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Angiogenesis and vascular function in the ovary

Running title: Ovarian angiogenesis

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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. The expression of fibroblast growth factor 2 (FGF2) however in the cow is more dynamic than that of VEGFA with a dramatic up-regulation during the follicle-luteal transition. This up-regulation 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, 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.

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. These vessels remain quiescent until there is an angiogenic stimulus such as hypoxia or wounding, which then up-regulates pro-angiogenic 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 pro-angiogenic 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 pro-angiogenic factors include fibroblast growth factor 2 (FGF2), VEGFA, plaletet 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 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 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 follicle-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).

It is however, unclear what stimulates VEGFA expression since hypoxia induced factor 1α ($HIF_{1\alpha}$) (a transcription factor induced by hypoxia and potent inducer of VEGFA) was absent from pre-antral follicles (Duncan *et al*. 2008). It is also unlikely to be gonadotrophins since 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 follicule 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 follicle 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, approximately 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 follicle 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 (Zeleznik *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, although 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 VEGF164 mRNA expression in bovine granulosa cells was up-regulated by oestradiol but down-regulated by progesterone, while these steroids had the opposite effect on VEGFA120 mRNA (Shimizu & Miyamoto, 2007). It is equally possible that the VEGF co-receptors, neurophilin-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). This 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 oestradiol 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 up-regulated during atresia in the marmoset (Thomas *et al*. 2008). Thus, the up-regulation of TSP1 might play a key role in follicular atresia by inhibited angiogenesis. Intriguingly, TSP1 expression was increased by LH in rat granulosa cells and were present in the early corpus luteum (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 corpus luteum 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 α1, β1 and β2 as well as nidogen, perlecan and focimatrix), while the ECM associated with the vasculature is principally composed of collagen IV and laminin β2 (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 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 vascular smooth muscle cells during the latter stages of follicular development. These cells through there contractile properties are likely to influence the follicular blood flow as well as stabilising the vasculature. Further investigation into on 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 up-regulates 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 up-regulate 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 peri-ovulatory follicle in preparation the intense angiogenesis that occurs after ovulation.

During the peri-ovulatory 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 up-regulation of eNOS and inducible NOS (iNOS) in the thecal vasculature (Zackrisson *et al*. 1996). However, this is more likely to be an oestradiol mediated up-regulation rather than the effect of LH since oestradiol 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, $HIF_{1\alpha}$ is up-regulated in the peri-ovulatory follicle of marmosets (Duncan *et al*. 2008) and in the collapsed follicle of pigs (Boonyaprakob *et al*. 2005) which suggests that the tissue is hypoxic. Since hCG was a more potent stimulator of $HIF_{1\alpha}$ than hypoxia itself in luteinising granulosa cells (van den Driesche *et al*. 2008), it is possible that the LH surge that induces $HIF_{1\alpha}$ expression directly. Thus it is possible that any increases in VEGFA following the LH surge are mediated through the induction of $HIF_{1\alpha}$ mRNA (Duncan *et al*. 2008). To date no studies have investigated $HIF_{1\alpha}$ expression in ruminants during the follicle-luteal transition.

Peri-ovulatory 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 up-regulated 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, Dow *et al*. 2002, Bakke *et al*. 2004, 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 peri-ovulatory 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 thrombospondin 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 peri-ovulatory follicle. In addition, it is increased by gonadotrophin stimulation (Madan *et al*. 2003) and this may occur through the $HIF_{1\alpha}$ pathway (Kim *et al*. 2009). ADAMTS1 might play a role in regulating endothelial cell invasion since it is transiently up-regulated 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 over-production 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 transmembrane 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 peri-vascular 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 peri-vascular 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, (Robinson *et al*. 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 neither case 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 follicle-luteal transition (Robinson *et al*. 2007), this emphasises the importance of FGF2 in controlling and possibly initiating luteal angiogenesis in the cow.

Pericytes and platelet-derived growth factor (PDGF) system

In a functional, mature vascular system, endothelial cells are supported by mural cells such as pericytes and vascular smooth muscle cells (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 platelet-derived growth factor BB (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 fore-front 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 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 (Chantrain *et al*. 2006). The second phase is when pericytes are recruited during vessel stablisation. 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 follicle-luteal transition in mice. Namely, mRNA expression of *Pdgfb* 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 (Robinson *et al*. 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 PDGFBB 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 PDGFBB alone stimulated CAM angiogenesis, when combined together, 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 corpus luteum 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, whilst 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 corpus luteum 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. Endothelial nitric oxide synthase (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 (ET-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 up-regulate *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 follicle-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?

