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Theca cells and the regulation of ovarian androgen production

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Summary

Theca cells are essential for female reproduction being the source of androgens that are precursors for follicular oestrogen synthesis and also signal through androgen receptors (AR) in the ovary and elsewhere. Theca cells arise from mesenchymal cells around the secondary follicle stage. Their recruitment, proliferation and cytodifferentiation are influenced, directly or indirectly, by paracrine signals from granulosa cells and oocyte although uncertainty remains over which are the critically important signals at particular stages. In a reciprocal manner, theca cells secrete factors that influence granulosa cell proliferation and differentiation at different follicle stages. Differentiated theca interna cells acquire responsiveness to luteinizing hormone (LH) and other endocrine signals and express components of the steroidogenic machinery required for androgen biosynthesis. They also express insulin-like peptide 3 (*INSL3*) and its receptor (*RXFP2*), levels of which increase during bovine antral follicle development. INSL3 signaling may play a role in promoting androgen biosynthesis since knockdown of either *INSL3* or its receptor (*RXFP2*) in bovine theca cells inhibits androgen biosynthesis while exogenous INSL3 can raise androgen secretion. Bone morphogenetic proteins (BMPs) of thecal or granulosal origin suppress thecal production of both INSL3 and androgen. Inhibin, produced in greatest amounts by granulosa cells of preovulatory follicles, reverses these BMP actions. Thus, BMP-induced inhibition of thecal androgen production may be mediated by reduced INSL3-RXFP2 signaling. Activins also inhibit androgen production in an inhibin-reversible manner and recent evidence in sheep indicates that theca cells synthesize and secrete activin, implying an autocrine role in suppressing androgen biosynthesis in smaller follicles, akin to that envisaged for BMPs.

Introduction

Ovarian androgens play an essential role in female reproductive physiology being obligatory substrates for ovarian oestrogen synthesis as well as having direct androgen receptor (AR)–mediated actions in the ovary and elsewhere. Indeed female mice lacking functional AR show defective follicle development and premature ovarian failure (Shiina *et al.* 2006). Ovary- and adrenal-derived androgens can also be aromatized to oestrogens by various peripheral tissues including brain, bone and adipose tissue (Simpson 2003) reflecting additional ‘non-reproductive’ roles.

Evidence that the mammalian ovary synthesises and secretes androgens first emerged in the 1930s (Deanesly 1938). It was subsequently revealed that theca interna cells of developing antral follicles are their principle source and that the capacity of ovarian follicles to synthesise oestrogens depended on the cooperation of theca interna and granulosa cells in accordance with the *two-gonadotrophin, two-cell theory* (Ryan & Petro 1966, Fortune & Armstrong 1977). This theory proposed that luteinizing hormone (LH) acts on theca interna cells to promote biosynthesis of androgens (androstenedione, testosterone), which then diffuse to neighbouring granulosa cells where the aromatase enzyme complex converts them to oestrogens (oestrone, oestradiol), under the influence of follicle stimulating hormone (FSH). Whilst the *two-cell, two-gonadotrophin theory* has stood the test of time remarkably well, it is increasingly evident that additional endocrine signals and a multitude of locally-produced signals also contribute to the regulation of thecal androgen production and granulosal oestrogen production at successive stages of follicle development.

The physiological importance of theca-derived androgens cannot be overstated since several key events in the female reproductive process (follicle maturation, preparation of reproductive tract, generation of preovulatory LH surge, ovulation, oestrus behaviour, libido) are reliant upon their timely production. Unfortunately, disorders that affect thecal androgen biosynthesis, such as polycystic ovarian syndrome (PCOS) in humans, are commonplace and are associated with impaired fertility and other co-morbidities (Baptiste *et al.* 2010). Given the above, it is perhaps surprising that theca cells have not commanded more attention by ovarian biologists over recent decades. Recent PubMed searches yielded cumulative hits totalling 63,492, 13,918 and 3,658 for the terms “oocyte”, “granulosa cell” and “theca cell” respectively, supporting this perception.

The aim of this review is to provide an update of the literature on ovarian theca cells and androgen production with an emphasis on studies involving domestic ruminants. We will also discuss in more detail recent findings from our own laboratory on the actions and interaction of bone morphogenetic proteins (BMPs) and insulin-like peptide 3 (INSL3) on androgen production by bovine theca cells. The reader will find much additional information on theca cells in excellent review articles (Erickson *et al.* 1985, Magoffin 2005, Tajima *et al.* 2007, Young & McNeilly 2010).

Formation of the theca layer

Recruitment of theca cells from ovarian stroma

It is generally accepted that theca cells are derived from mesenchymal progenitor cells within the cortical stroma. There is some evidence in the mouse that a definitive population(s) of thecal progenitor stem cells exists (Honda *et al.* 2007) although comparable studies are currently lacking in other species including ruminants. Under the influence of presumptive signals emitted by activated preantral follicles (i.e. primary and secondary stage), stromal progenitor cells congregate around the follicular basal lamina and align to form first one, and subsequently multiple layers of elongated cells surrounding the follicle (Erickson *et al.* 1985, Orisaka *et al.* 2006b, Itami *et al.* 2011). Thecal recruitment occurs independently of gonadotrophin action as the stromal progenitor cells do not express LH receptors and the theca layer still forms in FSH receptor-null mice (Kumar *et al.* 1997, Abel *et al.* 2000). Rather, evidence suggests that soluble factor(s) secreted by the oocyte and/or granulosa cells of activated follicles drive thecal recruitment (Magoffin 2002, Magoffin 2005, Orisaka *et al.* 2006b, Itami *et al.* 2011). Whilst the identity of the key factor(s) involved is still unknown, granulosa-derived kit ligand (KITLG) and hedgehog proteins, and oocyte-derived GDF9 are prime candidates. Theca layers fail to develop in the arrested follicles of GDF9-null mice (Elvin *et al.* 1999). GDF9 may act either directly on surrounding stromal (pre-theca) cells to upegulate *KIT* expression or indirectly by modulation of KITLG and IGF1 production by granulosa cells that, in turn, binds to their signaling receptors (KIT, IGFR) on pre-theca cells (Nilsson & Skinner 2002). Like GDF9, oocyte-derived BMP15 has also been shown to upregulate *KITLG* expression by granulosa cells (Otsuka & Shimasaki 2002).

