Corpus luteum development and angiogenesis

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Summary

Development of the corpus luteum (CL) is vital for the early increase in postovulatory progesterone that drives embryo development and the successful establishment of pregnancy. Following ovulation, rapid and dramatic cellular reorganisation and intense angiogenesis are required to support the timely transition to progesterone secretion. In addition to inherent physiological challenges, this process is sensitive to any imbalance in metabolism or health, particularly in the modern high yielding dairy cow. This makes luteal inadequacy a common cause of poor early embryo development and low conception rates in dairy cows. This review will explore the potential mechanisms that underlie this disorder. It will describe the impact that the pre-ovulatory follicle infrastructure, gonadotrophin support and size of the LH surge have on subsequent luteal development and function. A crucial component for rapid luteal growth is the formation of new blood vessels or angiogenesis. Several recent studies have highlighted the critical importance of fibroblast growth factor 2 (FGF2) in regulating this process. In particular, FGF2 has a profound influence on endothelial cell sprouting and branching. There is increasing evidence that pericytes, immune cells and platelets are also integral regulators of luteal angiogenesis and development. The complex and dynamic nature of luteal development means that it is likely to be sensitive to potential inadequacies particularly, in the high producing dairy cow.

Novel insights into the importance of adequate luteal function in the establishment of pregnancy

Conception rates of dairy cows are stubbornly less than 40% in many countries placing an enormous financial burden on dairy farmers. The majority of the losses that occur during the preimplantation period are due to poor embryonic development. The lagging embryo produces

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markedly reduced levels of the maternal recognition of pregnancy signal, interferon tau and fails to prevent luteolysis. Mann and Lamming (2001) eloquently showed that a delayed rise in circulatory progesterone (luteal inadequacy) resulted in the underdevelopment of embryos and lower concentrations of interferon tau. This demonstrated the crucial importance of the synchrony between the corpus luteum (CL), uterus and embryo that is necessary for the establishment of pregnancy (Wathes *et al*. 2002, Robinson *et al*. 2008b, Bridges *et al*. 2013).

The transition from a preovulatory follicle to a fully functioning CL is a remarkable and dynamic biological process requiring a tightly coordinated series of events following ovulation. These processes enable rapid luteal growth (0.5g to 5g within 8 days) and production of sufficient progesterone to support the developing embryo. This growth rate is only rivalled by the most aggressive tumours and is totally dependent on the rapid and extensive generation of new blood vessels, or angiogenesis (Reynolds and Redmer 1999). One important aspect of luteal development is that progesterone synthesis is 1000-fold greater than follicular oestradiol production (Wiltbank *et al*. 2012). The dramatically increased steroidogenic demand, at least in part, explains the necessity for phenomenal luteal growth rate. Interestingly, a major factor associated with plasma progesterone concentration on day 5 is luteal weight (Fig. 1a; Green *et al*. 2005, Mann 2009). This is, in turn, is influenced by the total luteal vasculature (Mann *et al*. 2007, Robinson *et al*. 2008b), which increases nearly 10-fold in size over the first 10 days (Fig. 1b). These steps occur in a tightly coordinated short timeframe and thus it is not surprising that in dairy cows producing >30 litres of milk a day that subtle deficiencies occur post ovulation resulting in the subsequent failure of pregnancy. Recently, a mathematical ordinal differential equation modelled bovine luteal development. This demonstrated that luteal growth required an appropriate balance of growth (proliferation and/or hypertrophy) between steroidogenic luteal and stromal cells. Moreover, this model showed that subtle changes in the proliferation rates of endothelial cells (low or high) would lead to 'pathological' luteal growth (Prokopiou *et al*. 2014).

The underlying mechanisms behind low circulatory progesterone concentrations have generated much controversy. This has centred on whether, in the high-producing dairy cow, it is due to increased metabolism of progesterone by the liver or the inability of the CL to synthesise progesterone. There is little doubt that progesterone can be metabolised more rapidly in high-yielding cows (Wiltbank *et al*. 2006). However, there are numerous studies linking low postovulatory progesterone concentrations to inadequate embryo development in non-lactating dairy cows (Mann and Lamming 2001, Robinson *et al*. 2005). Also, Endo *et al*. (2012) reported that lactating cows had larger CL and greater concentration of progesterone during the mid-luteal phase compared with non-lactating counterparts. These studies build on the work by Shelton *et al*. (1990), which demonstrated that cows with inherent subfertility had decreased progesterone synthetic capacity and reduced response to luteotrophic hormones *in vitro*. Collectively, this provides evidence that suboptimal circulatory progesterone concentration in lactating dairy cows cannot be solely due to increased metabolism of progesterone but is also the result of inherent inadequacies in the synthetic capacity of the luteal tissues.

Formation of the corpus luteum

Programming of the preovulatory follicle

The preovulatory follicle provides the structural and functional framework from which the CL rapidly develops. The concept of the preovulatory follicle programming the subsequent development of the CL has been suggested for several years (Inskeep 2004) but this idea is now

