogenesis following ovulation.

Perlecan is a large heparan sulphate proteoglycan (HSPG) that is a major constituent of both the follicular basal lamina and focimatrix that has been located between granulosa cells (Irving-Rodgers *et al.* 2006). It can sequester a number of angiogenic growth factors including FGF2. Heparanase is an endoglycosidase that cleaves polymeric heparan sulphate molecules from large HSPG. It was recently demonstrated that LH stimulated a rapid increase in heparanase mRNA and protein concentrations in the bovine granulosa cells (Klipper *et al.* 2009) and this could explain the disappearance of perlecan from collapsed follicles shortly after ovulation (Irving-Rodgers *et al.* 2006). This would then stimulate the release of sequestered factors such as FGF2 and heparan sulphates, thereby facilitating endothelial invasion. Moreover, FGF2 and VEGFA require not only their respective receptors but also

co-receptors such as heparan sulphates and neuropilin respectively for their full biological activity (Ferrara *et al.* 2003, Presta *et al.* 2005). The potential modulatory role of these co-receptors is currently poorly characterised and warrants further investigation.

Cell migration, the role of fibroblasts and the ovulatory 'wound' hypothesis

Endothelial cell migration is a cyclical process involving its polarisation towards an angiogenic stimulus, protrusion through filopodia-like structures, traction and then retraction. Traction requires the protruding tip cell to adhere through integrins to the surface (e.g. ECM) over which it is moving. The integrins consist of α and β chains that combine to form heterodimeric trans-membrane receptors that act as 'linker molecules' between the ECM and the cytoskeleton of endothelial cells. Meanwhile, the production and organisation of ECM components such as fibronectin create a scaffold on to which endothelial cells can migrate (Hughes 2008). In the developing bovine CL, fibronectin forms a delicate network of fibrils that are orientated along the main axis of the capillary sprout (Amselgruber *et al.* 1999, Silvester & Luck 1999) thereby acting as a 'pre-patterned' guide line for endothelial cell migration. Fibronectin also has a profound stimulatory effect on luteal-derived endothelial cell proliferation (Christenson & Stouffer 1996) and formation of endothelial cell networks *in vitro* (Robinson *et al.* 2008). Similarly, during wound healing, fibroblasts are activated to myofibroblasts under stimulation from transforming growth factor β and FGF2 (Hughes 2008). These myofibroblasts then play an integral role by secreting and organising the components of the ECM (e.g. collagen I, IV and fibronectin). Pericytes have a similar phenotype to myofibroblasts and can also deposit ECM (see below for more details). It has traditionally been believed that the luteal steroidogenic cells stimulate endothelial cell migration towards themselves by producing chemo-attractants. Indeed, in the collapsed follicle, FGF2 and VEGFA, are primarily localised to these steroidogenic luteal cells in several species (Berisha *et al.* 2000b, Wulff *et al.* 2000, Kaczmarek, *et al.* 2007, Robinson *et al.* 2007). This then creates directionality for endothelial cell migration. However, FGF2 and VEGFA have also been localised to perivascular cells albeit to a lesser extent, suggesting that this process is far more complex than simple migration towards steroidogenic cells. It could be that different isoforms of VEGFA and/or FGF2 (e.g. those that are cell associated) are expressed in these perivascular cells. Alternatively, there could be other migratory stimuli. It is possible that the blood clot formed during ovulation might play an active role by creating a stimulus for migration. Indeed, platelets were more potent stimulants of endothelial cell migration than granulosa cells (Furukawa *et al.* 2007). However, the blood clot forms

near to the ovarian surface and is relatively quickly removed (Duggavathi *et al.* 2003), although it could still create an environment whereby migration is supported. Intriguingly, we have observed that the endothelial cell clusters appear to migrate towards each other rather than to steroidogenic cells in our luteal angiogenic culture system that incorporates all cell types, (RS Robinson, KJ Woad, AJ Hammond, MG Hunter & GE Mann 2009, unpublished observations). This would indicate that it is the endothelial cells themselves (and not the steroidogenic cells) that produce the chemotactic factors, which then drive their migration and proliferation.

Endothelial proliferation and formation of vascular networks

The majority of the proliferating cells in the collapsed follicle are of vascular origin (Reynolds & Redmer 1999, Fraser & Lunn 2001). Both FGF2 and VEGFA are potent mitogens of endothelial cells and FGF2 and VEGFA stimulate bovine endothelial network formation *in vitro* (Robinson *et al.* 2008). Undoubtedly, VEGFA plays a fundamental role, since its blockade completely abolished endothelial proliferation, luteal vascularisation and progesterone production in the rat (Ferrara *et al.* 1998), primate (Wulff *et al.* 2001, Zimmermann *et al.* 2001, Hazzard *et al.* 2002) and mouse (Kuhnert *et al.* 2008). Recent studies in the cow have shown that local immunoneutralisation of VEGFA reduced luteal development and progesterone production (Yamashita *et al.* 2008) and the inhibition of VEGFA signalling suppressed the formation of endothelial networks *in vitro* (Woad *et al.* 2009). However, total inhibition was not achieved in both cases and whether this represents a species difference is unknown. Interestingly, treatment with the FGF receptor signalling inhibitor, SU5402, almost completely blocked endothelial network formation, by decreasing both the number of endothelial clusters and their size. This occurred even in the presence of exogenous VEGFA and indicates that FGF2 is critical for the formation of luteal endothelial networks. It also suggests that these factors must have complementary rather than redundant actions, since the remaining factors were unable to compensate for the loss of VEGF/FGF signalling (Woad *et al.* 2009). Combined with the dynamism of FGF2 during the follicular–luteal transition (Robinson *et al.* 2007), this emphasises the importance of FGF2 in controlling and possibly initiating luteal angiogenesis in the cow.