From the primary follicle stage in mice, granulosa cells begin to express hedgehog proteins (*Ihh, Dhh*) that induce target gene expression (*Ptch1, Gli1*) in surrounding mesenchymal stromal cells (i.e. pre-theca cells). This expression pattern persists in the theca layer until the preovulatory stage, perhaps implying a role in both theca cell recruitment and differentiation (Wijgerde *et al.* 2005). Cultured bovine theca cells from antral follicles also respond to hedgehog protein with upregulation of *Gli1* expression, increased proliferation and androgen production (Spicer *et al.* 2009). Other candidate theca recruitment factors include VEGFA (Yang & Fortune 2006, Yang & Fortune 2007) from granulosa cells and BMP15 and BMP6 from the oocyte. In addition, evidence suggests that established theca cells also secrete paracrine factors that indirectly influence surrounding stromal cells to 'amplify' recruitment including transforming growth factor a (TGFA), basic fibroblast growth factor (bFGF/FGF2), keratinocyte growth factor (KGF/FGF7), hepatocyte growth factor (HGF), IGFs and androgens. Both KGF and HGF have been shown to increase granulosal *KITLG* expression that, in turn, upregulates thecal FGF7and *HGF* expression (Parrott & Skinner 1998) as well as stromal *KIT* expression and cell proliferation (Parrott & Skinner 2000). Theca-derived androgens may also have an amplifying role since androgen can upregulate *KITLG* expression by mouse granulosa cells (Joyce *et al.* 1999) and promote the primary to secondary follicle transition in bovine ovarian cortical strips (Yang & Fortune 2006, Yang & Fortune 2007).

Proliferation and differentiation of theca cells

After congregating around the basal lamina, theca cells proliferate and differentiate into an inner theca interna and outer theca externa. Whilst the key signals responsible are largely unknown, proliferation and cytodifferentiation are presumably influenced by gradients of paracrine signaling molecules from the centrally located granulosa/oocyte compartment (i.e. KITLG, GDF9, BMP15, EGF, hedgehog proteins) in conjunction with endocrine signals (i.e. LH, insulin, IGFs) diffusing from new capillary vessels forming close to the basal lamina. Once established, theca cells may also secrete autocrine/paracrine factors that promote further proliferation and differentiation, including IGFs (Barbieri *et al.* 1986, Magoffin & Weitsman 1994, Spicer *et al.* 2004), bFGF (Nilsson *et al.* 2001) and androgens (Yang & Fortune 2006, Yang & Fortune 2007). Evidence in the mouse suggests that a radial signaling gradient of hedgehog proteins emitted by granulosa cells is involved in the differentiation of the more distantly located theca cells into theca externa cells that show a smooth muscle-like phenotype (Ren *et al.* 2009). In contrast, theca interna cells acquire LH receptors and begin to express components of the steroidogenic pathway (*NR5A1, STAR, CYP11A1,* *HSD3B1, CYP17A1*). Morphologically, theca interna cells display hallmark features of steroidogenically-active cells, including abundant smooth endoplasmic reticulum, numerous mitochondria with tubular cristae and lipid vesicles that store cholesterol esters as precursor for the synthesis of steroid hormones. As mentioned above, theca externa cells lack these features and have a morphology more akin to smooth muscle cells, indicative of a more structural or mechanical support role in the follicular unit. There is some evidence that theca externa cells exhibit contractile behaviour around the time of ovulation that may contribute to extrusion of the cumulus-oocyte and wound closure around the margin of the corpus haemorrhagicum (Hunter 2003).

As secondary follicles progress towards the antral stage they acquire their own vascular supply in the form of a sheath of capillaries coursing throughout the theca layer; these capillaries are excluded by the basal lamina from the avascular granulosal compartment, until follicle luteinisation (or atresia). A well-developed thecal capillary bed is essential for bidirectional transfer of substances to (e.g. gonadotrophins, nutrients) and from (e.g. steroids, metabolites) the follicular unit. VEGF and other pro-angiogenic factors expressed predominantly by granulosa cells play a prominent role in vascularization of the theca interna (Fraser 2006, Fraser & Duncan 2009, Robinson *et al.* 2009). Inhibition of VEGFA signaling leads to reduced proliferation of endothelial and theca cells, compromises follicle development and blocks ovulation (Fraser 2006). Treatment of bovine cortical strips with VEGFA promotes primary to secondary follicle transition (Yang & Fortune 2006, Yang & Fortune 2007). Recent evidence in cattle indicates that theca-derived BMP4 and BMP7 may contribute to thecal vascularization by upregulating VEGFA expression in granulosa cells (Shimizu *et al.* 2012).

