The expression, regulation and function of secreted protein, acidic, cysteine-rich in the follicle–luteal transition

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Abstract

The role of the tissue remodelling protein, secreted protein, acidic, cysteine-rich (SPARC), in key processes (e.g. cell reorganisation and angiogenesis) that occur during the follicle–luteal transition is unknown. Hence, we investigated the regulation of SPARC in luteinising follicular cells and potential roles of SPARC peptide 2.3 in a physiologically relevant luteal angiogenesis culture system. SPARC protein was detected mainly in the theca layer of bovine pre-ovulatory follicles, but its expression was considerably greater in the corpus haemorrhagicum. Similarly, SPARC protein (western blotting) was up-regulated in luteinising granulosa but not in theca cells during a 6-day culture period. Potential regulatory candidates were investigated in luteinising granulosa cells: LH did not affect SPARC (P > 0.05); transforming growth factor (TGF) B1 (P < 0.001) dose dependently induced the precocious expression of SPARC and increased final levels: this effect was blocked (P < 0.001) by SB505124 (TGF receptor 1 inhibitor). Additionally, fibronectin, which is deposited during luteal development, increased SPARC (P < 0.01). In luteal cells, fibroblast growth factor 2 decreased SPARC (P < 0.01) during the first 5 days of culture, while vascular endothelial growth factor A increased its expression (P < 0.001). Functionally, KGHK peptide, a SPARC proteolytic fragment, stimulated the formation of endothelial cell networks in a luteal cell culture system (P < 0.05) and increased progesterone production (P < 0.05). Collectively, these findings indicate that SPARC is intricately regulated by pro-angiogenic and other growth factors together with components of the extracellular matrix during the follicle–luteal transition. Thus, it is possible that SPARC plays an important modulatory role in regulating angiogenesis and progesterone production during luteal development.

Introduction

The transition from follicle to corpus luteum (CL) is a critical process in the reproductive cycle and is necessary for progesterone production in order to establish pregnancy (Robinson et al. 2008b). Moreover, even subtle inadequacies in the rise of post-ovulatory progesterone have detrimental effects on early embryo development and fertility (Mann & Lamming 2001). It is highly likely that the rate of luteal development (e.g. tissue reorganisation, rapid proliferation and extensive angiogenesis) will influence subsequent luteal function (Mann & Lamming 2001, Robinson et al. 2009).

The follicle–luteal transition is intricately controlled by the coordinated actions of endocrine and local auto/paracrine factors. LH is the key endocrine signal required for the initiation of the ovulatory process and activates a cascade of events that culminate in extracellular matrix (ECM) breakdown, follicular rupture, differentiation and remodelling (Richards 2001, Irving-Rodgers et al. 2006, Robinson et al. 2009). LH principally mediates this by activating adenyl cyclase, which then regulates numerous proteins including cyclooxygenase, amphiregulin and proteins involved in steroidogenesis as well as a plethora of growth factors (Richards et al. 1998, Espey & Richards 2002, McRae et al. 2005). Angiogenesis is another key process initiated by the LH surge and is essential for rapid luteal growth and function (Robinson et al. 2009). Much attention has focused on the role that vascular endothelial growth factor A (VEGFA) plays as VEGFA is transiently up-regulated during ovulation in rats (Koos 1995) and primates (van den Driesche et al. 2008). More importantly, VEGFA blockade prevents luteal vascularisation, growth and progesterone production (Wulff et al. 2001, Hazzard et al. 2002). However, there is now increasing evidence that, at least in the cow, fibroblast growth factor 2 (FGF2) plays the more dynamic role in the initiation of luteal angiogenesis (Berisha et al. 2006, Robinson et al. 2007, Woad et al. 2009). At the same time, there is extensive tissue and ECM remodelling; however, very little is known about its underlying mechanisms (Robinson et al. 2009). It is known that luteinising granulosa and theca cells are inter-dispersed during CL development in ruminants. Furthermore, components of the ECM (e.g. fibronectin) may actually
stimulate this remodelling (Irving-Rodgers et al. 2006). Indeed, fibronectin greatly increased luteal angiogenesis in vitro (Robinson et al. 2008a). As transforming growth factor B (TGFB) is a crucial factor regulating tissue remodelling in wound healing (Pepper 1997), TGFB is likely to play a similar role in luteal development. Indeed, bovine luteal cells secrete large amounts of TGFB1 (Gangrade et al. 1993), which is known to modulate granulosa cell proliferation and differentiation (Fazzini et al. 2006). These effects are mediated by the coordinated action of two TGF serine/threonine kinase receptors, namely type I (TGFBR1;ALK5) and type II (TGFBR2), which then activate SMAD proteins (Heldin et al. 1997). However, the targets of TGFB1 in the developing CL are relatively unknown.