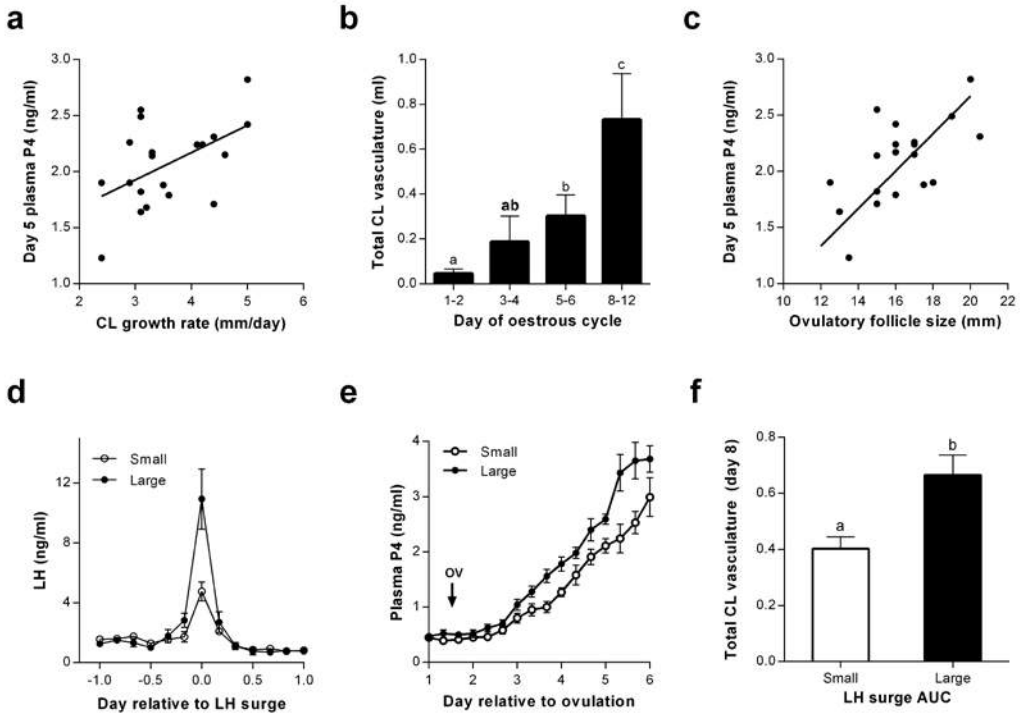

Fig. 1 The developing bovine corpus luteum and its potential influencing factors. (a) The rate of luteal growth during metoestrus was positively correlated with plasma progesterone concentrations ($r^2 = 0.27$, $n = 20$, $P < 0.05$). (b) shows the ten-fold increase in luteal vasculature during the formation of the CL (abc; $P < 0.05$). (c) Plasma progesterone concentrations on day 5 were correlated with the maximum size of the ovulatory follicle ($r^2 = 0.327$, $n = 18$, $P < 0.01$). (d-f) compares the effect of small *vs*. large LH surge on (e) subsequent plasma progesterone concentrations and (f) total vascularisation of the CL. Cows with a larger LH surge exhibited an earlier rise in postovulatory progesterone ($P < 0.01$) and increased total luteal vasculature (ab; $P < 0.01$). Data adapted from (Robinson *et al*. 2005, Robinson *et al*. 2006).

being revisited (Lonergan 2011). It is clear that "good" follicle quality for luteal function is not simply related to its size or oestradiol production capabilities (Wiltbank *et al*. 2011). For example, the relationship between peak oestradiol concentrations and subsequent luteal function remains inconclusive, with reports of positive associations (Jinks *et al*. 2013), while others have observed none (Robinson *et al*. 2005, Lynch *et al*. 2010, Bridges *et al*. 2012). However, the premature ovulation of dominant follicles with GnRH resulted in markedly reduced luteal size (Lamb *et al*. 2001, Vasconcelos *et al*. 2001, Mussard *et al*. 2007, Busch *et al*. 2008). More importantly, the manipulation of the follicular wave such that the preovulatory follicle naturally ovulates at a smaller size (Fig. 1c; Robinson *et al*. 2005) resulted in reduced postovulatory progesterone concentrations. Despite their being no difference in luteal diameter, these CL were lighter in weight (Robinson *et al*. 2005). It is important to note that a shorter follicular phase (< 4 days) is associated with proper timing of postovulatory luteinisation and progesterone production (Robinson *et al*. 2005, Starbuck *et al*. 2006) and increased likelihood of pregnancy (Gorzecka *et al*. 2011). However, reduced postovulatory plasma progesterone was noted if there was a very short (< 1.5 days) follicular phase (Bridges *et al*. 2010). Thus, it is much more likely

that a fully functional follicle requires an appropriate hormonal environment (e.g. supporting gonadotrophin) to reach physiological maturity at an optimal size (e.g. 13-16mm) and associated with a short follicular phase (Lynch *et al*. 2010, Wiltbank *et al*. 2011). Intriguingly, dairy heifers, which have good fertility, exhibit a shorter time from luteolysis to ovulation than dairy cows (Wiltbank *et al*. 2006). Collectively, this would suggest that the ovulatory follicular infrastructure and its subsequent ability to rapidly develop after ovulation will determine luteal competence. It could be that insulin-like growth factor 1 (IGF1) is an integral endocrine signalling molecule linking metabolic status with ovarian function. It is known that plasma IGF1 concentrations are also low in high milk-producing cows (Pushpakumara *et al*. 2003, Lucy 2008), and that IGF1 stimulates granulosa cell proliferation and oestradiol production (Webb *et al*. 2004). Furthermore, IGF1 increases progesterone secretion from luteal cells (Green *et al*. 2007). Thus, any deficiencies in plasma IGF1 are likely to compromise follicular quality and luteal function (Wathes *et al*. 2002).

Preovulatory vascularisation

It is feasible that the degree of follicular vascularisation plays a crucial role in creating this fully functional ovulatory follicle. Firstly, the follicular vascular bed in the theca layer provides the framework from which the luteal vasculature develops. Secondly, there is an active accumulation of pro-angiogenic growth factors in the follicular fluid (e.g. vascular endothelial growth factor A [VEGFA] and fibroblast growth factor 2 [FGF2]) during follicular maturation (Berisha *et al*. 2000, Robinson *et al*. 2007). Presumably, this accumulation provides the necessary stimulation for the intense period of angiogenesis that occurs after ovulation (Robinson *et al*. 2009b). Indeed, greater vascular perfusion in the preovulatory follicle was associated with increase oocyte competence, *in vitro* embryo development and ultimately, pregnancy rates (Siddiqui *et al*. 2009).