A brief life history of theca cells: from recruitment to luteal regression

It is clear that the capacity of stromal progenitor cells to differentiate into theca cells persists throughout the reproductive lifespan of a female (i.e. until the primordial follicle reserve is depleted). Whilst a theoretical possibility, we are not aware of any evidence that failure of thecal recruitment ever becomes a limiting factor in the supply of growing preantral follicles in females approaching the end of their reproductive lifespan. The subsequent fate of established theca cells (and their progeny) largely corresponds to the developmental trajectory of the individual follicle, >99% of which are lost through atresia during the 4-6 months it takes for a primary follicle to reach the preovulatory stage in sheep, cattle and humans (Lussier *et al.* 1994). Inadequate development and/or early regression of the thecal vasculature is reportedly a common feature of atretic follicles (Fraser 2006). A detailed discussion of follicle atresia in the bovine and how this relates to changes in theca cells, granulosa cells and oocyte may be found elsewhere (Rodgers & Irving-Rodgers 2010). Comprehensive reviews focussing on follicle vascularization include Robinson et al (2009) and Fraser and Duncan (2009).

Once follicles have acquired a well-developed capillary network it seems reasonable to assume that their theca interna cells are exposed to pituitary LH pulses and other endocrine signals, regardless of the stage of follicle development. However, androgen production remains at relatively low levels during preantral and early antral follicle stages, only increasing markedly during the mid- to late-antral stage. This implies the involvement of other, locally produced signals that suppress androgen production at earlier follicle stages whilst augmenting LH-dependent androgen production at later stages. Intraovarian factors implicated in the regulation of androgen production are discussed in more detail in the final section of this review.

For selected bovine antral follicles that achieve dominance around the time of luteal regression, exposure to the ovulation-inducing LH surge initiates luteinisation of both theca and granulosa cells, characterised by an abrupt loss of thecal *CYP17A1* expression and androgen-synthesizing capacity, and granulosal *CYP19A1* expression and oestrogen-synthesizing capacity (Voss & Fortune 1993). Instead, the proximal components of the steroidogenic pathways of both cell types are upregulated (i.e. *STAR, CYP11A1, HSD3B1*) and predominantly used for the synthesis of progesterone as theca cells transform into 'small' luteal cells and granulosa cells become 'large' luteal cells of the newly formed corpus luteum. Both 'small' and 'large' luteal cells actively secrete progesterone until corpus luteum regression (Berisha & Schams 2005, Miyamoto *et al.* 2010).

Paracrine effects of theca interna cells on granulosa cells and oocyte

In addition to supplying androgens to granulosa cells as substrates for aromatization to oestrogens, theca cells express an array of paracrine signaling molecules shown to influence the proliferation and differentiated function of granulosa cells at different stages of follicle development (Orisaka *et al.* 2006a). Prominent amongst these are androgens themselves that have been shown to act via AR to promote follicle development (Vendola *et al.* 1999, Shiina *et al.* 2006, Yang & Fortune 2006), upregulate *FSHR* and *CYP19A1* expression (Luo & Wiltbank 2006) and FSH-induced oestrogen production (Hillier & De Zwart 1981, Harlow *et al.* 1986, Weil *et al.* 1999). Thus, thecal androgens play a vital role in promoting granulosal *CYP19A1* expression/aromatase activity as well as providing substrate for the enzyme.

Many non-steroidal factors secreted by theca cells have likewise been shown to modify granulosa cell proliferation and/or function in ruminants and other species (**Fig. 1**). For example, *in vitro* studies on bovine/ovine follicles show that theca-derived KGF (FGF7) and HGF promote granulosa cell proliferation (Parrott *et al.* 1994, Parrott & Skinner 1998), TGFB1 down-regulates FSH-induced *CYP19A1* expression and oestradiol secretion (Ouellette *et al.* 2005, Zheng *et al.* 2008), IGF1 enhances cell proliferation and oestradiol secretion (Gutierrez *et al.* 1997, Glister *et al.* 2001, Monget *et al.* 2002), BMP4, BMP6 and BMP7 enhance basal and IGF-induced oestradiol secretion (Monget *et al.* 2002, Glister *et al.* 2004, Campbell *et al.* 2006). In contrast, FGF10 inhibits oestradiol secretion (Buratini *et al.* 2007) while FGF18 inhibits *FSHR* expression and steroidogenesis and promotes cell death (Portela *et al.* 2010). Since thecal expression of *FGF18* mRNA and FGF18 protein in follicular fluid were higher in subordinate than in dominant follicles, it was suggested that theca-derived

FGF18 might be an important atretogenic factor in bovine follicles (Portela *et al.* 2010). It should be cautioned that expression of many of the above proteins is not exclusive to theca cells and so the observed effects of purified/recombinant proteins on granulosa cells is not necessarily indicative of theca-granulosa interaction.

Thecal steroidogenesis and factors modulating androgen secretion

Endocrine factors

In response to pulses of GnRH from the hypothalamus, pituitary gonadotrophs secrete LH pulses that, in turn, promote transient increases in ovarian output of androgens and oestrogens (Baird & McNeilly 1981, Campbell *et al.* 1990). The frequency and amplitude of LH pulses are modulated by both extrinsic (e.g. photoperiod, socio-sexual cues) and intrinsic (e.g steroid feedback) influences (Martin 1984) and vary according to the stage of the reproductive cycle. LH plays a major role in promoting androgen production by theca interna cells, particularly those of antral follicles with a well-developed vascular system. It does so by upregulating the expression of several key genes involved in the steroidogenic pathway that converts cholesterol into androgen, including *STAR, CYP11A1 and CYP17A1*. As would be anticipated from this, treatment of cows with a GnRH antagonist (acyline) to block pulsatile LH secretion inhibited thecal *STAR* and *CYP17A1* mRNA levels and reduced androgen production (Luo *et al.* 2011).