Secreted protein, acidic, cysteine-rich (SPARC) protein is a matrix-associated glycoprotein mainly known for regulating cellular processes such as differentiation, cell–cell communication and cell migration (Yan & Sage 1999). Experimental evidence indicates that SPARC is present in tissues undergoing remodelling, wound healing, angiogenesis and morphogenesis (Sage et al. 1989, Lane et al. 1994, Phan et al. 2007). Thus, SPARC is a prime regulatory candidate for the continual and physiological remodelling observed in the ovary. This is further supported by observations that in other tissues, TGFB (Wrana et al. 1991, Fujita et al. 2002), VEGFA (Kato et al. 2001) and fibronectin (Fujita et al. 2002) up-regulated SPARC expression. However, there is very limited information on SPARC in the ovary and none on its regulation or potential function. An additional complexity in the biology of SPARC is that it is proteolytically degraded (e.g. by plasmin), which releases SPARC fragments with different biological functions (Lane et al. 1994, Yan & Sage 1999). For example, mature SPARC has an inhibitory effect on protein synthesis, proliferation and endothelial cell shape (Hasselaar & Sage 1992). While a particular proteolytic fragment of SPARC, namely peptide 2.3, stimulated endothelial cell proliferation and angiogenesis with the amino acid sequence Lys-Gly-His-Lys (KGHK) being critical for this activity (Lane et al. 1994). Proteolysis of SPARC could be a particularly important aspect in ovary, as during ovulation there is extensive production of proteases including plasmin (Dow et al. 2002).

Collectively, these studies led us to the overarching hypothesis that SPARC protein is up-regulated during the follicle–luteal transition that it plays a key role in cocooordinating the various cellular processes that occur during this period. Hence, we investigated the expression of SPARC at this time and tested the hypothesis that LH, TGFB1 and fibronectin up-regulate SPARC protein expression in luteinising follicular cells. While in luteal cells (from a recently formed CL), we tested the hypothesis that SPARC protein expression is differentially regulated by FGF2 and VEGFA. The final objective was to determine the degree to which the SPARC peptide, KGHK, would stimulate luteal endothelial cell network formation and progesterone production.

**Results**

**SPARC protein is up-regulated in the collapsed follicle**

In order to establish the presence of SPARC protein during the bovine follicle–luteal transition, an immunohistochemical assessment was performed. SPARC was mainly localised to the theca layer of large antral follicles and this immunostaining intensity decreased with increasing follicular size (Fig. 1A, B and C). In contrast, SPARC protein was generally absent from the granulosa cells but was present in three out of 12 large antral follicles of differing sizes (Fig. 1A, B and C). Interestingly, in the collapsed follicle, there was a visible increase in the intensity of SPARC immunostaining compared with large antral follicles (Fig. 1E, F), which was mainly localised to the small and large luteal cells. The control sections treated with mouse IgG were blank (Fig. 1D).

**Experiment 1: LH does not increase SPARC protein expression in luteinising follicular cells**

A specific band for mature SPARC protein (43 kDa) was detected by western blot (Fig. 2A). In luteinising granulosa cells, SPARC was absent or poorly expressed at 48 and 96 h but was maximally expressed at 144 h (P<0.001). A similar pattern was observed in LH (100 ng/ml)-treated cells (P>0.05). In contrast, forskolin (10 μM) reduced SPARC expression at 144 h compared with both control and LH-treated cells (P<0.001; Fig. 2B). Progesterone production by granulosa cells increased with time (P<0.001) in culture confirming that these cells were luteinising and was greatly increased by forskolin at all time points (P<0.001; Fig. 2C). There was also an overall effect of LH (P<0.01) on progesterone concentrations in the spent media of luteinising granulosa cells (55 and 31% greater at 96 and 144 h respectively; Fig. 2C).

