REPRODUCTION
REVIEW

Theca: the forgotten cell of the ovarian follicle

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Abstract

Theca cells function in a diverse range of necessary roles during folliculogenesis; to synthesize androgens, provide crosstalk with granulosa cells and oocytes during development, and provide structural support of the growing follicle as it progresses through the developmental stages to produce a mature and fertilizable oocyte. Thecal cells are thought to be recruited from surrounding stromal tissue by factors secreted from an activated primary follicle. The precise origin and identity of these recruiting factors are currently not clear, but it appears that thecal recruitment and/or differentiation involves not just one signal, but a complex and tightly controlled combination of multiple factors. It is clear that thecal cells are fundamental for follicular growth, providing all the androgens required by the developing follicle(s) for conversion into estrogens by the granulosa cells. Their function is enabled through the establishment of a vascular system providing communication with the pituitary axis throughout the reproductive cycle, and delivering essential nutrients to these highly active cells. During development, the majority of follicles undergo atresia, and the theca cells are often the final follicular cell type to die. For those follicles that do ovulate, the theca cells then undergo hormone-dependent differentiation into luteinized thecal cells of the corpus luteum. While the theca is an essential component of follicle development and ovulation, we do not yet fully understand the control of recruitment and function of theca cells, an important consideration since their function appears to be altered in certain causes of infertility.

Reproduction (2010) **140** 489–504

What are theca cells?

Reproduction is the result of a coordinated signaling network between the gonads, pituitary, and hypothalamus. The ovary is responsible for nurturing growing oocytes until an estradiol signal from primed preovulatory follicles induces GNRH, and a consequent luteinising hormone (LH) surge, to release a mature oocyte, which is then capable of being fertilized to produce an embryo. Within the ovary, there are a number of cell types that support the growth and development of oocytes until ovulation. The oocyte is surrounded by a layer of granulosa cells, which change morphology and proliferate when an oocyte begins the process of folliculogenesis. Activated follicles are thought to recruit precursor thecal cells from the stromal layer surrounding the granulosa cells and oocyte. Together, these form the follicle structure that synthesizes steroid hormones (Hillier *et al.* 1994). Thecal cells are not capable of producing estrogen but do produce androgens in response to LH, which are then converted into estrogen by follicle stimulating hormone (FSH)-induced aromatase in the neighboring granulosa cells of selected growing follicles. Over the past decade, research has focused on granulosa cells and their interaction with the oocyte, and the theca has been somewhat forgotten as a necessary and vital part of the developmental process.

The classification system for folliculogenesis has been well defined in numerous publications (McNatty *et al.* 1999, 2007, Montgomery *et al.* 2001, Barnett *et al.* 2006, Edson *et al.* 2009). Briefly (see Fig. 1), primordial follicles (type 1) are in the resting stage before being activated to start development and the oocyte is surrounded by one layer of flattened granulosa cells; type 1a are the follicles transitioning through to the primary (type 2) stage when the granulosa cells become cuboidal. Primary follicles have one layer of cuboidal granulosa cells, secondary follicles (type 3) have two to four layers of granulosa cells, large preantral (type 4) follicles have four to six layers of granulosa cells, and antral follicles (type 5) have more than five layers of granulosa cells. It is after secondary follicle formation that the thecal cells begin to emerge and form a layer around the granulosa–oocyte structure. Throughout folliculogenesis, the rates of atresia increase, and the early stages of folliculogenesis proceed very slowly (Gougeon 1986, Hirshfield 1994); therefore, most follicles are observed at early stages of development. At the antral stage, follicles become gonadotropin dependent and form large antral follicles (type 5+), most of which undergo atresia, and few are selected for ovulation (reviewed by Scaramuzzi *et al.* (1993) and Edson *et al.* (2009)).

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-10-0094
Online version via www.reproduction-online.org