Pericytes and PDGF system

In a functional, mature vascular system, endothelial cells are supported by mural cells such as pericytes and vSMC. These mural cells provide structural support and regulate blood flow through their contractile properties.

Pericytes share a basement membrane with the endothelial cells, but can make direct contact through peg-and-socket junctions. The final step in angiogenesis is vessel stabilisation, which occurs by the secretion of PDGFBB by endothelial cells, which acts in a paracrine manner to recruit pericytes (Gerhardt & Betsholtz 2003). Thus, for many years, pericytes were thought to have a passive role in angiogenesis and have been often neglected. There is now growing evidence that pericytes might play a more active role in initiating angiogenesis. This is not surprising since one of the first steps in angiogenesis is the detachment of pericytes from a sprouting vessel and once detached, pericytes can differentiate into collagen producing fibroblast-like cells (Gerhardt & Betsholtz 2003). Interestingly, during the ovulatory period, pericytes are located at what appears to be the forefront of the endothelial migratory path (Amselgruber *et al.* 1999, Redmer *et al.* 2001; Fig. 2A), whilst in the mature CL, they are closely associated with the endothelial cells (Fig. 2B). Furthermore, pericytes represent a large proportion of the proliferating cells in the early ovine CL (Redmer *et al.* 2001) and analysis of smooth muscle actin (a pericyte marker) staining during luteal development showed a biphasic pattern (Fig. 2C). It is possible that this represents two phases of pericyte activity: firstly that

Figure 2 The spatial associations between pericytes and endothelial cells during luteal development. Co-localisation of von Willebrand factor (VWF, green, endothelial cell marker) and smooth muscle actin (α -SMA, red, a pericyte marker) in (A) a collapsed follicle and (B) mature CL of cows. The pericytes appear to be at the leading edge of the endothelial cells (shown by arrows), whereas in the mature CL, pericytes are closely associated around endothelial cells (shown by arrowheads). The different layers are represented by G and T for granulosa and theca layers respectively. (C) Shows the quantification of % area of α -SMA staining during luteal development. This showed a biphasic pattern with peaks from day 3 to 4 (developing, early CL) and then day 8–12 (mature CL). The data are mean $\pm$ S.E.M.; $a < b$ $P < 0.05$.

pericytes act as guiding structures aiding the outgrowth of endothelial cells. This is supported by the fact that pericytes produce MMPs and might promote endothelial cell invasion by degrading ECM. Indeed, synthetic MMP inhibitors blocked the ability of vSMC to invade extracellular matrices but did not affect their motility (Chantraine *et al.* 2006). The second phase is when pericytes are recruited during vessel stabilisation. Collectively, these studies increase the evidence that pericytes playing a crucial and dynamic role during luteal angiogenesis.

The PDGF system plays an essential role in the activation of pericytes and the expression of components of this system is temporally regulated during the follicular–luteal transition in mice. Namely, mRNA

expression of *Pdgfrb* and its receptor PDGF receptor beta (*Pdgfrb*) increased after hCG-induced ovulation (Sleer & Taylor 2007). Moreover, mice treated with a PDGFRB inhibitor had fewer functional CL and widespread haemorrhage (Sleer & Taylor 2007), while the pre-ovulatory treatment of mice with a soluble ectodomain of PDGFRB prevented the recruitment of pericytes and reduced the area of endothelial staining in the CL (Kuhnert *et al.* 2008). Recently, we have shown that inhibition of PDGFR signalling reduced the formation of bovine luteal endothelial networks *in vitro* (Woad *et al.* 2009). In culture, pericytes and endothelial cells were in close proximity with each other and the pericytes appeared to be ahead of the endothelial cluster (RS Robinson, KJ Woad, AJ Hammond,

Figure 3 A working hypothesis of the angiogenic processes and their regulation during luteal vascularisation in the cow. (A) In the periovulatory follicle, the LH surge induces protease activity, hypoxia and the FGF2 production. There are further increases in FGF2 following the release of sequestered FGF2 (green) from the basement membrane (in blue) during its breakdown. FGF2 then stimulates the scatter and migration of endothelial cells from the existing thecal vasculature and activates fibroblasts, in conjunction with transforming growth factor β (TGFB) into myofibroblasts. The LH surge, at the same time, stimulates the production of angiopoietin 2 (ANGPT2), which causes the destabilisation of the pericyte–endothelial interactions and hence pericyte detachment. Throughout this period there is continual production of VEGFA, which activates endothelial cells and degradation of the extracellular matrix between pericytes and endothelial cells. (B) In the collapsed follicle, the myofibroblasts and/or pericytes migrate ahead of the endothelial tip cell (pink) towards the angiogenic stimulus (e.g. luteal cell and/or other endothelial cells) and in so doing ‘lay down’ an ECM scaffold that contains fibronectin. This provides a structural framework on which endothelial cells are guided along. The proliferation of these cells is stimulated by both VEGFA and FGF2. During this remodelling period, the high VEGFA concentrations support endothelial cell survival. (C) In the developing CL, the endothelial cells reconnect and align to form tubules under the influence of FGF2 (now endothelial-derived) and VEGFA. The newly formed vessels are stabilised by the recruitment of pericytes, through the production of platelet-derived growth factor B (PDGFB) and the activity of ANGPT1. Consequently, the luteal cells are now fully vascularised and so the levels of hypoxia decline. VEGFA concentrations remain high throughout luteal development to maintain the survival of the immature vessels.