Thecal androgen production is also enhanced by insulin, as revealed by *in vitro* studies on theca cells from several species including cattle and sheep and human (Spicer & Echternkamp 1995, Campbell *et al.* 1998, Franks *et al.* 1999). This has given rise to the theory that raised insulin levels in women with insulin resistance could be a contributory factor in the aetiology of polycystic ovarian syndrome, a condition usually associated with ovarian androgen excess and arrested antral follicle development (Baptiste *et al.* 2010). Like insulin, IGF1 can also stimulate thecal androgen production (Velazquez *et al.* 2008) while the adipokines leptin (Spicer 2001) and adiponectin (Lagaly *et al.* 2008) have been shown to inhibit thecal androgen production by cultured bovine theca cells.

Intra-ovarian factors

In concert with LH and other endocrine factors, numerous locally-produced growth factors have been implicated as intra-ovarian regulators of thecal androgen production. These include KITLG (Parrott & Skinner 1997), IGFs (Campbell *et al.* 1998, Spicer *et al.* 2004), bFGF/FGF2 (Hurwitz *et al.* 1990, Scaramuzzi & Downing 1995), FGF9 (Schreiber *et al.* 2012), EGF (Scaramuzzi & Downing 1995, Campbell *et al.* 1998), TGFA (Roberts & Skinner 1991, Campbell *et al.* 1994) TNFA (Spicer 1998), interleukins (Hurwitz *et al.* 1991) and multiple TGFβ superfamily members (reviews: (Woodruff & Mather 1995, Shimasaki *et al.* 2004, Knight & Glister 2006) (**Fig. 2**).

Theca cells express a full complement of receptors and intracellular signal transduction components for TGFβ superfamily members and are responsive to multiple ligands including TGFβ, activins, BMP2, BMP4, BMP6, BMP7 and AMH, all of which are expressed at the intrafollicular level and have been shown to attenuate basal and/or LH-induced androgen production in several species including rat, human, cattle and sheep (Brankin *et al.* 2005, Glister *et al.* 2005, Campbell *et al.* 2006, Glister *et al.* 2010, Glister *et al.* 2011, Campbell *et al.* 2012, Young *et al.* 2012, Young & McNeilly 2012). The suppressive effect of activin-A is antagonised by follistatin and inhibin (Wrathall & Knight 1995, Young *et al.* 2012, Young & McNeilly 2012). Activin-B also suppresses androgen production by sheep theca cells to a similar extent as activin-A, and the effects of both are effectively reversed by inhibin-A (Young *et al.* 2012).

In addition to blocking activin signaling, inhibin-A was shown to antagonise the suppressive effects of BMP2, 4, 6 and 7 on bovine theca cells (Glister *et al.* 2010). This was accompanied by a reversal of a marked BMP-induced decline in expression of *CYP17A1* and, to a lesser extent, *LHCGR, STAR, CYP11A1* and *HSD3B1* expression. In sheep theca cells, the inhibitory effect of activin-A on androgen production was associated with a decline in *STAR* and *HSD3B1* expression while CYP17A1 expression was unaffected (Young & McNeilly 2012). However, co-treatment with inhibin-A to reverse the activin-induced suppression of thecal androgen production, led to an increase in expression of *CYP17A1* and *HSD3B1*. Furthermore, treatment with inhibin alone raised *CYP17A1* expression and androgen production indicating that sheep theca cells produce an endogenous ligand whose action is opposed by inhibin. The finding that follistatin treatment alone also raised androgen secretion indicates that activin is the endogenous ligand produced by sheep theca cells (Young & McNeilly 2012). Indeed, the same group reported expression of *INHBA* and *INHBB* mRNAs in the theca layer of sheep antral follicles (Young *et al.* 2012). In contrast, whilst studies in the authors' laboratory have also documented expression of *INHBA* and *INHBB* mRNAs in bovine theca layers (Glister *et al.* 2010), we found no stimulatory effect of follistatin treatment on androgen secretion by isolated theca interna cells (Glister & Knight, unpublished data). Therefore, we interpret the ability of inhibin alone to raise androgen production as being due to antagonism of endogenous BMPs, that are also expressed by theca cells (Glister *et al.* 2010).

It was recently reported that AMH also exerts a suppressive effect on LH-induced androgen production by cultured sheep theca cells (Campbell *et al.* 2012). Moreover, AMH immunoreactivity in granulosa cells declined during follicle development and was inversely associated with aromatase immunoreactivity. The study also found that active immunization of sheep against AMH was associated with raised intrafollicular androgen concentrations in small antral follicles, supporting a physiological role for granulosa-derived AMH as an additional paracrine factor that can suppress thecal androgen production.

Taken together, the above evidence indicates that multiple intra-follicular TGFβ family members including activins, BMPs and AMH negatively regulate basal and LH-induced androgen production. In contrast, inhibins, follistatin and likely several BMP binding proteins (e.g. chordin, gremlin, noggin) secreted by granulosa cells (Glister *et al.* 2011) oppose these signals and upregulate androgen production. Granulosal production of inhibin and follistatin, as well as thecal expression of the inhibin co-receptor, betaglycan (Glister *et al.* 2010), increases in growing preovulatory follicles and, by counteracting activin/BMP signaling on theca cells, this would serve to enhance the ability of theca cells to deliver sufficient androgen to granulosa cells for aromatization to oestrogen. It should be noted that recent evidence in sheep (Young *et al.* 2012) and cattle (Glister *et al.* 2010) indicates that theca cells also express mRNAs for inhibin/activin subunits. This raises the possibility that theca cells, as well as granulosa cells, secrete functional inhibin/activin proteins that contribute to the regulation of thecal androgen production and other intrafollicular events. Further work is needed to investigate this aspect of intrafollicular regulation.