The vasculature is limited to the theca layer with an abundance of capillaries lying adjacent to the basement membrane (Robinson *et al*. 2009b). The morphology of the follicular vasculature changes considerably during its development. The initial vascularisation of the theca occurs through the budding and sprouting of endothelial cells (EC) from the interstitial blood vessels. In antral follicles, vessels enlarge (driven by increased metabolic demands) through elongation, duplication and sinusoidalisation. In the final stages of follicular maturation, there is a shift toward functional changes such as vessel stabilisation (e.g. pericyte recruitment) and capillary dilation (Macchiarelli *et al*. 2006). This is then likely to enhance the endocrine function of the preovulatory follicle in preparation for ovulation. However, there are no studies describing the relationship between the degree of follicular vascularisation and subsequent ability of the CL to develop.

Gonadotrophin support during follicular phase

FSH and LH are the principal regulators of antral follicular development and are key components for creating the appropriate supporting hormone environment for follicular development (Scaramuzzi *et al*. 2011). There is strong evidence that LH pulse rate is reduced when cows are in negative energy balance (Canfield and Butler 1990). Furthermore, it is the absolute level of gonadotrophic support rather than its pulsatile secretion that is important for ovulatory follicle development (Campbell *et al*. 2007). There has been extensive research into the use of gonadotrophin-based hormones to synchronise ovulation or promote luteal function (Peters 2005). However, there are only a few studies that have investigated the effects of supplementing the follicular phase with low doses of gonadotrophins. Those studies, though, have shown that administering low doses of crude gonadotrophic preparations (e.g. eCG) during the follicular phase can improve postovulatory progesterone concentrations and conception rates (Baruselli *et*

al. 2004, Souza *et al*. 2009). However, Pulley *et al*. (2013) reported that similar use of low-dose eCG before AI had no effect on progesterone and pregnancy rates. Thus, further investigations into the therapeutic benefits of treating cows with pure gonadotrophin preparations at non ovulatory doses during the follicular phase on subsequent luteal function are required.

Antral follicle count

There is increasing evidence that the antral follicle count (AFC) is an indication of ovarian reserve in cattle. Furthermore, heifers with a low AFC have antral follicles with reduced oestradiol-producing competence (Ireland *et al*. 2009) and these ovulatory follicles develop into similar-sized CL but with suboptimal function in terms of progesterone production (Jimenez-Krassel *et al*. 2009). This luteal inadequacy was associated with diminished LH responsiveness, lower StAR protein concentrations and an inherent deficiency in the capacity of granulosa cells to undergo luteinisation (Jimenez-Krassel *et al*. 2009). It is unknown whether these CL have an inadequate degree of vascularisation due to reduced follicular and luteal angiogenesis.

Role of the LH surge

The LH surge is the crucial process that triggers oocyte maturation, ovulation and the conversion of the follicle into progesterone-producing luteal tissue. The latter event involves numerous, highly orchestrated cellular and molecular processes (e.g. cell proliferation, differentiation of granulosa and theca cells into luteal cells, basement membrane degradation, changes in steroidogenesis and initiation of angiogenesis). Thus, it is highly feasible that the size of the endogenous LH surge could influence subsequent luteal development and function. Further analysis of the data in Robinson *et al*. (2005) revealed that those cows naturally ovulating with a larger LH surge (area under LH peak) had a much earlier rise in postovulatory progesterone than those with a 'smaller' LH surge (Fig. 1d-e). This is further supported by similar observations by Wolfenson *et al*. (2004). Moreover, cows treated with 25mg porcine LH (pLH) in late proestrous had greater luteal area and 50% increased progesterone concentrations when compared to those given 8 mg pLH (Ree *et al*. 2009).

The LH surge upregulates a plethora of genes (eg. prostaglandin endoperoxide synthase and progesterone receptor) that induce a series of cellular and biochemical processes that culminate in ovulation. Several events, (eg. breakdown of the basement membrane, immune-like responses) are integral for initiating luteal angiogenesis (Robinson *et al*. 2009b). Furthermore, LH dramatically increases the mRNA and protein concentrations of the pro-angiogenic growth factor, FGF2 within the follicle during the ovulatory period in cows (Berisha *et al*. 2006, Robinson *et al*. 2007, Gilbert *et al*. 2011). Similarly, LH increased luteal production of FGF2 *in vitro* (Robinson *et al*. 2007, Laird *et al*. 2013). The current evidence indicates that the other principal pro-angiogenic growth factor, vascular endothelial growth factor (VEGFA), is less influenced by the LH surge in the cow (Robinson *et al*. 2007, Berisha *et al*. 2008). This is in contrast to that reported in humans (van den Driesche *et al*. 2008) and mice (Kim *et al*. 2009). Intriguingly, it was noticed that dairy cows with a larger LH surge, subsequently had increased total luteal vascularisation on day 4 after ovulation (Fig. 1f).

Impact of disease on luteal function

Postpartum uterine disease has inevitable consequences for fertility. As well as any direct effects within the uterus, it is well established that endometritis can exert adverse effects on