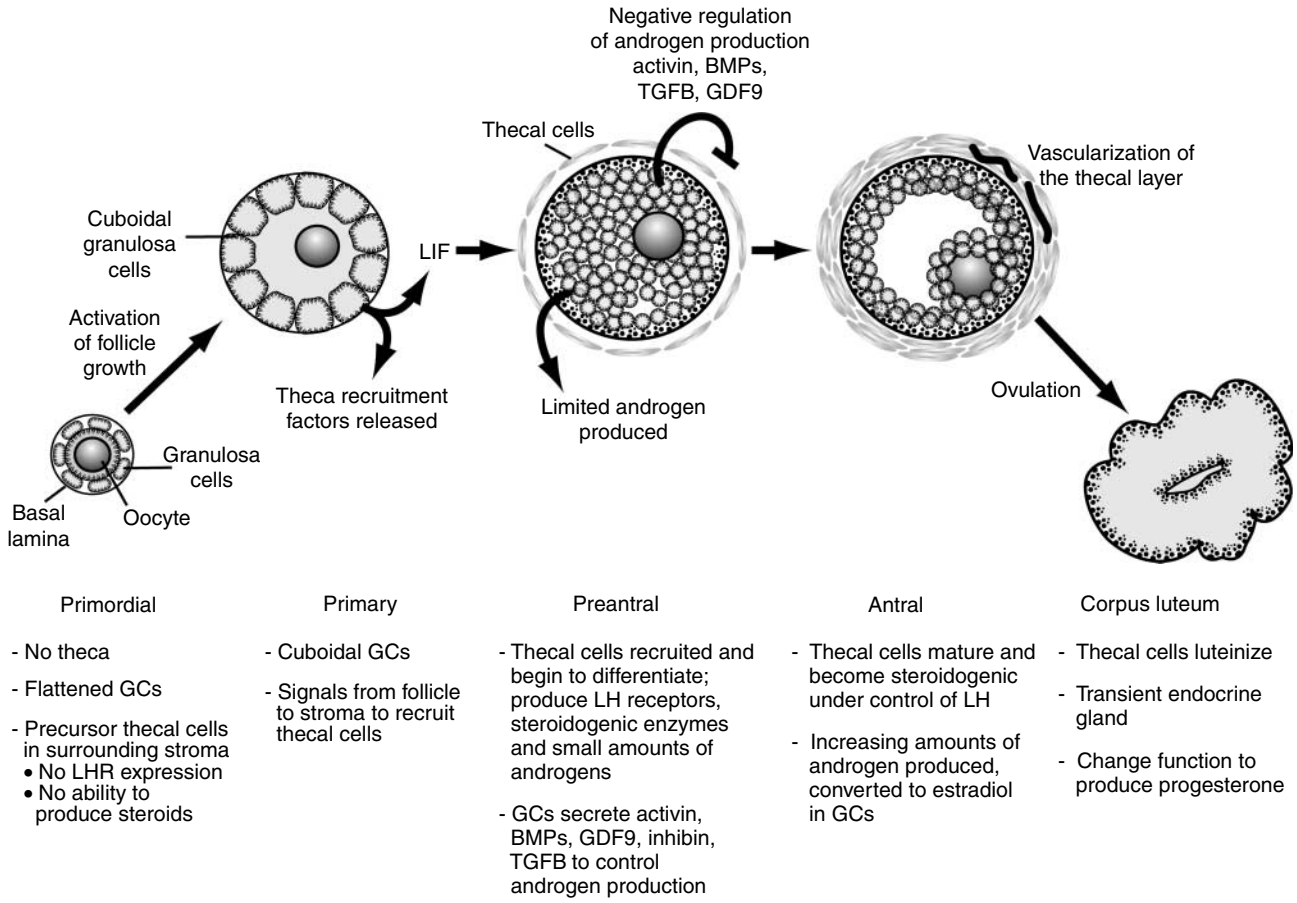

Figure 1 Thecal cell development and function during folliculogenesis. Thecal cells are vital for successful folliculogenesis. A primordial follicle consists of an oocyte and surrounding granulosa cells (GCs), and thecal layers are not formed until the follicle is activated and reaches the secondary stage of development. Thecal cells are required for the production of androgens to provide a structural scaffold, and they form the network of cells that support the vascular system, and after ovulation, thecal cells luteinize and form cells of the corpus luteum.

Origin of theca cells

Theca cells are first observed once a follicle has two or more layers of granulosa cells, which is around the time when thecal cells become LH responsive and steroidogenic enzymes are activated (Magoffin & Weitsman 1994). These specialized cells have long been thought to originate from fibroblast-like precursor cells within the ovarian stroma (Erickson *et al.* 1985, Orisaka *et al.* 2006, Honda *et al.* 2007). The putative undifferentiated progenitor theca cells do not express LH receptors (LHRs) or steroidogenic enzymes and are therefore not LH responsive, showing that initiation of theca cell differentiation is gonadotropin independent (Magoffin & Weitsman 1994). As theca cells are only associated with growing follicles, one would assume that the follicle itself produces factors that signal to the stroma to recruit cells that form the theca. Very few studies have investigated the factors that recruit thecal cells to the activated primary and secondary follicles. It is not currently clear whether cells surrounding the activated follicle differentiate into the theca layer, or whether they are in fact recruited from the stroma to form the theca layers. However, results from mature thecal cells cultured *in vitro* do provide clues about the origin of some recruiting factors and, more importantly, the complexity of this system and provide evidence of steroidogenic regulators with potential differentiative roles. Selected factors are discussed later in the review.

Structure of theca cells

Electron microscopic analysis of normal thecal development in ovine follicles throughout folliculogenesis showed that thecal layers from small follicles (<3 mm diameter) were composed of flattened theca cells together with capillaries and bundles of collagen lying next to the basal lamina (O'Shea *et al.* 1978). The thecal cells were either fibroblast-like cells or presumed steroidogenic cells with large amounts of smooth endoplasmic reticulum. As the follicles grew, the thecal

cells hypertrophied, became less flattened and richer in endoplasmic reticulum, eventually producing pseudopodia, and contained many droplets of lipid but showed no signs of degradation.