In vitro studies on bovine (Roberts & Skinner 1990, Wrathall & Knight 1995) and human (Gilling-Smith *et al.* 1997) theca cells have shown that oestradiol itself, at physiological concentrations (i.e. similar to peak concentrations in antral fluid) can upregulate thecal androgen production. This indicates that an additional intra-follicular positive feedback loop operates to ensure an adequate supply of androgen for conversion to oestrogen in the late follicular phase. A recent study in rats also provided evidence that, in addition to inhibin, another FSH-dependent paracrine factor from granulosa cells (oestradiol?) was capable of upregulating thecal *CYP17A1* expression (Hoang *et al.* 2013).

With regard to potential regulatory roles of oocyte-derive factors on thecal androgen production, GDF9 was found to enhance forskolin-stimulated androgen production by rat theca-interstitial cells (Solovyeva *et al.* 2000) while GDF9-induced upregulation of androgen secretion and *CYP17A1* expression in rat preantral follicles was blocked by intra-oocyte injection of GDF9 antisense nucleotide (Orisaka *et al.* 2009). However, other studies showed that GDF9 inhibits forskolin-induced androgen production by human theca cells (Yamamoto *et al.* 2002) and both LH- and IGF1-induced androgen production by bovine theca cells (Spicer *et al.* 2008). Whether these discordant findings reflect species differences, or differences in experimental methodology is not known at this time. Nonetheless, the likelihood is that oocyte-derived factors do exert direct regulatory actions on surrounding theca cells, as well as on granulosa cells.

Evidence for interactions between BMP and insulin-like peptide 3 (INSL3) signaling in regulating theca androgen production

As mentioned above studies in the authors' laboratory using bovine theca cells in primary culture showed that bone morphogenetic proteins (BMPs) are powerful suppressors of thecal androgen production and that granulosa-derived inhibin can antagonise this effect of BMPs and raise androgen production (Glister *et al.* 2005, Glister *et al.* 2010). Likewise, several BMP-binding proteins (gremlin, noggin) can reverse the inhibitory effect of BMPs (**Fig. 3**), and multiple BMP-binding proteins are expressed in the bovine ovary, particularly by granulosa cells (Glister *et al.* 2011). In a subsequent microarray study (Glister *et al.* 2013) we showed that BMP treatment down-regulates expression of several hundred genes in theca cells including multiple components of the steroidogenic pathway leading to androgen biosynthesis, most prominently *CYP17A1* but also NR5A1, *STAR, CYP11A1* and *HSD3B1*. Intriguingly, thecal expression of insulin-like peptide 3 (*INSL3*) was profoundly suppressed by BMP treatment and this prompted a series of experiments that revealed a hitherto unknown functional link between BMP and INSL3 pathways in the regulation of ovarian androgen production (Glister *et al.* 2013, Satchell *et al.* 2013). INSL3 was initially identified as a testicular product, but it has become evident that the ovary also synthesizes substantial amounts of INSL3 (review: (Ivell & Anand-Ivell 2011). In the bovine ovary, both *INSL3* and its cognate receptor (*RXFP2*) are predominantly expressed by theca cells and expression levels of both increase during antral follicle development (Satchell *et al.* 2013). In rat preantral follicles expression of *RXFP2* was also detected in oocytes and INSL3 was shown to upregulate *GDF9* expression, follicle growth and androgen production (Xue *et al.* 2014). Using cultured bovine theca cells, RNAi-mediated knockdown of either *INSL3* or its receptor *RXFP2* was shown to suppress androgen production (**Fig. 4)** whereas exogenous synthetic human INSL3 promoted a modest increase in androgen production (Glister *et al.* 2013). During the synchronized bovine oestrous cycle plasma INSL3 levels increase during the preovulatory period and then decline after the LH surge, paralleling the changes in plasma oestradiol (Satchell *et al.* 2013)(**Fig. 5**). This suggests that the peak in circulating INSL3 reflects the output of theca cells of the dominant ovulatory follicle and that the subsequent fall in INSL3 after the LH surge reflects diminished thecal output associated with follicle luteinisation. In support of this, in vitro culture of theca cells with a luteinizing concentration of LH promoted a marked decline in *INSL3* mRNA expression and INSL3 secretion accompanied by an upregulation of *STAR* and *CYP11A1* expression and progesterone secretion (Satchell *et al.* 2013).

Collectively, these findings revealed the importance of another intraovarian growth factor, INSL3, for maintaining androgen production by ovarian theca cells and showed that the suppressive action of BMPs on androgen production is intimately linked to their inhibition of INSL3 signaling. On the basis of these findings we propose that a functional deficit in thecal BMP signaling promotes excess thecal INSL3-RXFP2 signaling and that this could be a contributory factor in ovarian androgen excess disorders such as PCOS. Indeed, circulating INSL3 levels are raised in women with PCOS (Gambineri *et al.* 2011, Anand-Ivell *et al.* 2013). Conversely, a functional excess of thecal BMP signaling could contribute to androgen insufficiency by reducing INSL3-RXFP2 signaling. Both situations compromise normal follicle development and lead to subfertility or infertility in animals and human. Moreover, the extra-ovarian actions of androgens, either acting directly or after peripheral conversion to oestrogens, will be perturbed by over- or under-secretion of ovarian androgen.

Concluding Remarks

In summary, theca interna cells have an indispensible role in the ovary, not only contributing to preantral and antral follicle development mediated by androgen receptor interaction, but also in the timely provision of androgen substrate required for granulosal oestrogen biosynthesis, particularly in the final preovulatory stage of follicle development. It has become apparent that theca cells are closely regulated by an array of intra-ovarian factors that operate in concert with LH and other endocrine signals to modulate follicular androgen biosynthesis. Intra-ovarian BMPs and the INSL3-RXFP2 system are recent additions to this list and, based on findings from the authors' laboratory, a schematic model depicting their proposed involvement is presented in **Fig. 6**. Dysregulation of ovarian androgen production is a likely consequence of perturbations in one or more of these local signaling mechanisms at any stage of follicle development. Recognising that most of the experimental evidence thus far has arisen from in vitro studies, the challenge remains to define which are the most important local signaling mechanisms in terms of physiological regulation in the whole animal context.