ovarian function through a range of mechanisms, including the action of infection-induced endotoxins in the ovary (Sheldon *et al*. 2009). Uterine infection has been associated with reduced follicular development, oestradiol and progesterone concentrations (Sheldon *et al*. 2002, Williams *et al*. 2008). Intriguingly, lipopolysaccharide (LPS), a component of the bacterial cell wall, is present in follicular fluid (Herath *et al*. 2007). Furthermore, LPS and associated Pam3CysSerLys4 acting via toll-like receptor (TLR) 2 and 4 disrupt bovine granulosa cell steroidogenesis (Herath *et al*. 2007, Price *et al*. 2013) without affecting granulosa cell survival (Shimizu *et al*. 2012). Importantly, Holstein Friesian cows with increased vaginal discharge (indicative of uterine infection) exhibited increased incidence of aberrant reproductive cyclicity and reduced milk progesterone concentrations on day 5 at 1st insemination (Fig. 2; Mann GE, unpublished observations). Thus, it is clear that the reproductive consequences of endometritis are much broader than local effects within the uterus. Similar effects have been observed during infection by other diseases. For example, experimental infection with bovine viral diarrhoea (BVD) virus impaired follicular development (Grooms *et al*. 1998), and reduced circulating oestradiol and progesterone concentrations (Fray *et al*. 1999, Fray *et al*. 2002). The dramatic effect of an experimental BVD infection, timed to induce peak viraemia during the ovulatory period, on subsequent progesterone production is shown in Fig. 3. Furthermore, experimental treatment of lactating cows with endotoxins during the follicular phase resulted in a delayed postovulatory progesterone rise (Lavon *et al*. 2008), while intravenous infusion of LPS during the mid luteal phase temporarily reduced luteal size, progesterone concentrations and luteal blood flow (Herzog *et al*. 2012). Thus, a number of different diseases have been demonstrated to exert substantial negative effects on luteal function in the cow. However, the mechanisms by which these distant bacterial infections affect luteal function remained unresolved. One possibility is that LPS, which is known to affect endothelial cell function (Aplin *et al*. 2014), could affect luteal angiogenesis and warrants further research.

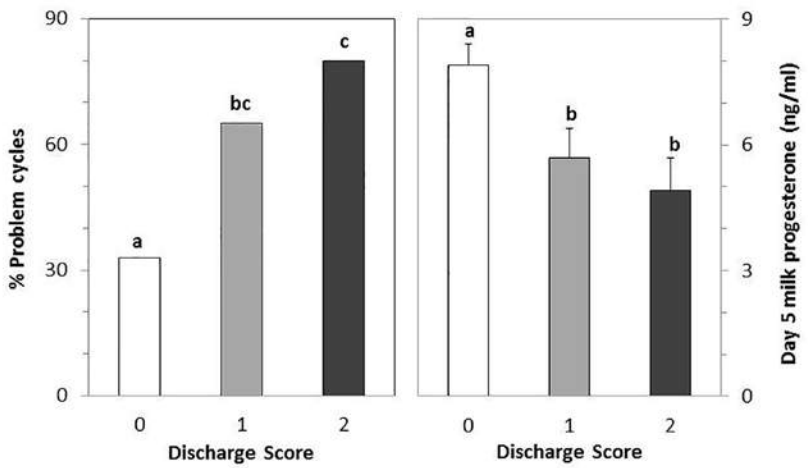

Fig. 2 Post-partum uterine infections adversely affect subsequent ovarian function. There is increased incidence of a) reproductive cycle problems and b) compromised post-ovulatory luteal function in lactating Holstein Friesian dairy cows with vaginal discharge classified as either 0 (absent or clear with no pus or fetid aroma; n = 61), 1 (present with some pus but no fetid odour; n = 50) or 2 (present with considerable pus and a fetid odour; n = 10). ab $P < 0.05$; ac $P < 0.01$.

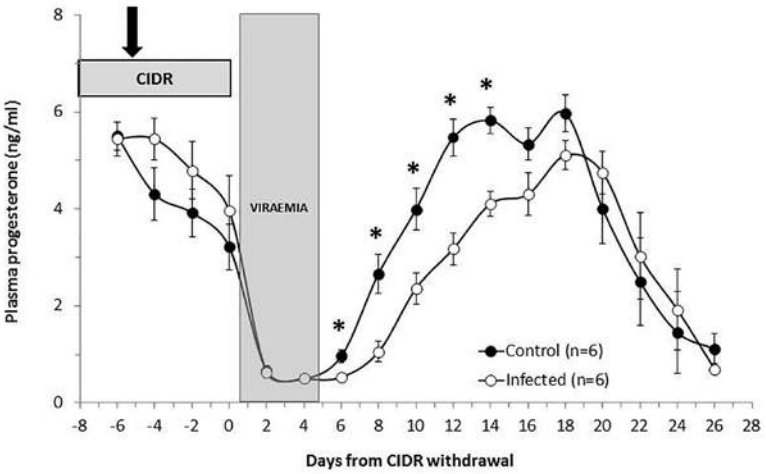

Fig. 3 Effect of viral infection on luteal function. There was a significant reduction in post-ovulatory progesterone concentrations in Holstein Friesian cows acutely infected with bovine viral diarrhoea virus (block arrow) at a time designed to induce peak viraemia during the ovulatory period. Data is adapted from Fray *et al*. (2002).

Control of luteal development and angiogenesis

Local regulation of luteal angiogenesis and critical importance of FGF2

Luteal angiogenesis is the product of detailed cell-cell communication between several cell types, resulting in the creation of an extensive and complex vascular network that is critical to tissue function. VEGFA is a potent mitogen that promotes the growth of vascular endothelial cells (EC) and is thought to be an important regulator of luteal angiogenesis. VEGFA also stimulates EC migration, survival and vascular permeability (Ferrara *et al*. 1998). In the bovine CL, VEGFA protein is expressed by luteal steroidogenic cells (Berisha *et al*. 2000) throughout the luteal phase (Robinson *et al*. 2007). Its importance to luteal angiogenesis is underlined by the results of neutralisation experiments, whereby inhibition of VEGFA activity postovulation reduced luteal vascularisation and progesterone production in non-human primates (Wulff *et al*. 2001). Similarly in the cow, VEGFA antibody delivered directly into the ovulated follicle reduced subsequent luteal size and steroidogenic function by up to 50% (Yamashita *et al*. 2008).