MG Hunter & GE Mann 2009, unpublished observations). It is likely that VEGFA and FGF2 may further influence PDGF signalling and pericyte function. For example, VEGFA promoted PDGFB while FGF2 increased PDGFRB production (Kano *et al.* 2005, Zhang *et al.* 2009). Further to this, FGF2 acted synergistically with PDGFB to promote angiogenesis in the cornea

(Zhang *et al.* 2009) and chorioallantoic membrane (CAM) of chick embryos (Greenberg *et al.* 2008). However, it has been proposed that VEGFA may act as a negative regulator of pericyte function and vessel maturation (Greenberg *et al.* 2008). This was based on the observations that while VEGFA and PDGFB alone stimulated CAM angiogenesis, when combined together,

Figure 4 Proposed different mechanisms by which the CL is vascularised in ruminants and primates. The granulosa and theca cells are shown in orange and blue respectively with the vasculature in red. In the pre-ovulatory follicle, the granulosa layer remains avascular, while there is extensive vascularisation in the theca. During follicular development, VEGFA and FGF2 accumulate. Proteolytic activity (e.g. matrix metalloproteinase (MMP) and plasminogen activators (PA)) increases following the LH surge, as well as heparanase expression. These proteases degrade the basement membrane which releases sequestered angiogenic factors such as FGF2 which enables vascular cells to migrate under the influence of the VEGFA gradient. At the same time, there is hyperaemia of the theca vasculature and increases in VEGFA and FGF2 concentrations, at least in the cow. The large, transient increase in FGF2 may induce a different pattern of vascularisation following ovulation between primates and ruminants. Namely, FGF2 stimulates disassembly of the existing vasculature and scattering of endothelial cells. At the same time, there is extensive intermingling of all luteal cell types in ruminants. In primates, however, the luteinising theca and granulosa cells largely remain separate. Thus, the initial angiogenic step is the sprouting from the existing thecal vasculature towards the luteinising granulosa cells producing VEGFA. After a short period of angiogenesis, these sprouts then start to connect with each other, form tubules and recruit pericytes and blood flow is reinitiated. As the CL continues to develop, there is further extension and maturation of the vasculature. In contrast, in the ruminant, following the disassembly of the theca vasculature, there is extensive endothelial cell proliferation and migration in order to re-establish connections with other endothelial and luteal cells. This is promoted by the laying down of fibronectin. Once the endothelial cells are reconnected, there is a decrease in FGF2 concentrations and then capillary beds are reconstructed. Consequently, blood flow can increase and plasma concentrations of progesterone increase.

angiogenesis was suppressed and pericyte coverage of vascular sprouts was ablated. However, VEGFR2 inhibitors reversed this effect. This emphasises the complexity of inter-play between the different pro-angiogenic factors and our current working hypothesis is summarised in Fig. 3.

Comparative luteal angiogenesis

The formation of the CL involves luteinisation of follicular cells, endothelial cell invasion and tissue remodelling in all species. There are however, considerable differences in the histology of corpora lutea from primates, rodents and ruminants. For example, in primates and ruminants, small and large luteal cells differentiate from theca and granulosa cells respectively but do not necessarily in rodents (Stocco *et al.* 2007). Moreover, in ruminants, there is extensive mixing and intermingling between large and small luteal cells, while in primates the granulosa and theca-derived layers remain largely separated (Stocco *et al.* 2007). Thus, it is feasible that the manner in which the luteal tissue is vascularised differs among these groups. In primates, angiogenesis might occur via the traditional mechanism of sprouting from the existing theca vasculature into the granulosa-lutein layer. This would imply that endothelial cells invade, migrate and proliferate towards an angiogenic stimulus produced by the granulosa cells (e.g. VEGFA). In contrast, in ruminants, more extensive tissue remodelling occurs and it is feasible that this causes more disassembly and scattering of existing vasculature in the collapsed follicle. This would be more akin to vasculogenesis. This mechanism was also suggested by Kaessmeyer & Plendl (2009) who described endothelial cells as forming 'vascular initiation points'. Once these initiation points are formed, there is then rapid endothelial proliferation and migration along the ECM scaffold, which is followed by the reconstruction of the vascular bed (Fig. 4). This is supported by the fact that the degree of vascularisation (as determined by % area of von Willebrand factor (VWF) staining) was higher in the early than mid CL in marmosets (Young *et al.* 2000), whereas, the degree of vascularisation increased continually through luteal development in the cow (Robinson *et al.* 2006). Furthermore, the pattern of VWF staining changed from being disorganised and widely dispersed on day 5 to highly organised and structured on day 8 (Robinson *et al.* 2006). Interestingly, transiently high concentrations of FGF2 stimulate the scattering of endothelial cells, while prolonged exposure to lower concentrations support endothelial proliferation and cell adhesion (Presta *et al.* 2005). Thus, the induction of FGF2 during the ovulatory period only seen in ruminants might explain why the manner of vascularisation is different from that in primates.