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Figure Legends

Fig. 1 Theca cells are recruited from cortical stromal cells and proliferate and differentiate under the influence of paracrine factors secreted by the granulosa cells and/or oocyte of activated follicles. Differentiated theca interna cells are responsive to LH and other endocrine and intra-ovarian factors. In turn, they secrete factors (steroids and proteins) that exert autocrine/paracrine effects on theca cells and paracrine effects on granulosa cells. They also deliver androgens to granulosa cells as substrate for oestrogen synthesis. Abbreviations: AMH, anti-mullerian hormone; BMP, bone morphogenetic protein; IGF, inslulin-like growth factor; INSL3, insulin-like peptide 3; GDF, growth and differentiation factor; HGF, hepatocyte growth factor; HH proteins, hedgehog proteins; KITL, kit ligand (stem cell factor); TGF, transforming growth factor. Black arrows indicate paracrine effects while orange arrows indicate autocrine effects. Grey arrows and grey dashed arrows indicate endocrine effects.

Fig. 2 Systemic and intra-ovarian factors shown to modulate thecal androgen production *in vitro*. So far, only a minority of these factors (highlighted in bold) has been shown to modulate ovarian androgen production *in vivo* (evidenced by experiments involving direct administration, immunoneutralization, spontaneous mutations or targeted deletions of ligand/receptor genes). More *in vivo* studies are required to strengthen the physiological relevance of *in vitro* observations relating to local autocrine/paracrine interactions. However, such experiments are challenging, not least due to multiple sites of action and complex hypothalamic-pituitary-ovarian feedback interactions operating *in vivo*. Superscript letters indicate the main source(s) of each factor: e, endocrine; g, granulosa; m, macrophage; o, oocyte; t, theca. For other abbreviations see Fig. 1 legend.

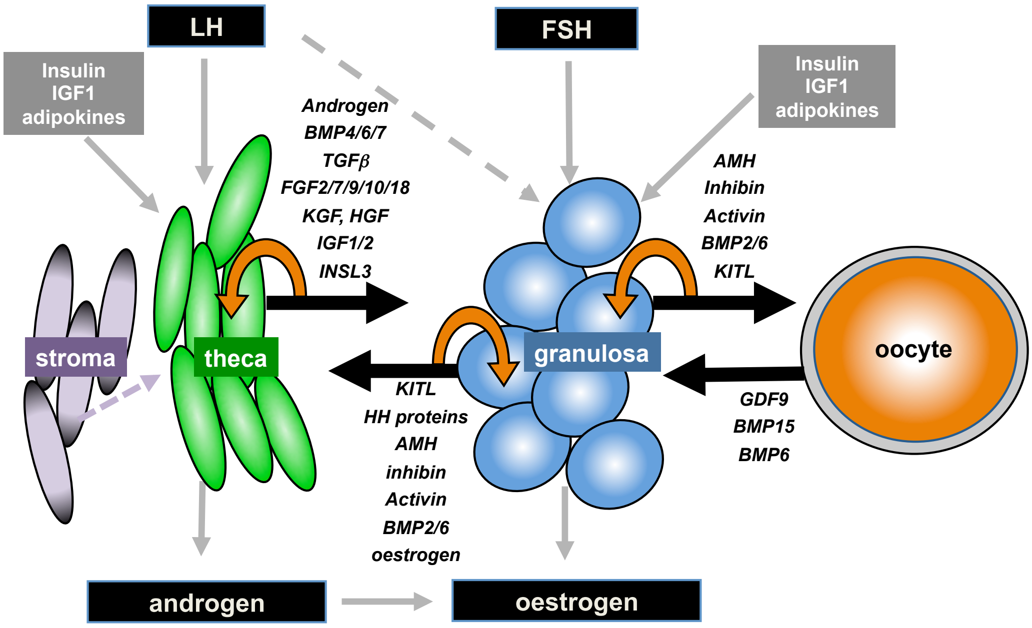
Fig. 3 The BMP-binding proteins gremlin and noggin are expressed in bovine antral follicles and can selectively antagonize BMP-induced suppression of androstenedione secretion by bovine theca cells *in vitro.* Note that gremlin also raises 'basal' androstenedione secretion in the absence of BMP treatment, suggesting neutralization of an endogenous ligand, likely BMP4. (Glister, Satchell & Knight, unpublished data)

Fig. 4 RNAi knockdown of INSL3 or its receptor (RXFP2) in cultured bovine theca cells reduces CYP17A1 expression and androstenedione secretion indicating that INSL3 signaling is required for maintaining androgen synthesis. Values are means ± SEM (n=4 independent cultures). **$P<0.01$ versus control. (redrawn from Glister et al 2013)

Fig. 5 Changes in mean (±SEM) plasma concentrations of progesterone, oestradiol-17β and INSL3 during PG-synchronized oestrous cycles in heifers. Samples are aligned to the time of PG administration (day 0) indicated by the arrow. Statistical analysis was performed by repeated measures ANOVA. (replotted from Satchell et al 2013)

Fig. 6 Putative model of BMP-INSL3 pathway interaction in the regulation of thecal androgen production. According to the model (a) both INSL3 and its cognate receptor RXFP2 are primarily expressed by theca cells (TC); (b) expression of INSL3 and RXFP2 increase during antral follicle development and decline after the preovulatory LH surge; (c) INSL3-RXFP2 signalling is required to sustain TC androgen production since knockdown of either suppresses CYP17A1 expression and androgen production; (d) in a feed forward manner androgens positively regulate INSL3-RXFP2 signaling since pharmacological blockade of androgen synthesis reduces INSL3 and RXFP2 expression; (e) BMPs from granulosa cells (GC) and/or TC suppress INSL3 expression and this is accompanied by a loss of their androgen-synthesizing capacity; (f) BMP signalling, in turn, is negatively regulated by GC-derived inhibin and extracellular BMP-binding proteins; (g) diminished BMP signalling could contribute to raised INSL3 and androgen production in conditions such as polycystic ovarian syndrome in humans.