In contrast to granulosa cells (Fig. 2D), SPARC (43 kDa) was detectable throughout culture in luteinising theca cells. Quantification showed that SPARC protein levels tended to be lower at 48 h compared with 96 and 144 h in culture (P=0.07). Similarly, levels of SPARC protein were numerically lower in theca cells treated with forskolin, although this failed to reach significance (P=0.09; Fig. 2E). Moreover, LH had no effect on levels of SPARC protein levels in luteinising theca cells. Progesterone production, however, that increased with time (P<0.05) was also increased by treatment with both LH (P<0.01) and forskolin (P<0.001; Fig. 2F).
Experiment 2: TGFB1 acting via the receptor TGFBR1 up-regulates SPARC protein in luteinising granulosa cells

In control cells, SPARC protein (43 kDa; Fig. 3A) increased over time (P < 0.001) and was increased by TGFB1 (P < 0.001) at all time points (Fig. 3B). Furthermore, there was a TGFB1 × time interaction (P < 0.01), which indicated that the stimulatory effect of TGFB1 was greater at 48 and 96 h than at 144 h (Fig. 3B). In the absence of TGFB1, SB505124 reduced (P < 0.001) SPARC protein expression by two- and four-fold at 96 and 144 h respectively (Fig. 3C). Importantly, SB505124 prevented the TGFB1 (1 ng/ml)-induced up-regulation of SPARC protein (P < 0.001), but this effect was less at the higher dose of 10 ng/ml TGFB1 (Fig. 3C). Overall, progesterone production by luteinising granulosa cells increased twofold with time in culture (P < 0.001) but was reduced by treatment with TGFB1 (P < 0.01) and, surprisingly, also with the inhibitor to TGFBR1 (i.e. SB505124; P < 0.001; Fig. 3D and E). Indeed, there was a TGFB1 × SB505124 interaction (P < 0.001), which showed that the dose-dependent reduction in progesterone production caused by TGFB1 treatment was only evident in the absence of SB505124 (Fig. 3D).

Experiment 3: fibronectin increases SPARC protein in luteinising granulosa cells

Western blot analysis demonstrated that fibronectin coating of the culture well had a profound stimulatory effect on the level of mature SPARC protein (43 kDa; P < 0.001; Fig. 4) and this effect being dose dependent at all points. An additional band for SPARC protein at 37 kDa (possible proteolytic fragment) was detected, which has been previously reported (Lane et al. 1994). Interestingly, its appearance increased with time and fibronectin (particularly at 10 µg/well) induced an earlier detection of this band. There was a small but consistent stimulatory effect of fibronectin coating (10 µg) on progesterone production (P < 0.05; data not shown).

Experiment 4: FGF2 and VEGFA differentially regulate SPARC protein expression in bovine luteal cells

FGF2 and VEGFA are the principal factors stimulating ovarian angiogenesis (Robinson et al. 2009), thus their effects on SPARC protein were investigated. SPARC protein (43 kDa) was detected in bovine luteal cells on day 1 of culture (Fig. 5A) and increased with time.
There was a FGF2 × culture time interaction (P < 0.001; Fig. 5B) such that FGF2 treatment reduced the mature SPARC protein levels on days 1, 3 and 5 in comparison to control wells (P < 0.001) but had minimal effects on days 7 and 9. There was also a VEGFA × culture time interaction (P < 0.05) but, in this case, SPARC protein levels were similar to controls in the first few days but were slightly increased during the later stages of culture. The analysis also showed that there was a FGF2 × VEGFA × culture time interaction (P < 0.05; Fig. 5B). Namely, in the absence of VEGFA, SPARC protein was decreased by FGF2 on days 1, 3 and 5, while in its presence, SPARC protein was only decreased by FGF2 on day 1 of culture.

**Experiment 5: KGHK peptide stimulates endothelial cell network formation and progesterone production in bovine luteal cells**

In all treatments, there was extensive proliferation as cells reached confluence by 7–9 days of culture. Under basal conditions, limited endothelial cell networks were observed in control wells but treatment with KGHK peptide increased the formation and number of small tubule-like structures (Fig. 6A and C). There were extensively branched endothelial cell networks across all treatments under angiogenic stimulated conditions (Fig. 6B and D). Quantification of von Willebrand factor (VWF) staining showed that, under basal conditions, KGHK peptide increased the total area of endothelial cell network formation (P < 0.05; Fig. 6E) at both doses. The total length of endothelial networks was numerically increased with KGHK peptide treatment but this did not reach significance (P = 0.07). Combined FGF2 and VEGFA treatment increased the area of endothelial cell networks by threefold, and under these conditions, KGHK peptide had no effect on total area of endothelial cell networks (Fig. 6E). In control wells, progesterone production increased (P < 0.001) with time (by five- to six-fold; Fig. 7). Under basal conditions, treatment with KGHK peptide increased progesterone production by as much as twofold (P < 0.001; Fig. 7A), with similar effects at both doses. Combined FGF2 and VEGFA treatment further increased progesterone production, however, under these conditions; there was no significant effect of KGHK peptide treatment (Fig. 7B).