Blood flow and luteal function

The extensive vascularisation of the CL enables it to receive one of the highest blood flows per unit tissue mass. Luteal blood flow remains at pre-ovulation levels in the collapsed follicle, but thereafter gradually increases in parallel with increases in luteal volume and coincides with increases in progesterone (Acosta *et al.* 2003). Nitric oxide is an endothelial-derived mediator that regulates vascular tone. eNOS is localised to theca-derived cells and is highest during the early luteal phase in sheep (Grazul-Bilska *et al.* 2006) and cows (Rosiansky-Sultan *et al.* 2006), while expression of endothelin-1, a vasoconstrictor, is minimal during the early luteal phase. This would presumably mean that the luteal blood vessels would be predominantly vasodilated, thereby maximising the blood flow. VEGFA has been shown to upregulate eNOS expression (Grazul-Bilska *et al.* 2006), while nitric oxide dose-dependently increased the expression of FGF2 and VEGFA in luteal pericytes (Beckman, *et al.* 2006). This, in turn, could further promote luteal angiogenesis.

Conclusion

The controlled, physiological angiogenesis that accompanies folliculogenesis, ovulation and luteal development requires the co-ordinated activity of multiple cell types and different angiogenic factors. It appears that VEGFA regulates angiogenesis by stimulating endothelial proliferation, migration and survival and is required at all stages from a secondary follicle right through to the mature CL. However, the often overlooked FGF2 plays a more dynamic role and is likely to be critical during the follicular-luteal transition. The transient increase in FGF2 at this time might be important to stimulate the extensive tissue remodelling that accompanies rapid angiogenesis after ovulation in the cow. Finally, there is increasing evidence that pericytes and PDGF have multiple and critical roles in luteal angiogenesis. The successful development of our culture system in which endothelial cell networks are formed *in vitro* heralds a new era in elucidating the physiological control of the angiogenic process in the developing CL, enabling us to solve those questions still unanswered such as how do blood vessels develop? What is the role of the other luteal cell types? What stops the angiogenic process?

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

Some of this work has been funded by BBSRC and Pfizer.

Acknowledgements

We greatly appreciate the technical assistance of staff at the University of Nottingham without which this work would not have been possible.