Fig. 1


LH
FSH
Insulin
IGF1
adipokines
Insulin
IGF1
adipokines
Androgen
BMP4/6/7
TGFβ
FGF2/7/9/10/18
KGF, HGF
IGF1/2
INSL3
AMH
Inhibin
Activin
BMP2/6
KITL
stroma
theca
granulosa
oocyte
KITL
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inhibin
Activin
BMP2/6
oestrogen
GDF9
BMP15
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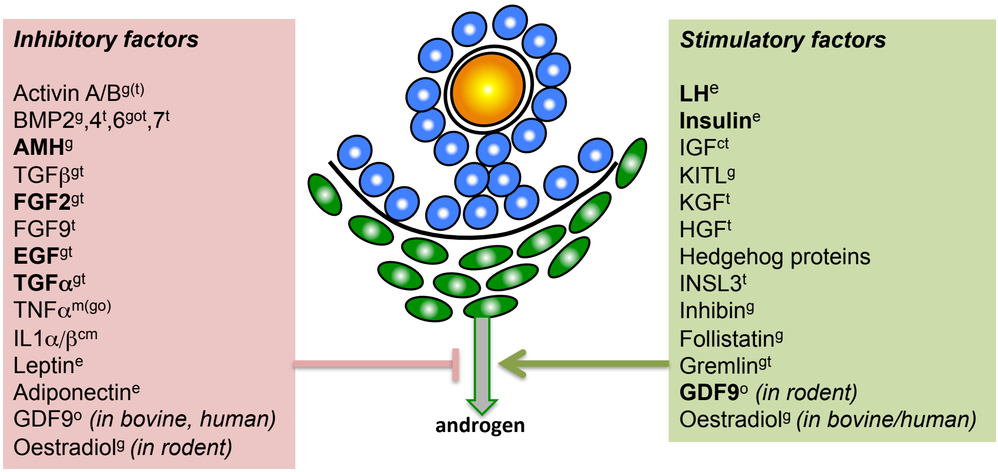
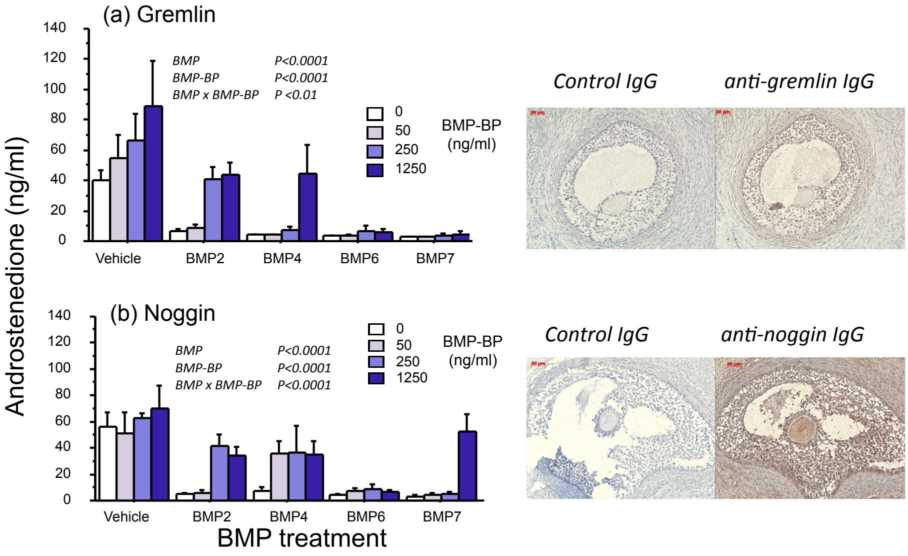
androgen
oestrogen

Fig. 2



Inhibitory factors
Activin A/B$^{g(t)}$
BMP2^{g},4^{t},6^{got},7^{t}
AMH^{g}
TGFβ^{gt}
FGF2^{gt}
FGF9^{t}
EGF^{gt}
TGFα^{gt}
TNFα$^{m(go)}$
IL1α/β^{cm}
Leptin^{e}
Adiponectin^{e}
GDF9^{o} (in bovine, human)
Oestradiol^{g} (in rodent)
androgen
Stimulatory factors
LH^{e}
Insulin^{e}
IGF^{ct}
KITL^{g}
KGF^{t}
HGF^{t}
Hedgehog proteins
INSL3^{t}
Inhibin^{g}
Follistatin^{g}
Gremlin^{gt}
GDF9^{o} (in rodent)
Oestradiol^{g} (in bovine/human)

Fig. 3



(a) Gremlin
Androstenedione (ng/ml)
140
120
100
80
60
40
20
0
BMP P<0.0001
BMP-BP P<0.0001
BMP x BMP-BP P <0.01
0
50
250
1250
BMP-BP (ng/ml)
Vehicle
BMP2
BMP4
BMP6
BMP7
Control IgG
anti-gremlin IgG
(b) Noggin
BMP P<0.0001
BMP-BP P<0.0001
BMP x BMP-BP P<0.0001
Control IgG
anti-noggin IgG
BMP treatment