**Discussion**

Any inadequacies in the formation of the CL will lead to reduced progesterone production and fertility. The current study has demonstrated that SPARC might play an important role in the regulation of luteal development and function. For the first time, we have shown that SPARC protein is present in large antral follicles but is markedly up-regulated in the newly forming CL. Moreover, TGFB1 and fibronectin, but not LH, are potential candidate up-regulators of SPARC in luteinisng...
granulosa cells. Furthermore, SPARC was differentially regulated by the angiogenic factors, FGF2 and VEGFA. Treatment with the proteolytic fragment of SPARC (KGHK) promoted the formation of luteal endothelial cell networks and surprisingly it also increased progesterone production.

SPARC protein has been previously detected in the developing murine (Bagavandoss et al. 1998) and bovine CL (Robinson et al. 2007). However, this study showed for the first time that SPARC protein expression is markedly increased immediately post-ovulation in cows. This supports previous observations that Sparc mRNA was increased by several-fold during the hCG-induced luteinisation of murine granulosa cells (McRae et al. 2005). Collectively, these data indicate that SPARC may play an important role in luteal development. This is not surprising as SPARC is frequently associated with tissue remodelling (Sage et al. 1989, Phan et al. 2007) and SPARC and its proteolytic fragments have numerous different cellular roles such as contra-adhesion, proliferation and migration.

LH was investigated as a potential regulator of SPARC. However, in vitro LH had no effect on SPARC expression in either luteinising granulosa or theca cells despite LH-stimulating progesterone output. As the expression of LHCGR mRNA and protein is greatly reduced during luteinisation (Mamluk et al. 1998, Okuda et al. 1999) and LH had no effect on SPARC protein, it was important to determine LHCGR mRNA levels in luteinising granulosa cells. Indeed, LHCGR mRNA levels declined with time and were virtually undetectable by the end of culture when grown in 1% FBS (data not shown). Thus, we performed an additional experiment in which FBS concentrations were increased to 10%, which maintained LHCGR mRNA at higher levels and for a longer period of time. Even under these conditions, LH had still no effect on SPARC protein expression in luteinising granulosa cells (data not shown). Moreover, the addition of forskolin, which bypasses the LHCGR to directly increase intracellular cAMP, greatly enhanced progesterone production but actually decreased SPARC protein levels. Collectively, these observations strongly indicate that LH and/or the cAMP cascade are not directly responsible for the up-regulation of SPARC expression during luteinisation of bovine granulosa and theca cells.

An interesting observation was that SPARC protein was initially absent in luteinising granulosa cells, but after a few days of culture, its expression was induced. It is feasible that SPARC is switched on in granulosa cells once they have completed luteinisation, although treatment with forskolin actually suppressed SPARC protein expression. Alternatively, SPARC is up-regulated once granulosa cells have reached confluence. Indeed, increasing the FBS concentration to 10% (v/v) induced SPARC protein expression (data not shown), which may
have occurred by promoting cell proliferation and thus confluence. This hypothesis is supported by Magee et al. (2000) who showed that SPARC protein expression was induced by increasing cell density and thus confluence in human retinal pigment epithelial cells. SPARC is well known for its contra-adhesive properties and regulates the changeover from strongly adherent state to an intermediate state during the initiation of cell migration (Rempel et al. 2001). Thus, it is tempting to speculate that up-regulation of SPARC during follicle–luteal transition assists in loosening the strong adhesive connections between granulosa cells and allowing cells to invade (Goldblum et al. 1994). This would then enable the extensive spatial reorganisation and intermingling of granulosa and theca cells observed during luteal development in ruminants (Robinson et al. 2009).

Luteinisation can be considered as a wound repair process, leading to the formation of new tissue, rich in fibronectin (Silvester & Luck 1999). Indeed, in the early bovine CL, fibronectin forms a delicate network of fibrils that orientates the main axis of the capillary sprout (Amselgruber et al. 1999, Silvester & Luck 1999).