References

- Acosta TJ, Hayashi KG, Ohtani M & Miyamoto A 2003 Local changes in blood flow within the preovulatory follicle wall and early corpus luteum in cows. *Reproduction* **125** 759–767.
- Acosta TJ, Gastal EL, Gastal MO, Beg MA & Ginther OJ 2004 Differential blood flow changes between the future dominant and subordinate follicles precede diameter changes during follicle selection in mares. *Biology of Reproduction* **71** 502–507.
- Acosta TJ, Hayashi KG, Matsui M & Miyamoto A 2005 Changes in follicular vascularity during the first follicular wave in lactating cows. *Journal of Reproduction and Development* **51** 273–280.
- Amselgruber WM, Schafer M & Sinowatz F 1999 Angiogenesis in the bovine corpus luteum: an immunocytochemical and ultrastructural study. *Anatomia, Histologia, Embryologia* **28** 157–166.
- Armstrong L & Bornstein P 2003 Thrombospondins 1 and 2 function as inhibitors of angiogenesis. *Matrix Biology* **22** 63–71.
- Bakke LJ, Dow M, Cassar CA, Peters MW, Pursley JR & Smith GW 2002 Effect of the preovulatory gonadotropin surge on matrix metalloproteinase (MMP)-14, MMP-2, and tissue inhibitor of metalloproteinases-2 expression within bovine periovulatory follicular and luteal tissue. *Biology of Reproduction* **66** 1627–1634.
- Bakke LJ, Li QL, Cassar CA, Dow MPD, Pursley JR & Smith GW 2004 Gonadotropin surge-induced differential upregulation of collagenase-1 (MMP-1) and collagenase-3 (MMP-13) mRNA and protein in bovine preovulatory follicles. *Biology of Reproduction* **71** 605–612.
- Beckman JD, Grazul-Bilska AT, Johnson ML, Reynolds LP & Redmer DA 2006 Isolation and characterization of ovine luteal pericytes and effects of nitric oxide on pericyte expression of angiogenic factors. *Endocrine* **29** 467–476.
- Berisha B, Schams D, Kosmann M, Amselgruber W & Einspanier R 2000a Expression and localisation of vascular endothelial growth factor and basic fibroblast growth factor during the final growth of bovine ovarian follicles. *Journal of Endocrinology* **167** 371–382.
- Berisha B, Schams D, Kosmann M, Amselgruber W & Einspanier R 2000b Expression and tissue concentration of vascular endothelial growth factor, its receptors, and localization in the bovine corpus luteum during estrous cycle and pregnancy. *Biology of Reproduction* **63** 1106–1114.
- Berisha B, Steffl M, Amselgruber W & Schams D 2006 Changes in fibroblast growth factor 2 and its receptors in bovine follicles before and after GnRH application and after ovulation. *Reproduction* **131** 319–329.
- Berisha B, Steffl M, Welter H, Kliem H, Meyer HH, Schams D & Amselgruber W 2008 Effect of the luteinising hormone surge on regulation of vascular endothelial growth factor and extracellular matrix-degrading proteinases and their inhibitors in bovine follicles. *Reproduction, Fertility, and Development* **20** 258–268.
- Boonyaparakob U, Gadsby JE, Hedgpeth V, Routh PA & Almond GW 2005 Expression and localization of hypoxia inducible factor-1 alpha mRNA in the porcine ovary. *Canadian Journal of Veterinary Research* **69** 215–222.
- Cavender JL & Murdoch WJ 1988 Morphological studies of the microcirculatory system of periovulatory ovine follicles. *Biology of Reproduction* **39** 989–997.
- Chantraine CF, Henriot P, Jodele S, Emonard H, Feron O, Courtoy PJ, DeClerck YA & Marbaix E 2006 Mechanisms of pericyte recruitment in tumour angiogenesis: a new role for metalloproteinases. *European Journal of Cancer* **42** 310–318.
- Christenson LK & Stouffer RL 1996 Isolation and culture of microvascular endothelial cells from the primate corpus luteum. *Biology of Reproduction* **55** 1397–1404.
- Curry TE & Smith MF 2006 Impact of extracellular matrix remodeling on ovulation and the folliculo-luteal transition. *Seminars in Reproductive Medicine* **24** 228–241.
- Dow MPD, Bakke LJ, Cassar CA, Peters MW, Pursley JR & Smith GW 2002 Gonadotropin surge-induced up-regulation of the plasminogen activators (tissue plasminogen activator and urokinase plasminogen activator) and the urokinase plasminogen activator receptor within bovine periovulatory follicular and luteal tissue. *Biology of Reproduction* **66** 1413–1421.
- van den Driesche S, Myers M, Gay E, Thong KJ & Duncan WC 2008 HCG up-regulates hypoxia inducible factor-1 alpha in luteinized granulosa cells: implications for the hormonal regulation of vascular endothelial growth factor A in the human corpus luteum. *Molecular Human Reproduction* **14** 455–464.
- Duggavathi R, Bartlewski P, Barrett D & Rawlings N 2003 Use of high-resolution transrectal ultrasonography to assess changes in numbers of small ovarian antral follicles and their relationships to the emergence of follicular waves in cyclic ewes. *Theriogenology* **60** 495–510.
- Duncan WC, van den Driesche S & Fraser HM 2008 Inhibition of vascular endothelial growth factor in the primate ovary up-regulates hypoxia-inducible factor-1 alpha in the follicle and corpus luteum. *Endocrinology* **149** 3313–3320.
- Ferrara N, Chen H, Davis-Smyth T, Gerber HP, Nguyen TN, Peers D, Chisholm V, Hillan KJ & Schwall RH 1998 Vascular endothelial growth factor is essential for corpus luteum angiogenesis. *Nature Medicine* **4** 336–340.
- Ferrara N, Gerber HP & LeCouter J 2003 The biology of VEGF and its receptors. *Nature Medicine* **9** 669–676.
- Fraser HM & Duncan WC 2009 Regulation and manipulation of angiogenesis in the ovary and endometrium. *Reproduction, Fertility, and Development* **21** 377–392.
- Fraser HM & Lunn SF 2001 Regulation and manipulation of angiogenesis in the primate corpus luteum. *Reproduction* **121** 355–362.
- Fredriksson L, Li H & Eriksson U 2004 The PDGF family: four gene products form five dimeric isoforms. *Cytokine & Growth Factor Reviews* **15** 197–204.
- Furukawa K, Fujiwara H, Sato Y, Zeng B, Fujii H, Yoshioka S, Nishi E & Nishio T 2007 Platelets are novel regulators of neovascularization and luteinization during human corpus luteum formation. *Endocrinology* **148** 3056–3064.
- Gerhardt H & Betsholtz C 2003 Endothelial-pericyte interactions in angiogenesis. *Cell and Tissue Research* **314** 15–23.
- Goede V, Schmidt T, Kimmina S, Kozián D & Augustin H 1998 Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. *Laboratory Investigation* **78** 1385–1394.
- Gottsch ML, Van Kirk EA & Murdoch WJ 2002 Role of matrix metalloproteinase 2 in the ovulatory folliculo-luteal transition of ewes. *Reproduction* **124** 347–352.
- Grazul-Bilska A, Navanukraw C, Johnson M, Arnold D, Reynolds L & Redmer D 2006 Expression of endothelial nitric oxide synthase in the ovine ovary throughout the estrous cycle. *Reproduction* **132** 579–587.
- Grazul-Bilska AT, Navanukraw C, Johnson ML, Vonnahme KA, Ford SP, Reynolds LP & Redmer DA 2007 Vascularity and expression of angiogenic factors in bovine dominant follicles of the first follicular wave. *Journal of Animal Science* **85** 1914–1922.
- Greenaway J, Gentry PA, Feige JJ, LaMarre J & Petrik JJ 2005 Thrombospondin and vascular endothelial growth factor are cyclically expressed in an inverse pattern during bovine ovarian follicle development. *Biology of Reproduction* **72** 1071–1078.
- Greenberg JI, Shields DJ, Barillas SC, Acevedo LM, Murphy E, Huang JH, Scheppe L, Stockmann C, Johnson RS, Angle N *et al.* 2008 A role for VEGF as a negative regulator of pericyte function and vessel maturation. *Nature* **456** 809–813.
- Hayashi KG, Berisha B, Matsui M, Schams D & Miyamoto A 2004 Expression of mRNA for the angiopoietin-Tie system in granulosa cells during follicular development in cows. *Journal of Reproduction and Development* **50** 477–480.