The important pro-angiogenic properties of fibroblast growth factor 2 (FGF2) are also key to luteal function. FGF2 is more potent than VEGFA at stimulating the proliferation of corpus luteum-derived capillary EC in culture (Gospodarowicz *et al*. 1986, Zalman *et al*. 2012) and increased progesterone secretion when infused into bovine CL (Liebermann *et al*. 1996). Furthermore, the luteal FGF2 content was very high (eg. $<$100ng/g) in the recently ovulated, collapsed follicle but returned to basal concentrations within a few days (Robinson *et al*. 2007, Zalman *et al*. 2012). Simultaneously, there is a spatial shift in the expression FGF2 from endothelial to steroidogenic cells and back again (Schams *et al*. 1994, Berisha *et al*. 2006). This all indicates that FGF2 plays a dynamic and crucial role in the initiation of luteal angiogenesis.

We have developed a novel culture system that simulates luteal angiogenesis (Robinson *et al*. 2008a, Laird *et al*. 2013) and provides an invaluable tool to study its regulation (Fig. 4). In this physiologically-relevant culture system, multiple luteal cell types (steroidogenic cells, ECs,

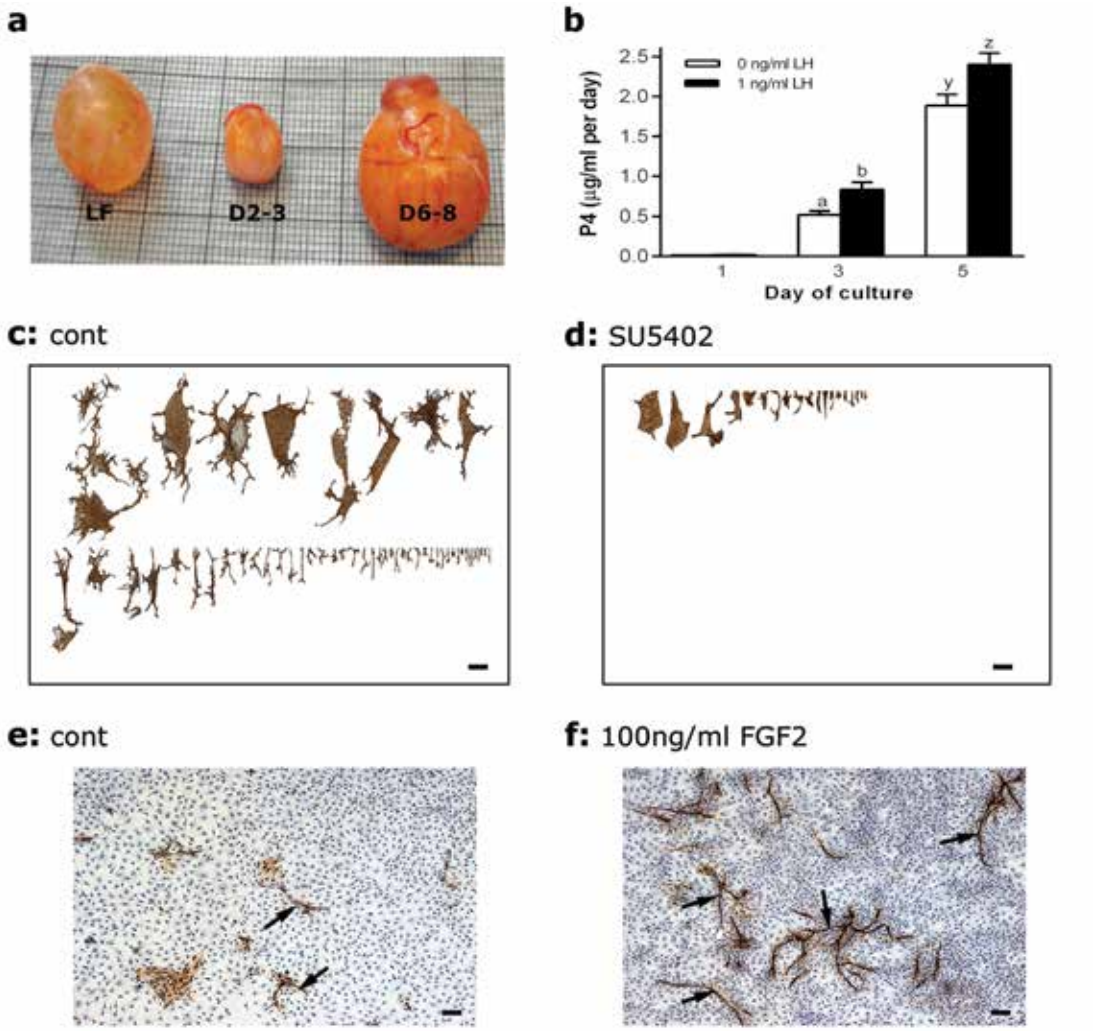

Fig. 4 A physiologically-relevant culture system that mimics luteal angiogenesis and function. (a) shows the dynamic ovarian changes that occur during the follicular-luteal transition in cows. A large antral follicle (LF) following ovulation will form a corpus haemarrhagicum (D2-3), which will then undergo rapid growth (D6-8). (b) shows increased progesterone production by bovine luteinising follicular cells over time in culture. LH (1ng/ml) further increased progesterone concentrations (ab; yz; $P < 0.05$). (c) and (d) show bovine luteal cells that were treated with (c) control or (d) 1μM SU5402 [FGFR1 inhibitor] on days 3-6 of culture. The EC networks/islands (brown staining), present on day 6, were selected and then sorted according to size. SU5402 markedly reduced the degree of EC sprouting. (e) and (f) show endothelial cell (EC) network (brown staining) formation in bovine luteinising follicular cells treated with (e) control medium or (f) 100ng/ml FGF2. On day 5 of culture, FGF2 treatment stimulated the earlier branching and elongation of EC networks (arrows) while EC localised to undeveloped islands (asterisks) in the control. The scale bars represent 100μm. Data and images adapted from (Woad *et al*. 2012, Laird *et al*. 2013).

fibroblasts and pericytes) isolated from large antral follicles or corpora haemorrhagica are co-cultured. Importantly, progesterone production increased over time, and was further stimulated by LH (Fig. 4b) confirming that the luteal cells remain steroidogenically active. Critically, EC migration, proliferation and intricate network formation is supported in this system and the

degree of network formation is highly responsive to angiogenic stimuli (Robinson *et al*. 2008a, Woad *et al*. 2009). In these EC networks, there are multiple branch points and interconnections that develop over time (Woad *et al*. 2012, Laird *et al*. 2013).