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Figure Legends

Fig 1 Schematic representation of the temporal changes in the levels of angiogenic factors during the bovine follicle-luteal transition. The data are based on observations from (Goede *et al*. 1998, Berisha *et al*. 2000b, Hayashi *et al*. 2004, Berisha *et al*. 2006, Rosiansky-Sultan *et al*. 2006, Robinson *et al*. 2007, 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 is no data available in the cow, the [1]$HIF_{1\alpha}$ and [2]PDGFB 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.

Fig 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 on day 3-4 (developing, early CL) and then day 8-12 (mature CL). The data are mean + sem; a<b; $P<0.05$.

Fig 3 A working hypothesis of the angiogenic processes and their regulation during luteal vascularisation in the cow. (A) In the peri-ovulatory 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 (TGFβ) 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 re-connect 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.

Fig 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 connect with each other, form tubules and recruit pericytes and blood flow is re-initiated. 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 re-connected, there is a decrease in FGF2 concentrations and then capillary beds are reconstructed. Consequently, blood flow can increase and plasma concentrations of progesterone increase.

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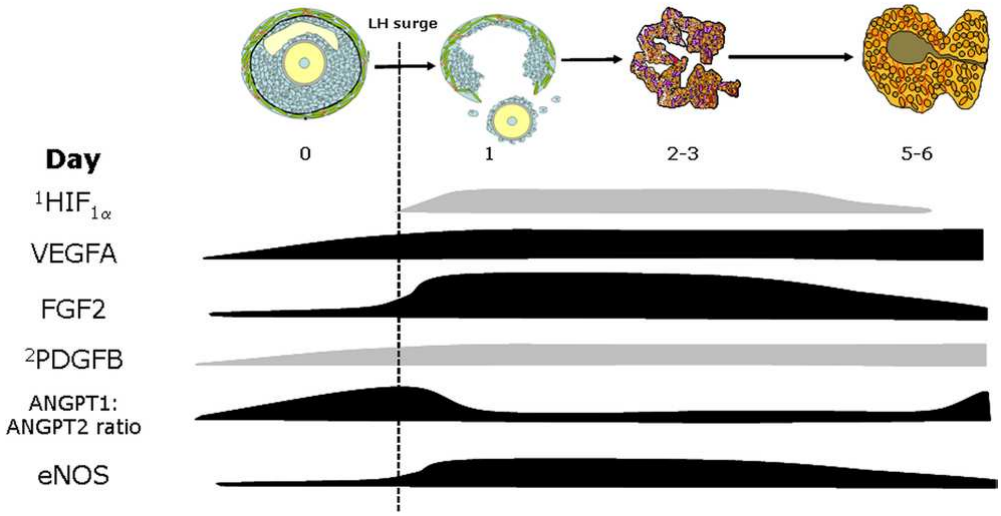
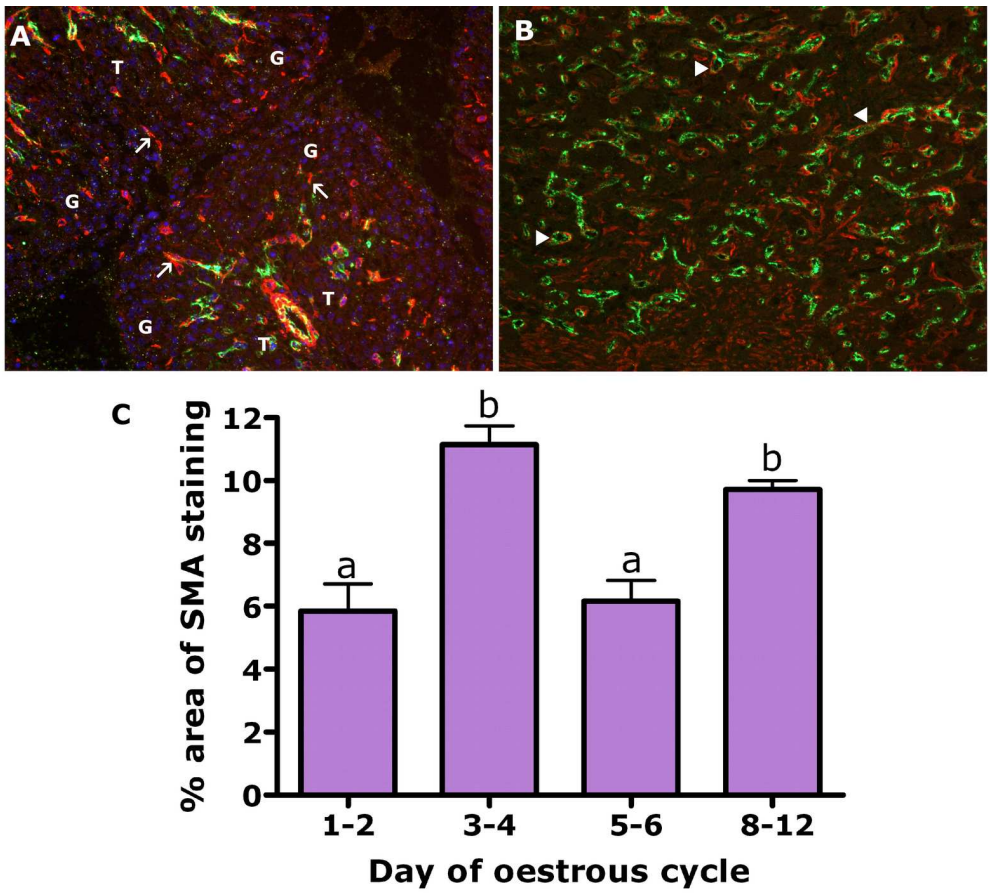
Table 1: The principal angiogenic factors and their associated properties.