- Hazzard TM, Molskness TA, Chaffin CL & Stouffer RL 1999 Vascular endothelial growth factor (VEGF) and angiopoietin regulation by gonadotrophin and steroids in macaque granulosa cells during the peri-ovulatory interval. *Molecular Human Reproduction* **5** 1115–1121.
- Hazzard TM, Xu FH & Stouffer RL 2002 Injection of soluble vascular endothelial growth factor receptor 1 into the preovulatory follicle disrupts ovulation and subsequent luteal function in rhesus monkeys. *Biology of Reproduction* **67** 1305–1312.
- Hughes C 2008 Endothelial–stromal interactions in angiogenesis. *Current Opinion in Hematology* **15** 204–209.
- Hunigen H, Bisplinghoff P, Plendl J & Bahramsoltani M 2008 Vascular dynamics in relation to immunolocalisation of VEGF-A, VEGFR-2 and Ang-2 in the bovine corpus luteum. *Acta Histochemica* **110** 462–472.
- Irving-Rodgers HF, Catanzariti KD, Aspden WJ, D'Occhio MJ & Rodgers RJ 2006 Remodeling of extracellular matrix at ovulation of the bovine ovarian follicle. *Molecular Reproduction and Development* **73** 1292–1302.
- Jiang JY, Macchiarelli G, Tsang BK & Sato E 2003 Capillary angiogenesis and degeneration in bovine ovarian antral follicles. *Reproduction* **125** 211–223.
- Kaczmarek MM, Kowalczyk AE, Wacławik A, Schams D & Ziecik AJ 2007 Expression of vascular endothelial growth factor and its receptors in the porcine corpus luteum during the estrous cycle and early pregnancy. *Molecular Reproduction and Development* **74** 730–739.
- Kaessmeyer S & Plendl J 2009 Angiogenesis and vasculogenesis in the corpus luteum *in vitro*. *Clinical Hemorheology and Microcirculation* **41** 83–101.
- Kano MR, Morishita Y, Iwata C, Iwasaka S, Watabe T, Ouchi Y, Miyazono K & Miyazawa K 2005 VEGF-A and FGF-2 synergistically promote neoangiogenesis through enhancement of endogenous PDGF-B-PDGRF beta signaling. *Journal of Cell Science* **118** 3759–3768.
- Karamysheva AF 2008 Mechanisms of angiogenesis. *Biochemistry* **73** 751–762.
- Kim KH, Moriarty K & Bender JR 2008 Vascular cell signaling by membrane estrogen receptors. *Steroids* **73** 864–869.
- Kim J, Bagchi I & Bagchi M 2009 Signaling by hypoxia-inducible factors is critical for ovulation in mice. *Endocrinology* **50** 3392–3400.
- Kliem H, Welter H, Kraetzl WD, Steffl M, Meyer HH, Schams D & Berisha B 2007 Expression and localisation of extracellular matrix degrading proteases and their inhibitors during the oestrous cycle and after induced luteolysis in the bovine corpus luteum. *Reproduction* **134** 535–547.
- Klipper E, Tatz E, Kisliouk T, Vlodavsky I, Moallem U, Schams D, Lavon Y, Wolfenson D & Meidan R 2009 Induction of heparanase in bovine granulosa cells by luteinizing hormone: possible role during the ovulatory process. *Endocrinology* **150** 413–421.
- Kuhnert F, Tam BYY, Sennino B, Gray JT, Yuan J, Jocson A, Nayak NR, Mulligan RC, McDonald DM & Kuo CJ 2008 Soluble receptor-mediated selective inhibition of VEGFR and PDGFR beta signaling during physiologic and tumor angiogenesis. *PNAS* **105** 10185–10190.
- Macchiarelli G, Jiang JY, Nottola SA & Sato E 2006 Morphological patterns of angiogenesis in ovarian follicle capillary networks. A scanning electron microscopy study of corrosion cast. *Microscopy Research and Technique* **69** 459–468.
- Madan P, Bridges PJ, Komar CM, Beristain AG, Rajamahendran R, Fortune JE & MacCalman CD 2003 Expression of messenger RNA for ADAMTS subtypes changes in the periovulatory follicle after the gonadotropin surge and during luteal development and regression in cattle. *Biology of Reproduction* **69** 1506–1514.
- Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand S, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N *et al.* 1997 Angiopoietin-2, a natural antagonist for Tie2 that disrupts *in vivo* angiogenesis. *Science* **277** 55–60.
- Martelli A, Berardinelli P, Russo V, Mauro A, Bernabo N, Gioia L, Mattioli M & Barboni B 2006 Spatio-temporal analysis of vascular endothelial growth factor expression and blood vessel remodelling in pig ovarian follicles during the periovulatory period. *Journal of Molecular Endocrinology* **36** 107–119.
- Martelli A, Palmerini MG, Russo V, Rinaldi C, Bernabò N, Di Giacinto O, Berardinelli P, Nottola SA, Macchiarelli G & Barboni B 2009 Blood vessel remodeling in pig ovarian follicles during the periovulatory period: an immunohistochemistry and SEM-corrosion casting study. *Reproductive Biology and Endocrinology* **16** 72.