Additionally, TGFβ1 is a key regulator of proliferation, differentiation as well as wound healing (Pepper 1997). While the exact regulation of TGFβ1 during the ovulatory period remains unresolved, TGFβ1 was present in peri-ovulatory granulosa cells in pigs (Sriperumbudur et al. 2010), and bovine luteal cells produce an abundance of TGFβ1 protein (Gangrade et al. 1993). Hence, these factors were investigated as potential regulators of SPARC in luteinising granulosa cells. Indeed, both TGFβ1 and fibronectin coating dose dependently increased SPARC protein expression in granulosa cells as well as inducing its earlier appearance. This is supported by similar observations in non-ovarian cells (Wrana et al. 1991, Fujita et al. 2002). This study also showed that i) TGFβ1 is acting primarily via TGFβR1, as its specific inhibitor, SB505124, completely attenuated the stimulatory effects of 1 ng/ml TGFβ1. ii) Luteinising bovine granulosa cells produce endogenous TGFβ1 (or an alternative TGFβR1 ligand) as in the absence of exogenous TGFβ, SB505124 blocked the up-regulation of SPARC. iii) TGFβ1 decreased progesterone production in luteinising granulosa cells, which is in agreement with several studies in ruminants (Knight & Glister 2006, Zheng et al. 2009). However,
contrary to expectation, SB505124 also decreased progesterone production. The reasons for this are unclear, but SB505124 is also a partial inhibitor of p38-MAPK (Vogt et al. 2011), which is known to stimulate progesterone production in human granulosa cells (Seto-Young et al. 2011). iv) Fibronectin coating promoted the consistent appearance of an additional SPARC band at 37 kDa. Iruela-Arispe et al. (1995) also found the presence of a similar band (35 kDa) in the chorioallantoic membrane angiogenesis model and showed that this was due to plasmin. It is known that follicular plasmin concentrations increase dramatically during the LH surge in cows (Dow et al. 2002). Thus, the relationship between fibronectin and activity of specific proteases such as plasmin on SPARC biology warrants further investigation. Collectively, these results provide clear evidence that TGFβ1 and fibronectin are factors that are capable of up-regulating SPARC protein expression observed during luteinisation of granulosa cells.

The follicle–luteal transition period is characterised by extensive production of FGF2, VEGFA and intense angiogenesis (Robinson et al. 2009). Interestingly, SPARC protein was decreased by FGF2 during the earliest stages of the luteal cultures which agree with observations in human pulp cells (Shiba et al. 1998, Motamed et al. 2003). It is feasible that FGF2 stimulates angiogenesis by inducing the release of proteases (Flaumenhaft et al. 1992), which then induce the proteolysis of mature SPARC into its pro-angiogenic fragments. The high levels of VEGFA that are also present in the early CL (Robinson et al. 2007) might complement this by up-regulating SPARC protein expression (Fig. 5). This is supported by previous observations in which exogenous VEGFA increased SPARC protein expression.

Figure 6 KGHK peptide stimulates the development of luteal endothelial cell networks in vitro. (A, B, C and D) The formation of endothelial cell networks (as indicated by arrows) under basal (no exogenously added FGF2 or VEGFA; A and C) and angiogenic stimulated (1 ng/ml FGF2 with 1 ng/ml VEGFA; B and D) conditions. Treatment with KGHK peptide (100 μg/ml; C and D) increased the number of small endothelial cell clusters under basal conditions. Endothelial cells were immunostained brown for VWF (arrows) with scale bar, 100 μm. (E) Image analysis showed that KGHK peptide (μg/ml; P<0.05) increased total area of endothelial cells (μm²) under basal but not angiogenic stimulated conditions. Values are mean±s.e.m. (n=4) and statistical significance was tested by randomised block ANOVA.

Figure 7 KGHK peptide stimulates progesterone production (μg/ml per day) by bovine luteal cells. In both (A) basal and (B) angiogenic stimulated conditions, progesterone production increased over time (P<0.001). Under basal conditions, there was a time×treatment interaction (P<0.05) with KGHK peptide stimulating progesterone production on all days, but the effect was greater on days 5 and 9. However, under angiogenic stimulated (1 ng/ml FGF2 and 1 ng/ml VEGFA) conditions, KGHK peptide had no effect. Values are mean±s.e.m. (n=4) and statistical significance was tested by randomised block ANOVA.
in human umbilical vein endothelial cells (Kato et al. 2001). From these findings, it can be speculated that the regulation of SPARC protein involves a complex and intricate interplay between FGF2 and VEGFA.

For the first time, we show that the plasmin proteolytic fragment of SPARC (KGHK) increased endothelial cell network formation in CL-derived cells. This was due, in part, to increased number of endothelial cell clusters but also an increase in their size. Similar increased formation of endothelial cords has been reported in bovine aortic endothelial cells (Lane et al. 1994). The mechanism by which KGHK stimulated angiogenesis is unclear. SPARC binds to endothelial cells, although the exact identification of its cell surface receptor remains elusive. There is growing evidence to suggest that SPARC actions can be mediated through integrin, β1 (Weaver et al. 2008). Intriguingly, the copper binding domain of SPARC (which contains the KGHK sequence) interacts with integrin β1 (Weaver et al. 2008). When integrin β1 dimerises with integrin α subunits, it specifically binds to ECM proteins (e.g. fibronectin), which has profound stimulatory effects on luteal angiogenesis in vitro (Robinson et al. 2008a). Further investigations are required to elucidate the potential role of integrin β1 in mediating the stimulatory effects of KGHK peptide.