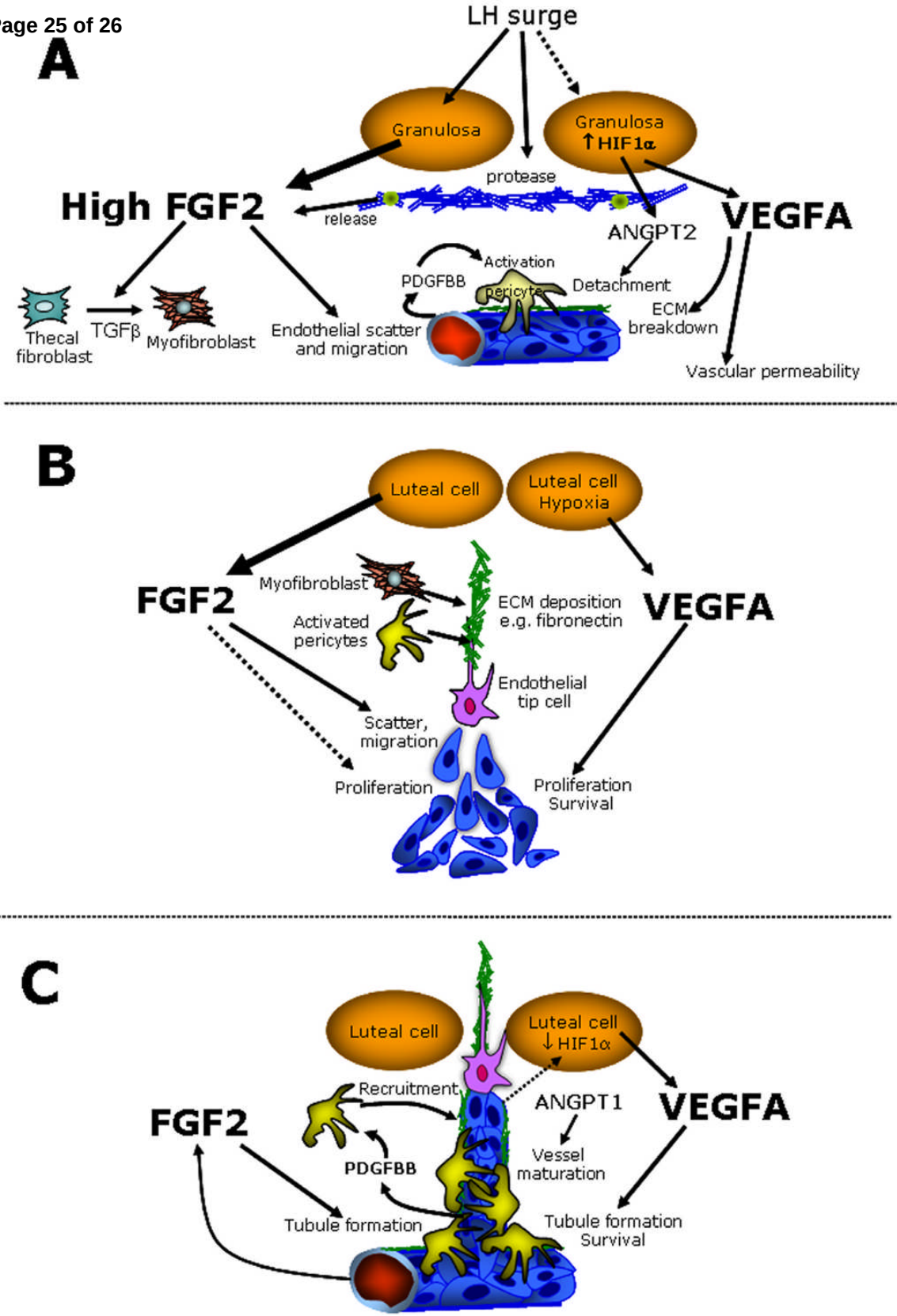
Ligand	Isoforms	Biochemical properties	Receptor/cellular target	Functions	References
Pro-angiogenic growth factors					
VEGFA	$VEGFA_{121}$ $VEGFA_{145}$ *$VEGFA_{165}$ $VEGFA_{189}$ $VEGFA_{206}$	Binds to heparin and ECM (except $VEGFA_{121}$) VEGFA121, 145 and 165 are secreted and soluble while VEGFA189, 206 are cell associated	VEGFR1 (Flt) (signalling capability are unresolved) VEGFR2 (KDR) (tyrosine kinase activity)	Stimulates endothelial proliferation, migration and tube formation. Vascular permeability factor	Ferrara *et al.* 2003
FGF2	18kD (cytoplasmic) 22-34kD (nuclear)	Heparin binding	FGFR1-4 (tyrosine kinase activity)	Stimulates endothelial proliferation and migration Mitogen for fibroblasts Cell differentiation	Presta *et al*. 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 *et al.* 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 *et al.* 1997
Anti-angiogenic factors					
Thrombospondin	TSP1 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	$\alpha v\beta 3$ integrin	Inhibits endothelial cell proliferation and migration	Wahl *et al*. 2005