Specific inhibition of VEGF receptor 2 (VEGFR2) and FGF receptor (FGFR) activity decreased EC network formation and this was most dramatic in response to FGFR inhibition, whereby EC networks were reduced by 95% (Woad *et al*. 2009). Similarly, the sequestration of FGF2 with small molecule 27 (a fragment of thrombospondin) inhibited FGF2-induced EC proliferation and migration (Zalman *et al*. 2012). Further investigation by Woad *et al*. (2012) showed that EC were most sensitive to FGFR inhibition during the early stages of EC network development, which further supports a role for FGF2 in the initiation of luteal angiogenesis (Fig. 4c-d). This corresponds to the period characterised by EC sprouting. Indeed, blocking the activity of FGFR1 altered the pattern of EC growth as well as its extent and resulted in a dramatic reduction (90%) in EC branching (Fig. 4c-d; (Woad *et al*. 2012). More importantly, FGF2 promoted the precocious transition of undeveloped EC islands into organised EC networks with an increased number of branch points (Fig. 4e-f; (Laird *et al*. 2013). In order for an endothelial cell to sprout away from the established vasculature, it needs to form a tip EC, with a different phenotype. The tip EC then migrates toward the angiogenic stimulus. This would suggest that FGFR signalling is crucial for endothelial tip cell formation and thus directional EC migration and vascular sprouting (De Smet *et al*. 2009). However, there is still remarkably little known about how FGF2 could induce endothelial tip cells. The Notch signalling pathway plays an essential role in EC tip formation with tip cells expressing the membrane-bound delta-like ligand 4 (DLL4) (Gridley 2007). The DLL4 binds to Notch receptors on adjoining EC and in so doing converts them into stalk cells (Eilken and Adams 2010). Recently, we have detected DLL4 and Notch4 receptor in the bovine preovulatory follicle and CL (Robinson *et al*., unpublished observations). Interestingly, in marmosets, when DLL4 was inhibited during the periovulatory period a hypervascularised CL developed that showed decreased progesterone production (Fraser *et al*. 2012). The patterning of EC observed by Fraser *et al*. (2012) showed several similarities to that induced by high concentrations of FGF2 (Laird *et al*. 2013). Inhibition of Notch signalling using γ-secretase inhibitors reduced EC network formation and progesterone production (Robinson *et al*., unpublished observations). Thus, further investigation into the interplay between FGF2 and the Notch signalling system is certainly warranted.

It is tempting to speculate that since FGF2 promotes precocious luteal angiogenesis *in vitro* (Woad *et al*. 2009, Woad *et al*. 2012) and luteal *FGF2* mRNA are reduced in lactating cows (Pretheeban *et al*. 2010) that inadequate local concentrations of FGF2 limit luteal angiogenesis *in vivo*. Thus, there is an urgent need to determine whether the direct FGF2 infusion into the collapsed follicle could promote luteal angiogenesis, blood flow and progesterone production. This proof-of-concept study would provide evidence as to whether stimulation of luteal angiogenesis can overcome luteal inadequacy *in vivo*.

One other factor that we have investigated is secreted protein, acidic, cysteine-rich (SPARC) protein. SPARC is a matrix-associated glycoprotein that regulates differentiation, cell-cell communication and cell migration whose function is changed by targeted proteolytic degradation (Yan and Sage 1999). In bovine luteinising granulosa cells, transforming growth factor B (TGFB) and fibronectin dose-dependently induced the precocious expression of SPARC (Joseph *et al*. 2012). Furthermore, SPARC protein expression is markedly increased immediately post ovulation in cows (Joseph *et al*. 2012) and is abundantly present in luteal and endothelial cells of the developing bovine CL (Robinson *et al*. 2007, Wiltbank *et al*. 2012). Similarly, *Sparc* mRNA expression was increased seven-fold during the hCG-induced luteinisation of murine granulosa cells (McRae *et al*. 2005). Functionally, a plasmin proteolytic fragment of SPARC

(KGHK) increased EC network formation *in vitro,* which was due to both increased number and size of the EC clusters. Unexpectedly, KGHK peptide also stimulated progesterone production in these cells (Joseph *et al*. 2012). In fact, this stimulation was greater than that observed with LH. Based on this, it is likely that SPARC and/or KGHK containing peptides play an integral role in luteal development and function and might be novel targets for the treatment of luteal inadequacy.