Fig. 4

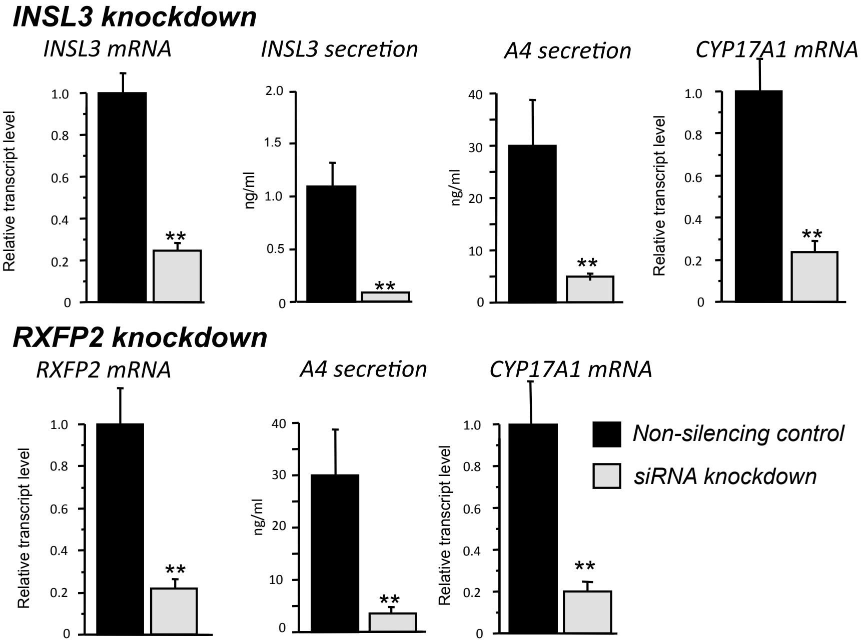

INSL3 knockdown
INSL3 mRNA
INSL3 secretion
A4 secretion
CYP17A1 mRNA
Relative transcript level
ng/ml
1.0
0.8
0.6
0.4
0.2
0
2.0
1.5
0.5
40
30
20
10
**
RXFP2 knockdown
RXFP2 mRNA
A4 secretion
CYP17A1 mRNA
Non-silencing control
siRNA knockdown

Fig. 5

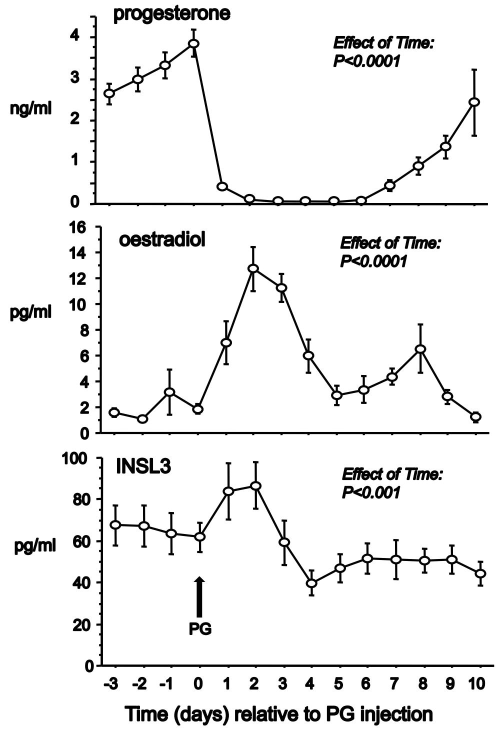
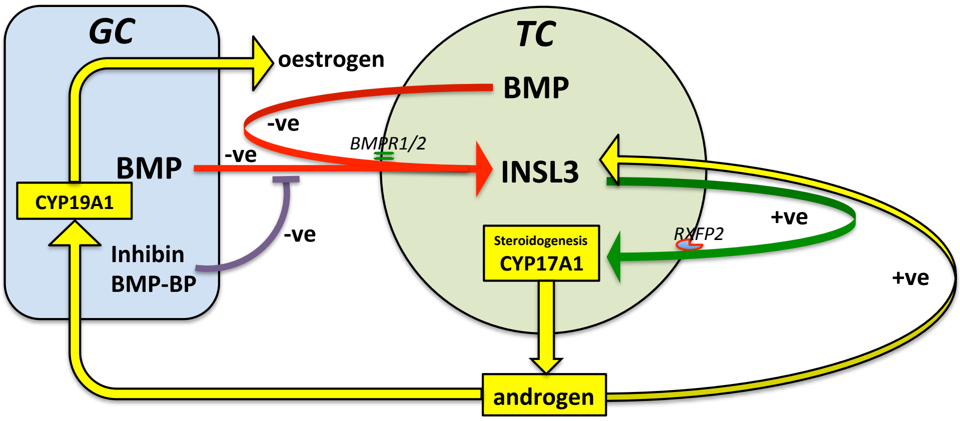

progesterone
ng/ml
0
1
2
3
4
Effect of Time:
P<0.0001
oestradiol
pg/ml
0
2
4
6
8
10
12
14
16
Effect of Time:
P<0.0001
INSL3
pg/ml
0
20
40
60
80
100
Effect of Time:
P<0.001
PG
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
Time (days) relative to PG injection

Fig. 6


GC
TC
oestrogen
BMP
-ve
-ve
BMPR1/2
INSL3
CYP19A1
BMP
-ve
Inhibin
BMP-BP
+ve
RXFP2
Steroidogenesis
CYP17A1
+ve
androgen