An unexpected and novel finding was that KGHK peptide stimulated progesterone production. Again, the mechanisms are unclear, although it is possible that KGHK peptide is acting through integrin β1 to promote the luteinisation of steroidogenic cells and/or their survival. Indeed, in this study, fibronectin increased progesterone production in luteinising granulosa cells and furthermore the copper-binding domain of SPARC promoted cell survival in murine lens epithelial cells (Weaver et al. 2008). Collectively, these observations strengthen our hypothesis that SPARC and/or KGHK containing peptides might play an important role in the development and function of the CL and might be novel targets for the treatment of luteal inadequacy.

In summary, the findings reported here demonstrate that SPARC protein expression is induced by TGFβ1 and fibronectin in granulosa cells undergoing luteinisation. Furthermore, SPARC was intracellularly regulated by the key angiogenic factors controlling luteal angiogenesis (FGF2 and VEGFA) in bovine luteal cells. Moreover, KGHK peptide stimulated the formation of luteal endothelial networks and progesterone production in vitro, suggesting a potential role for SPARC protein in regulating ovarian angiogenesis and steroidogenesis. In conclusion, these studies have identified SPARC as a potential novel regulator of luteal development.

Materials and Methods

All reagents were obtained from Sigma–Aldrich unless otherwise stated. LH (APP1743B, biopotency 1.06×oLH NIDDK-I-2) was a gift from Dr A F Parlow, NIDDK, California, USA, while TGFβ1 was purchased from R&D Systems (Reading, UK). Bovine ovaries were collected from a local abattoir and transported to the laboratory in PBS at ambient temperature.

**Immunohistochemical localisation of SPARC protein in bovine ovary**

Large antral (9–16 mm diameter; n = 12) and collapsed follicles (n = 9) were fixed in Bouin’s fixative for 6 h and embedded in paraffin wax. Tissue sections (5 μm) were dewaxed and rehydrated before antigen retrieval by boiling (0.01 M sodium citrate buffer, pH 6.0) for 10 min. Next, sections were washed twice for 5 min in PBS and endogenous peroxidase activity was blocked by incubating with 0.03% (v/v) hydrogen peroxide in methanol for 10 min. Then, tissue was blocked in 50 mg/ml BSA for 30 min and incubated with 2.5 μg/ml monoclonal mouse anti-bovine SPARC (Invitrogen) in a humidified chamber overnight at 4 °C. Negative controls were incubated with 2.5 μg/ml mouse IgG. The primary antibody was detected using the DAKO EnVision system (Dako, Ely, UK) and visualised with 3,3-diaminobenzidine (Dako). Sections were counterstained with haematoxylin and bright field pictures were taken using a Leica DM4000B microscope. The intensity of the immunostaining for SPARC was determined on a scale of 0 (absent) to 4 (strong) by assessment in an unbiased manner by a single individual.

**Experiment 1: effect of LH and forskolin on SPARC expression in luteinising follicular cells**

LH is the principal hormone regulating the follicle–luteal transition and thus was the most likely candidate to regulate SPARC expression during this time. Hence, this experiment focused on the effects of LH (100 ng/ml) and forskolin (10 μM) on SPARC expression in vitro in luteinising granulosa and theca cells collected from large antral follicles. A total of three cultures were performed as described below with each treatment performed in duplicate.

**Luteinising granulosa and theca cell culture**

For each culture, approximately ten large, healthy antral follicles (9–16 mm, clear fluid and visible vascularisation) were dissected, follicular fluid removed and follicles hemisected. Granulosa cells were harvested with an inoculation loop into DMEM/Ham F12 (1:1) medium and then manually dispersed and filtered through a 70 μm mesh filter. The theca sheet was peeled from the follicle and dispersed in 20 ml DMEM/Ham F12 (1:1) medium containing 100 mg/ml collagenase type IA, 50 mg/ml hyaluronidase type IS and 0.25 units/ml of DNase type IV in a shaking water bath at 37 °C. After 30 min, the digestion was inhibited by adding 1 ml FBS and cell clumps removed by filtration through a 70 μm mesh filter. Granulosa and theca cells were seeded separately in 12-well plates at 4×10^5 viable cells (determined by trypan blue exclusion (Tennant 1964)) per well in 2 ml DMEM/Ham F12 (1:1) medium containing 1% (v/v) FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 39 °C in a humidified incubator with 5% CO₂ in air. DMSO
was required to dissolve forskolin and equivalent concentrations of DMSO (0.02% v/v) were added to all other treatments. The spent medium was collected, stored at −20 °C for progesterone analysis and replaced every 48 h for up to a total of 144 h. At 48, 96 and 144 h, the cells were collected and prepared as described below and then analysed for SPARC protein by western blotting.