Emerging role of other cell types in luteal angiogenesis

Pericytes (mural cells) are an integral component of the microvasculature and form intimate contacts with endothelial cells. A key component in the activation of pericytes during angiogenesis is the induction of platelet-derived growth factor receptor (PDGFR) B expression. The potential for pericytes to regulate ovarian function in rodents was demonstrated by a reduction in number of CL and progesterone production in response to intra-ovarian PDGFR blockade (Sleer and Taylor 2007, Kuhnert *et al*. 2008). It is highly likely that pericytes are active players in the early as well as later stages of angiogenesis (Ozerdem and Stallcup 2003). This is particularly noticeable during the follicle-to-luteal transition, where pericytes appear to migrate into luteinising-granulosa layer ahead of EC in sheep (Redmer *et al*. 2001) and cattle (Amselgruber *et al*. 1999, Robinson *et al*. 2009b). Furthermore, pericytes form a large proportion of proliferating cells in the early CL and secrete VEGFA (Redmer *et al*. 2001). It has been suggested that these pericytes are synthetic and lay down fibronectin strands along which EC can migrate (Robinson *et al*. 2009b). The inherent plasticity of pericytes has hindered research into their exact role in regulating angiogenesis. However, it is recognised that FGF2 stimulates their proliferation (Laird *et al*. 2013) and suppresses their contractile vascular smooth muscle cell phenotype (Papetti *et al*. 2003). Moreover, FGF2 can activate pericytes by inducing *Pdgfrb* expression (Nissen *et al*. 2007). This is likely to result in pericytes having an elongated, contracted appearance (Laird *et al*. 2013) that is associated with a highly migratory phenotype. It is likely that TGFB which is produced by bovine luteinising follicular cells (Joseph *et al*. 2012) also influences the phenotypic appearance of these mural cells (Shirakihara *et al*. 2011). The activated pericytes are then recruited to the developing vasculature. Intriguingly, using an *in vitro* culture system, smooth muscle actin (SMA)-positive mural cells are often localised in close proximity of EC islands (Fig. 5). These *in vitro* dual localisation studies have shown that (1) the abundance of mural cells around the EC islands increases over time, (2) some mural cells become an integral component of the EC islands, (3) there are several distinct mural cell phenotypes with some having finger-like projections connecting to EC and other mural cells, and (4) in the latter stages of culture, the mural cells have a more flattened, contracted shape and surround EC islands (Fig. 5; Laird *et al*. 2013). In order to further elucidate these complex cell-cell communications it will be necessary to differentially label EC and mural cells in culture and track their interactions using time-lapse microscopy.

Importantly, Woad *et al*. (2009) demonstrated that *in vitro* PDGFRB blockade with a receptor tyrosine kinase inhibitor greatly attenuated the ability of EC to develop into networks. Moreover, the early stages of *in vitro* luteal angiogenesis were the most sensitive to PDGFRB inhibition. Namely, the migration and formation of the initial EC islands (Robinson *et al*. 2009a). These observations are supported by previous experiments showing that luteal PDGFRB signalling blockade using an adenoviral approach reduced microvessel EC density by nearly 50% as well as pericyte coverage of those vessels (Kuhnert *et al*. 2008). Preliminary observations have shown that PDGF-BB dose-dependently increased EC network formation by 3-fold but only in the absence of exogenously added FGF2 and VEGFA (Robinson *et al*. unpublished observations).

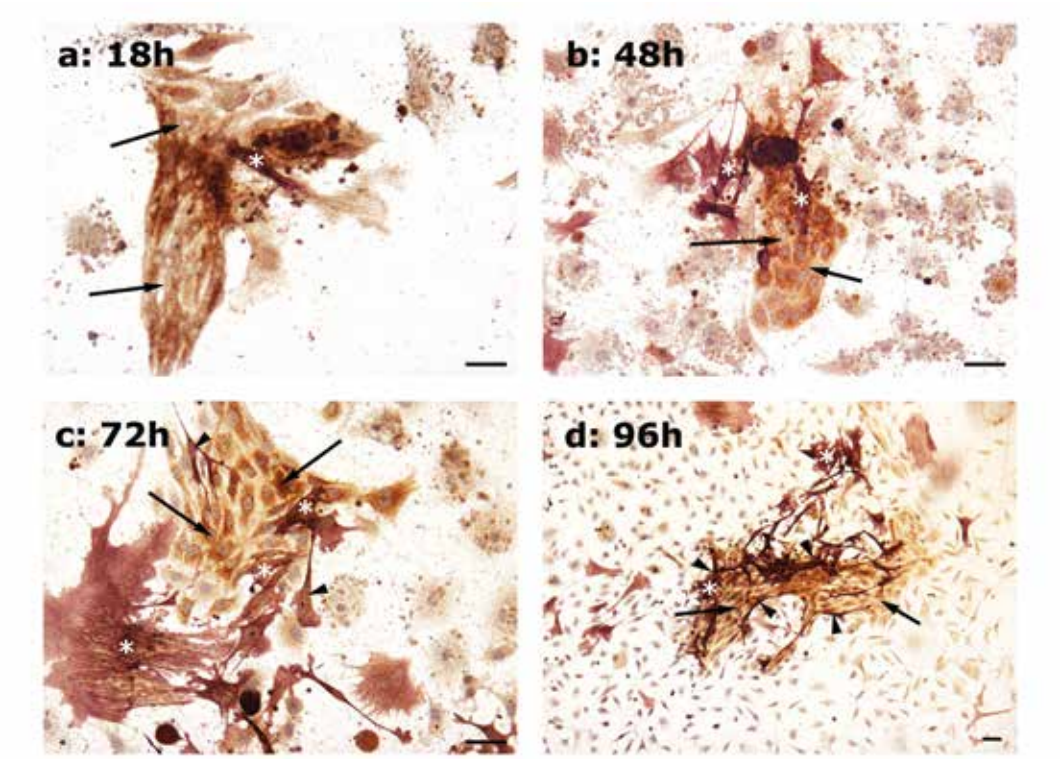

Fig. 5 The dual localisation of endothelial and mural cells in a luteal angiogenesis culture system. Cells were fixed and dual immunostained for von Willebrand factor (endothelial cell (EC) marker; brown) and smooth muscle actin (mural cell marker; red) at (a) 18h; (b) 48h; (c) 72h and (d) 96h. Initially, small islands of EC (indicated by black arrow) with a few mural cells (singular flattened cells with cell projection; indicated by white asterisk). These mural cells increased in abundance over time across the whole well, but were frequently associated with EC islands. Often, the morphological appearance of the mural cells was different when they were aligned around or within an EC island. Namely, they had a contracted, elongated phenotype (arrowheads) and this was more noticeable as time progressed. The scale bars represent 100μm.