- Martinez-Chequer JC, Stouffer RL, Hazzard TM, Patton PE & Molskness TA 2003 Insulin-like growth factors-1 and -2, but not hypoxia, synergize with gonadotropin hormone to promote vascular endothelial growth factor-A secretion by monkey granulosa cells from preovulatory follicles. *Biology of Reproduction* **68** 1112–1118.
- Matos MHT, van den Hurk R, Lima-Verde IB, Luque MCA, Santos KDB, Martins FS, Bao SN, Lucci CM & Figueiredo JR 2007 Effects of fibroblast growth factor-2 on the *in vitro* culture of caprine preantral follicles. *Cells, Tissues, Organs* **186** 112–120.
- Mitsube K, Zackrisson U & Brannstrom M 2002 Nitric oxide regulates ovarian blood flow in the rat during the periovulatory period. *Human Reproduction* **17** 2509–2516.
- Nilsson EE, Detzel C & Skinner MK 2006 Platelet-derived growth factor modulates the primordial to primary follicle transition. *Reproduction* **131** 1007–1015.
- Petrik JJ, Gentry PA, Feige JJ & LaMarre J 2002 Expression and localization of thrombospondin-1 and -2 and their cell-surface receptor, CD36, during rat follicular development and formation of the corpus luteum. *Biology of Reproduction* **67** 1522–1531.
- Phan B, Rakenius A, Pietrowski D, Bettendorf H, Keck C & Herr D 2006 hCG-dependent regulation of angiogenic factors in human granulosa lutein cells. *Molecular Reproduction and Development* **73** 878–884.
- Presta M, Dell'Era P, Mitola S, Moroni E, Ronca R & Rusnati M 2005 Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine & Growth Factor Reviews* **16** 159–178.
- Redmer DA, Doraiswamy V, Bortnem BJ, Fisher K, Jablonka-Shariff A, Grazul-Bilska AT & Reynolds LP 2001 Evidence for a role of capillary pericytes in vascular growth of the developing ovine corpus luteum. *Biology of Reproduction* **65** 879–889.
- Reynolds LP & Redmer DA 1999 Growth and development of the corpus luteum. *Journal of Reproduction and Fertility Supplement* **54** 181–191.
- Robinson RS, Hammond AJ, Nicklin LT, Schams D, Mann GE & Hunter MG 2006 Endocrine and cellular characteristics of corpora lutea from cows with a delayed post-ovulatory progesterone rise. *Domestic Animal Endocrinology* **31** 154–172.
- Robinson RS, Nicklin LT, Hammond AJ, Schams D, Hunter MG & Mann GE 2007 Fibroblast growth factor 2 is more dynamic than vascular endothelial growth factor A during the follicle–luteal transition in the cow. *Biology of Reproduction* **77** 28–36.
- Robinson RS, Hammond AJ, Mann GE & Hunter MG 2008 A novel physiological culture system that mimics luteal angiogenesis. *Reproduction* **135** 405–413.
- Rosiansky-Sultan M, Klipper E, Spanel-Borowski K & Meidan R 2006 Inverse relationship between nitric oxide synthases and endothelin-1 synthesis in bovine corpus luteum: interactions at the level of luteal endothelial cell. *Endocrinology* **147** 5228–5235.
- Schams D, Kosmann M, Berisha B, Amselgruber WM & Miyamoto A 2001 Stimulatory and synergistic effects of luteinizing hormone and insulin like growth factor 1 on the secretion of vascular endothelial growth factor and progesterone of cultured bovine granulosa cells. *Experimental and Clinical Endocrinology & Diabetes* **109** 155–162.
- Seli E, Zeyneloglu HB, Senturk LM, Bahtiyar OM, Olive DL & Arici A 1998 Basic fibroblast growth factor: peritoneal and follicular fluid levels and its effect on early embryonic development. *Fertility and Sterility* **69** 1145–1148.
- Shimizu T & Miyamoto A 2007 Progesterone induces the expression of vascular endothelial growth factor (VEGF) 120 and Flk-1, its receptor, in bovine granulosa cells. *Animal Reproduction Science* **102** 228–237.
- Shimizu T, Jiang IY, Sasada H & Sato E 2002 Changes of messenger RNA expression of angiogenic factors and related receptors during follicular development in gilts. *Biology of Reproduction* **67** 1846–1852.
- Shimizu T, Jayawardana BC, Nishimoto H, Kaneko E, Tetsuka M & Miyamoto A 2006 Hormonal regulation and differential expression of neuropilin (NRP)-1 and NRP-2 genes in bovine granulosa cells. *Reproduction* **131** 555–559.
- Shimizu T, Berisha B, Schams D & Miyamoto A 2007 Expression of angiopoietin (ANPT)-1, ANPT-2 and their receptors in dominant follicles during periovulatory period in GnRH-treated cow. *Reproduction in Domestic Animals* **42** 221–224.