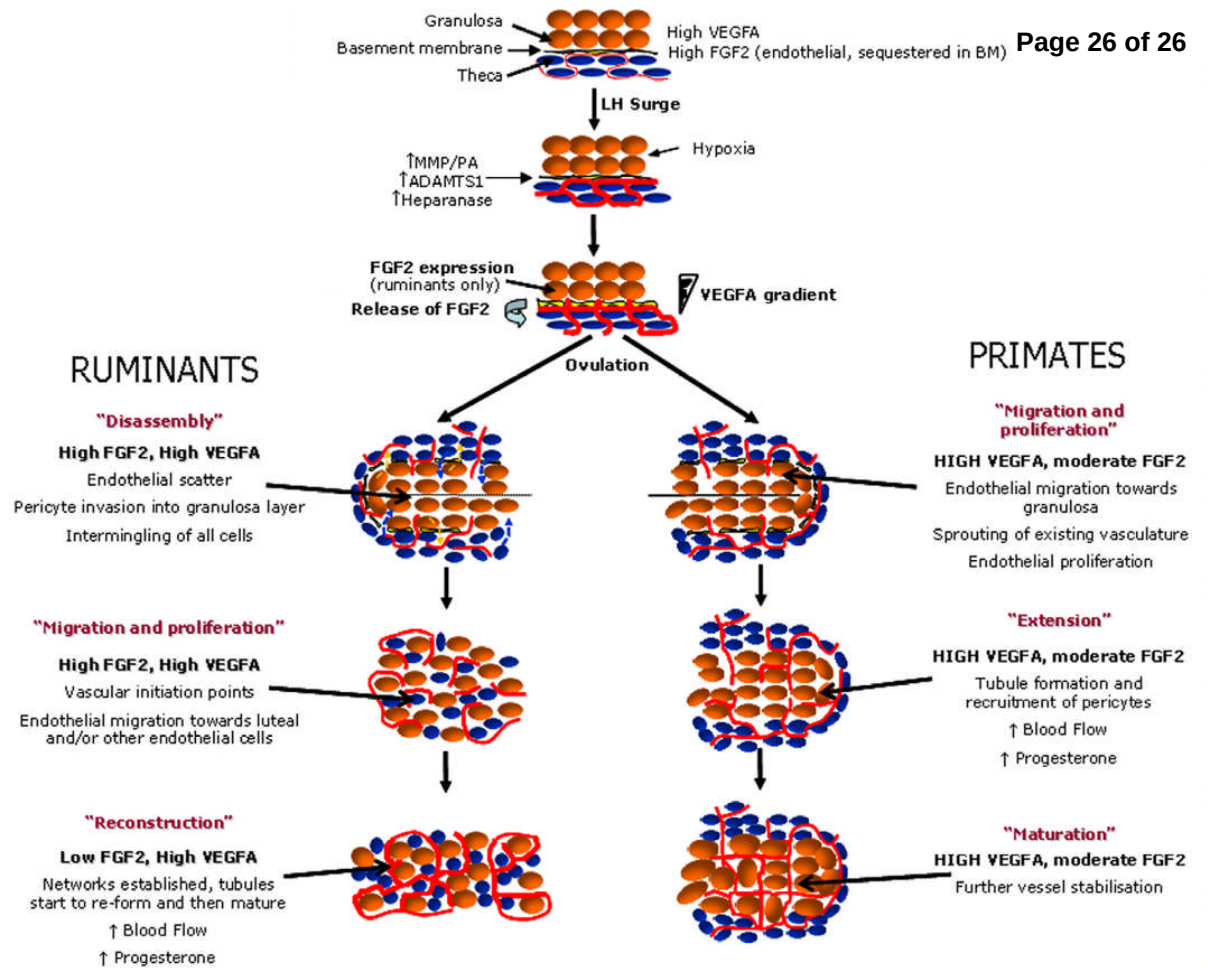


Schematic representation of the temporal changes in the levels of angiogenic factors during the bovine follicle-luteal transition. The data are based on observations from (Goede et al. 1998, Berisha et al. 2000b, Hayashi et al. 2004, Berisha et al. 2006, Rosiansky-Sultan et al. 2006, Robinson et al. 2007, 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 is no data available in the cow, the 1HIF1α 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.
42x22mm (600 x 600 DPI)



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 on day 3-4 (developing, early CL) and then day 8-12 (mature CL). The data are mean + sem; a<b; P<0.05.
66x60mm (600 x 600 DPI)


A
LH surge
Granulosa
Granulosa
↑HIF1α
protease
release
High FGF2
VEGFA
ANGPT2
Activation
PDGFBB
pericyte
Detachment
ECM
breakdown
Thecal
fibroblast
TGFβ
Myofibroblast
Endothelial scatter
and migration
Vascular permeability
B
Luteal cell
Luteal cell
Hypoxia
Myofibroblast
FGF2
ECM deposition
e.g. fibronectin
VEGFA
Activated
pericytes
Endothelial
tip cell
Scatter,
migration
Proliferation
Proliferation