**Cell sample preparation for protein analysis**

At the end of culture, cells were removed by scraping and centrifuged at 13,000 g for 20 min at 4 °C. Cell pellets were re-suspended in 20 μl PBS containing protease inhibitor complete cocktail (Roche) and stored at −80 °C until analysis. Protein concentration was determined by Bradford assay (Bio-Rad).

**Western blot analysis for SPARC**

Protein samples (20 μg) were subjected to SDS–PAGE using a 12% (w/v) resolving gel. Samples were loaded in a random sequence with a positive control (theca cells) on each gel and the separated proteins were then electrotransferred to PVDF membrane (Bio-Rad). These were blocked with 5% (w/v) milk in PBS–TWEEN (PBS-T) for 2 h and then were blotted with 2 μg/ml monoclonal mouse anti-bovine SPARC (Invitrogen) and rabbit anti-human histone H3 (1:18,000; Abcam, Cambridge, UK) overnight at 4 °C. Membranes were then washed five times in PBS-T for 10 min each and then incubated with goat anti-rabbit (1:8000, Sigma) and sheep anti-mouse IgG (1:50,000, GE Healthcare, Amersham, UK) peroxidase conjugate for 1 h at RT. Following a PBS-T wash (5 × 10 min), membranes were visualised with an ECL system (GE Healthcare). The bands (SPARC and histone H3) were detected using the Molecular Imager FX (Bio-Rad) and quantified by densitometry using Quantity One Version 4.2 (Bio-Rad). The results are presented as SPARC:histone H3 ratio.

**Progesterone analysis**

Progesterone concentration in spent media was determined by ELISA (Ridgeway Science, Gloucestershire, UK) as described previously (Robinson et al. 2008a). The samples were analysed in duplicate and diluted 40- to 500-fold in PBS as appropriate. For luteinising granulosa and theca cells, the mean progesterone production was determined from duplicate wells at each time point with intra- and inter-assay coefficients of variation (CV) of 8.5 and 11.9% respectively.

**Experiment 2: effect of TGFβ on SPARC in luteinising granulosa cells**

In order to determine which factors were potentially up-regulating SPARC during the follicle–luteal transition, granulosa cells (n=3 cultures) were treated with TGFβ1 (at 1 or 10 ng/ml) in the presence or absence of SB505124 (2 μM). This compound specifically inhibits the ATP binding site of TGFBR1 and has been used extensively to block TGFBR1 activation (DaCosta Byfield et al. 2004). DMSO was required to dissolve SB505124 and equivalent concentrations of DMSO (0.1% v/v) were added to all other treatments. Each treatment was performed in duplicate and luteinising granulosa cells were collected at 48, 96 and 144 h and analysed for SPARC protein by western blotting.

**Experiment 3: effect of fibronectin on SPARC expression in luteinising granulosa cells**

In this experiment (n=3 cultures), we assessed the effect of fibronectin coating on SPARC protein, as it is a key ECM component laid down during luteal development (Silvester & Luck 1999). This required the culture wells to be coated with fibronectin, which was performed by incubating each well with bovine fibronectin (1 or 10 μg; dissolved in 1 ml water) in a humidified incubator at 39 °C for 4 h and then they were dried at 39 °C. On the next day, wells were washed with distilled water and equilibrated with DMEM/Ham F12 medium until plating. Each treatment was performed in duplicate and luteinising granulosa cells were collected at 48, 96 and 144 h and analysed for SPARC protein by western blotting.