- Shozu M, Minami N, Yokoyama H, Inoue M, Kurihara H, Matsushima K & Kuno K 2005 ADAMTS-1 is involved in normal follicular development, ovulatory process and organization of the medullary vascular network in the ovary. *Journal of Molecular Endocrinology* **35** 343–355.
- Silvester LM & Luck MR 1999 Distribution of extracellular matrix components in the developing ruminant corpus luteum: a wound repair hypothesis for luteinization. *Journal of Reproduction and Fertility* **116** 187–198.
- Sleer LS & Taylor CC 2007 Platelet-derived growth factors and receptors in the rat corpus luteum: localization and identification of an effect on luteogenesis. *Biology of Reproduction* **76** 391–400.
- Stocco C, Telleria C & Gibori G 2007 The molecular control of corpus luteum formation, function, and regression. *Endocrine Reviews* **28** 117–149.
- Stouffer RL, Martinez-Chequer JC, Molskness TA, Xu FH & Hazzard TM 2001 Regulation and action of angiogenic factors in the primate ovary. *Archives of Medical Research* **32** 567–575.
- Su SC, Mendoza EA, Kwak HI & Bayless KJ 2008 Molecular profile of endothelial invasion of three-dimensional collagen matrices: insights into angiogenic sprout induction in wound healing. *American Journal of Physiology. Cell Physiology* **295** C1215–C1229.
- Tamanini C & De Ambrogi M 2004 Angiogenesis in developing follicle and corpus luteum. *Reproduction in Domestic Animals* **39** 206–216.
- Thomas FH, Wilson H, Silvestri A & Fraser HM 2008 Thrombospondin-1 expression is increased during follicular atresia in the primate ovary. *Endocrinology* **149** 185–192.
- Wahl M, Kenan D, Gonzalez-Gronow M & Pizzo S 2005 Angiostatin's molecular mechanism: aspects of specificity and regulation elucidated. *Journal of Cellular Biochemistry* **96** 242–261.
- Wahlberg P, Boden I, Paulsson J, Lund LR, Liu K & Ny T 2007 Functional corpora lutea are formed in matrix metalloproteinase inhibitor-treated plasminogen-deficient mice. *Endocrinology* **148** 1226–1234.
- van Wezel IL, Umapathysivam K, Tilley WD & Rodgers RJ 1995 Immunohistochemical localization of basic fibroblast growth factor in bovine ovarian follicles. *Molecular and Cellular Endocrinology* **115** 133–140.
- Woad KJ, Hammond AJ, Hunter M, Mann GE, Hunter MG & Robinson RS 2009 FGF2 is crucial for the development of bovine luteal endothelial networks *in vitro*. *Reproduction* **138** 581–588.
- Wulff C, Wilson H, Largent P, Duncan WC, Armstrong DG & Fraser HM 2000 Angiogenesis in the human corpus luteum: localization and changes in angiopoietins, Tie-2, and vascular endothelial growth factor messenger ribonucleic acid. *Journal of Clinical Endocrinology and Metabolism* **85** 4302–4309.
- Wulff C, Wilson H, Rudge JS, Wiegand SJ, Lunn SF & Fraser HM 2001 Luteal angiogenesis: prevention and intervention by treatment with vascular endothelial growth factor Trap(A40). *Journal of Clinical Endocrinology and Metabolism* **86** 3377–3386.
- Wulff C, Wilson H, Wiegand SJ, Rudge JS & Fraser HM 2002 Prevention of thecal angiogenesis, antral follicular growth, and ovulation in the primate by treatment with vascular endothelial growth factor trap R1R2. *Endocrinology* **143** 2797–2807.
- Xu FH & Stouffer RL 2005 Local delivery of angiopoietin-2 into the preovulatory follicle terminates the menstrual cycle in rhesus monkeys. *Biology of Reproduction* **72** 1352–1358.
- Yamashita H, Kamada D, Shirasuna K, Matsui M, Shimizu T, Kida K, Berisha B, Schams D & Miyamoto A 2008 Effect of local neutralization of basic fibroblast growth factor or vascular endothelial growth factor by a specific antibody on the development of the corpus luteum in the cow. *Molecular Reproduction and Development* **75** 1449–1456.
- Yang MY & Fortune JE 2007 Vascular endothelial growth factor stimulates the primary to secondary follicle transition in bovine follicles *in vitro*. *Molecular Reproduction and Development* **74** 1095–1104.
- Young FM, Rodger FE, Illingworth PJ & Fraser HM 2000 Cell proliferation and vascular morphology in the marmoset corpus luteum. *Human Reproduction* **15** 557–566.
- Zackrisson U, Mikuni M, Wallin A, Delbro D, Hedin L & Brannstrom M 1996 Cell-specific localization of nitric oxide synthases (NOS) in the rat ovary during follicular development, ovulation and luteal formation. *Human Reproduction* **11** 2667–2673.
- Zeleznik AJ, Schuler HM & Reichert LE 1981 Gonadotropin-binding sites in the rhesus-monkey ovary – role of the vasculature in the selective distribution of human chorionic-gonadotropin to the preovulatory follicle. *Endocrinology* **109** 356–362.
- Zhang JH, Cao RH, Zhang Y, Jia TH, Cao YH & Wahlberg E 2009 Differential roles of PDGFR-alpha and PDGFR-beta in angiogenesis and vessel stability. *FASEB Journal* **23** 153–163.
- Zimmermann RC, Hartman T, Bohlen P, Sauer MV & Kitajewski J 2001 Preovulatory treatment of mice with anti-VEGF receptor 2 antibody inhibits angiogenesis in corpora lutea. *Microvascular Research* **62** 15–25.

Received 7 July 2009

First decision 17 August 2009

Revised manuscript received 18 September 2009

Accepted 28 September 2009