It has been long-recognised that ovulation has numerous characteristics similar to an inflammatory response (Silvester and Luck 1999). A recent review by Shirasuna *et al*. (2012) highlighted the exciting and potential role of immune cells in ovarian angiogenesis. While, there are some definite species differences, the current evidence strongly indicates that immune cells (e.g. neutrophils, macrophages and/or lymphocytes) infiltrate into the developing bovine CL. This is likely due to both the increased vascular permeability and stimulation from proinflammatory signals (e.g. prostaglandin E2; interleukin 8 (IL8); (Jiemtaweeboon *et al*. 2011)) but also might be regulated by angiogenic factors (Shirasuna *et al*. 2012). There is increasing evidence that these immune cells are active players in luteal angiogenesis. For example, activated polynuclear neutrophils and IL8 stimulated the formation of luteal-derived endothelial cell capillary-like structures (Jiemtaweeboon *et al*. 2011). These observations provide mechanistic insight into induced lymphopaenia causing luteal dysfunction in cattle (Alila and Hansel 1984). Turner *et al*. (2011) showed that macrophages were required for maintaining vascular integrity during luteal development in mice. Similarly, bone marrow-derived vascular progenitor cells and

macrophages contributed to neovascularization during murine luteal formation (Kizuka *et al*. 2012). The role of macrophages might be the most important since these cells are the most abundant and they can be differentiated into the "tissue remodelling" M2 phenotype by the luteal microenvironment. In turn, M2 macrophages would synthesise various pro-angiogenic factors including VEGFA and FGF2 to promote angiogenesis. Collectively, this highlights the need for more research to better understand the cell–cell communication that occurs between immune and endothelial cells during luteal development.

Platelets are the initial responders to any vascular damage (e.g. wound) and in so doing provide a flexible delivery system of an enormous reservoir of angiogenic factors (Nurden 2011). Indeed, numerous platelets are transiently present and adhere to the damaged vasculature in the newly formed CL (Cavender and Murdoch 1988, Furukawa *et al*. 2007) and are capable of stimulating EC migration (Furukawa *et al*. 2007, Battinelli *et al*. 2011). However, these studies failed to demonstrate whether platelets are activated and can promote luteal angiogenesis. Preliminary experiments in our laboratories have shown that platelet-rich plasma increased the area of EC networks. There is a further layer of complexity, in that platelets compartmentalise pro- and anti-angiogenic factors within their α-granules (Battinelli *et al*. 2011, Kamykowski *et al*. 2011). These angiogenic factors are then differentially released depending on the stimuli. For example, adenosine diphosphate (ADP) stimulates VEGFA release from platelets while thromboxane A2 induces the preferential release of anti-angiogenic endostatin (Battinelli *et al*. 2011). Intriguingly, cows in severe negative energy balance have markedly reduced platelet count and mean cell volume (Wathes *et al*. 2009). This offers the exciting potential that the targeted manipulation of platelets could be utilised to increase availability of pro-angiogenic factors within the ovulatory wound and enhance luteal development and function.

Blood flow and luteal development

The extensive vascularisation of the CL results in it receiving a very high blood flow (Reynolds and Redmer 1999). After ovulation, luteal blood flow, as measured by colour Doppler ultrasonography, increases in parallel with luteal volume and coincides with the rise in plasma progesterone (Acosta *et al*. 2003, Herzog *et al*. 2011) and this is dependent on a LH surge occurring (Hayashi *et al*. 2006). Indeed, it has been proposed that luteal blood flow is a better indicator of luteal function than its size (Matsui and Miyamoto 2009, Herzog *et al*. 2010). Moreover, Luttgenau *et al*. (2011) found that cows with lower mid-luteal phase progesterone concentrations had reduced luteal blood flow during this period. Treatment with hCG 6 days after ovulation transiently increased luteal blood flow and sustained increased progesterone concentrations (Beindorff *et al*. 2009). However, there was no difference in luteal blood flow during luteal development between those cows who became pregnant and those that did not (Herzog *et al*. 2011). This is in contrast to observations in the Mediterranean buffalo where pregnant buffalo cows had greater luteal blood flow and progesterone on days 10 and 15 after insemination than their non-pregnant counterparts (Vecchio *et al*. 2012). One explanation is that despite enormous technological advances, accurate measurement of luteal blood flow remains a challenge and any important differences between groups might be subtle. Furthermore, colour Doppler ultrasonography does not detect the smaller blood capillaries that are abundant throughout the internal luteal tissue (Beindorff *et al*. 2009). An enhanced approach to measure luteal microvasculature blood flow is using real-time contrast-enhanced ultrasound combined with a microbubble contrast agent (Russo *et al*. 2009, Sboros *et al*. 2011).

Conclusion

Luteal inadequacy is a common cause of poor conception rates in dairy cows. Rapid luteal development and associated intense angiogenesis requires the timely and coordinated interplay between different cell types and growth factors. There is increasing evidence that preovulatory follicular infrastructure and its LH support programme subsequent luteal development and progesterone production. This opens up new therapeutic strategies whereby improving the quality of the preovulatory follicle with, for example low doses of LH, could improve not only follicular, but also, luteal function.

Rapid and extensive angiogenesis is essential for luteal growth and function. Recent studies have elucidated that FGF2, rather than VEGFA, appears to play the dominant role in controlling luteal angiogenesis in cows. More importantly, FGF2 has a profound influence on EC sprouting and branching. There is increasing emerging evidence that pericytes, immune cells and platelets are also integral regulators of luteal angiogenesis and development. The complex and dynamic nature of luteal development makes it sensitive to any metabolic (e.g. low plasma IGF1 concentrations) or immune-related influences. Additionally, any inherent deficiency in local growth factor support, particularly in those associated with angiogenesis, could have profound effects on the ability of the CL to develop sufficiently. All these factors will be particularly pertinent in the high producing dairy cow.

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