**Experiment 4: the effect of FGF2 and VEGFA on SPARC expression in bovine luteal cells**

FGF2 and VEGFA are the principal factors stimulating ovarian angiogenesis (Robinson et al. 2009), thus their effects on SPARC protein were investigated. Luteal cells from recently ovulated follicles (day 1–4 of the oestrous cycle) were used to investigate this as VEGFA and, in particular, FGF2 are most abundant in this time period (Robinson et al. 2007). The luteal cells were prepared and cultured as described previously (Robinson et al. 2008a). Briefly, the different luteal cell types were dispersed with 2 mg/ml collagenase and 5 μg/ml DNase 1 and then plated (2 × 105 viable cells/well) onto fibronectin (10 μg)-coated coverslips in 12-well plates. The cells were then grown in specialised endothelial cell medium (EBM-2; Lonza, Wokingham, UK), which contained gentamicin, heparin, EGF, LR1-IGF1, hydrocortisone and ascorbic acid. To this EBM-2 media, 5 ng/ml LH, 100 units/ml penicillin, 10 μg/ml streptomycin, 10 μg/ml insulin, 5.5 μg/ml transferrin, 5 ng/ml selenium and 2% (v/v) FBS were added. Bovine luteal cells were treated with FGF2 (0 and 1 ng/ml) and VEGFA (0 and 1 ng/ml) in a 2 × 2 factorial design. Each treatment was performed in duplicate for a total of 4 cultures. The cells were collected by scraping and prepared for protein analysis on days 1, 3, 5, 7 and 9 of culture for subsequent analysis of SPARC expression by western blotting.

**Experiment 5: effect of KGHK on luteal endothelial cell network formation and progesterone production**

In order to determine the potential function of SPARC in the developing CL, the effect of a SPARC peptide fragment (KGHK) on endothelial cell network formation and progesterone production was investigated using a physiological luteal angiogenesis culture system. Thus, the effects of KGHK peptide (0, 20 and 100 μg/ml (0–0.21 mM); Bachem, Bubendorf, Switzerland) on luteal endothelial cell network formation under basal and angiogenic stimulated (1 ng/ml FGF2 and
1 ng/ml VEGFA (Robinson et al. 2008a); conditions were determined. The complex biochemistry of SPARC means that it is difficult to accurately determine tissue concentrations (Chlenski et al. 2011), and to the best of our knowledge, no study has determined tissue KGHK concentrations. Thus, the doses of KGHK peptide used were at the lower end of those previously studied (Funk & Sage 1993, Lane et al. 1994, Iruela-Arispe et al. 1995) and were below the dose of a scrambled SPARC peptide 2 sequence (up to 0.8 mM) shown to have no effect on proliferation of bovine aortic endothelial cells or bovine ligament fibroblasts (Funk & Sage 1993). The mixed luteal cell types prepared as described earlier and plated out (2 × 10^5 viable cells/well) onto fibronectin (10 μg)-coated coverslips in 12-well plates. The cells were then grown in the specialised endothelial cell medium as described earlier. Each treatment was performed in duplicate for a total of four cultures. The spent medium was collected after 1, 3, 5, 7 and 9 days for progesterone analysis and on day 9 cells were fixed in acetonemethanol (1:1) for 5 min for immunohistochemical analysis.

Detection of endothelial cell networks: immunohistochemistry and image analysis

Endothelial cells were immunostained with VWF and quantified as previously validated (Robinson et al. 2008a). The antibody used was polyclonal rabbit anti-human VWF (4 μg/ml; Dako) diluted in 2% (v/v) NGS, which was detected by the ABC method (Vector Laboratories, Peterborough, UK). Image analysis was performed using Image Pro-Plus 6.0 (Media Cybernetics, Wokingham, UK). Briefly, 20 randomised fields of view were captured and the area of VWF staining was highlighted to determine the total area and total length of endothelial cell networks. This was repeated for both coverslips per treatment.

Progesterone analysis

Progesterone concentration in spent media was determined by ELISA (Ridgeway Science) and progesterone production was expressed as its concentration in the spent media (adjusted to per day) during the continuous culture period with intra- and inter-assay CV of 7.4 and 11.0% respectively.

Statistical analysis

Statistical analyses were performed using Genstat 13 (VSN International, Hemel Hempstead, UK). The data were checked for normality and heterogeneity of variance using residual plots and Levene’s test, respectively, and log transformed when required. SPARC regulation: replicate cultures were treated as blocks; any treatment effects and their interactions on SPARC levels were analysed by multiple-way ANOVA with day and treatments as factors. Progesterone production: treatment and time effects on progesterone production were determined by randomised block two-way ANOVA (follicular cultures) or repeated measures ANOVA (luteal cultures). Effects of KGHK: the effects of treatment on endothelial cell network formation were determined using randomised block one-way ANOVA. Given the structured nature of the experiments (factorial in arrangement) reported in this study, it was not appropriate to use any post hoc test (Finney 1988, Lowry 1992), and it was more correct to base interpretations on the significance of main effects or interactions given by the ANOVA.

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.

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