Survival
C
Luteal cell
Luteal cell
↓HIF1α
Recruitment
ANGPT1
VEGFA
FGF2
Vessel
maturation
PDGFBB
Tubule formation
Tubule formation
Survival


Granulosa
Basement membrane
Theca
High VEGFA
High FGF2 (endothelial, sequestered in BM)
LH Surge
Hypoxia
↑MMP/PA
↑ADAMTS1
↑Heparanase
FGF2 expression
(ruminants only)
Release of FGF2
VEGFA gradient
Ovulation
RUMINANTS
PRIMATES
"Disassembly"
High FGF2, High VEGFA
Endothelial scatter
Pericyte invasion into granulosa layer
Intermingling of all cells
"Migration and proliferation"
HIGH VEGFA, moderate FGF2
Endothelial migration towards granulosa
Sprouting of existing vasculature
Endothelial proliferation
"Migration and proliferation"
High FGF2, High VEGFA
Vascular initiation points
Endothelial migration towards luteal and/or other endothelial cells
"Extension"
HIGH VEGFA, moderate FGF2
Tubule formation and recruitment of pericytes
↑ Blood Flow
↑ Progesterone
"Reconstruction"
Low FGF2, High VEGFA
Networks established, tubules start to re-form and then mature
↑ Blood Flow
↑ Progesterone
"Maturation"
HIGH VEGFA, moderate FGF2
Further vessel stabilisation