Theca cells are highly differentiated with structural features characteristic of steroid-secreting cells including abundant mitochondria with vesicular cristae, agranular endoplasmic reticulum, and lipid vesicles (reviewed by Magoffin (2005)). The mitochondria contain the first enzyme in the steroidogenic pathway, cholesterol side-chain cleavage cytochrome P450 (CYP11A), and the endoplasmic reticulum contains the remaining enzymes necessary to produce androgens. The lipid vesicles store the precursors for steroid hormone synthesis as cholesterol esters which are transported into the mitochondria by steroidogenic acute regulatory protein (STAR; reviewed by Manna *et al*. (2009)). Thecal cells are vital components of the follicle, providing both structural support and being the exclusive producer of ovarian androgens, which are necessary as substrates for estradiol production in the neighboring granulosa cells.

Steroidogenesis

Androgens synthesized in thecal cells are transported to the granulosa cells where P450 aromatase converts these androgens to estrone and 17β-estradiol. The steroidogenic enzymes are produced in a cell-specific manner (Wood & Strauss 2002), and in addition to the large amount of androgen receptor expressed in thecal and granulosa cells (but not in oocytes; Li *et al*. 2009*a*), studies indicate that a complete oocyte-independent system for androgen production exists within the growing follicle. Indeed, androgen and subsequent estradiol production can occur in mouse ovaries devoid of oocytes (McNeilly *et al*. 2000). Silencing 17α-hydroxylase (CYP17) in the rat ovary caused a decline in androstenedione, 17α-hydroxyprogesterone, and testosterone production, and also reduced progesterone levels (Li *et al*. 2009*b*); showing ovarian androgen biosynthesis can be inhibited by silencing CYP17 expression alone, and indicating a potential target for therapeutic development.

Androgen production from thecal cells in the gonadotropin-dependent stage is largely under the control of LH from the pituitary (Baird *et al*. 1981, Palermo 2007). LH is released in a pulsatile manner, and the frequency and amplitude of these pulses vary across the reproductive cycle in response to ovarian steroidogenic feedback. The pulse frequency of LH dictates the amounts of steroid hormones produced, where each pulse of LH is followed by an increase in androstenedione and estradiol secreted from the ovary in many species (Baird *et al*. 1976, 1981, Peluso *et al*. 1984, Schallenberger *et al*. 1984, Walters & Schallenberger 1984). When theca cells were cultured *in vitro*, low levels of LH also stimulated androgen production (Campbell *et al*. 1998, Ryan *et al*. 2008), whereas, at high doses, LH inhibited androstenedione production and stimulated progesterone secretion as well as changing cell morphology indicating that the high LH levels induced luteinization in these cells (Campbell *et al*. 1998). LH has been shown to increase levels of STAR and steroidogenic enzymes (CYP11A1, CYP17, and 3-β-hydroxysteroid dehydrogenase (HSD3B)) and *LHR* gene expression (Magoffin & Weitsman 1993*a*, 1993*b*, 1993*c*, 1994, Lavoie & King 2009; see Fig. 2).

Insulin also plays an important role in thecal cell function. *In vitro* studies using thecal cells from porcine, bovine, and ovine ovaries have shown that insulin induced dose-dependent cell proliferation, increased steroid production, and increased the expression of genes encoding STAR, CYP11A1, and CYP17, thus promoting steroidogenesis (Morley *et al*. 1989, Campbell *et al*. 1995, 1998, Wrathall & Knight 1995, Mamluk *et al*. 1999, Smith *et al*. 2005; see Fig. 2).

The onset of thecal steroidogenic enzyme gene expression is similar in those mammalian species studied in depth (Pollack *et al*. 1997, Kerban *et al*. 1999, Lundy *et al*. 1999, Watson *et al*. 2000, Logan *et al*. 2002). Theca cells are first able to produce steroids just prior to antrum formation, as shown by the onset of expression of STAR, CYP11A1, CYP17, HSD3B, and LHR in thecal cells of preantral (large type 4) follicles, and the mRNA and protein localization at specific stages mirrored one another (Logan *et al*. 2002) and does not require gonadotropins (Scaramuzzi *et al*. 1993). mRNA encoding steroidogenic enzymes were also observed in the theca of bovine preantral follicles, although, unlike sheep, STAR expression was limited to thecal cells (Bao & Garverick 1998). Steroidogenic factor 1, a well-studied transcription factor regulating P450 enzymes and STAR, was expressed by granulosa cells, and protein was observed in both thecal and granulosa cells (Logan *et al*. 2002). Overall, the expression patterns found in the sheep ovary are similar to those observed in other mammalian species.

Angiogenesis

Small primordial follicles are located in the avascular region of the ovarian cortex, especially in larger species, and do not have their own vascular system. Once follicle growth is activated and a thecal layer has been recruited, a follicle develops its own vascular network within the surrounding thecal layer (see Fig. 1). Thecal cell proliferation begins early in the secondary stage of follicle development, although endothelial cell staining is still absent at this point showing that the theca forms before vascularization begins (Fraser & Duncan 2009). There are many potential factors involved in controlling angiogenesis in the developing follicle but vascular endothelial growth factor (VEGF) has a central role and has been studied extensively. VEGF, a potent mitogen for endothelial cells (Ferrara & Davis-Smyth 1997), stimulates vascular permeability (Connolly 1991,

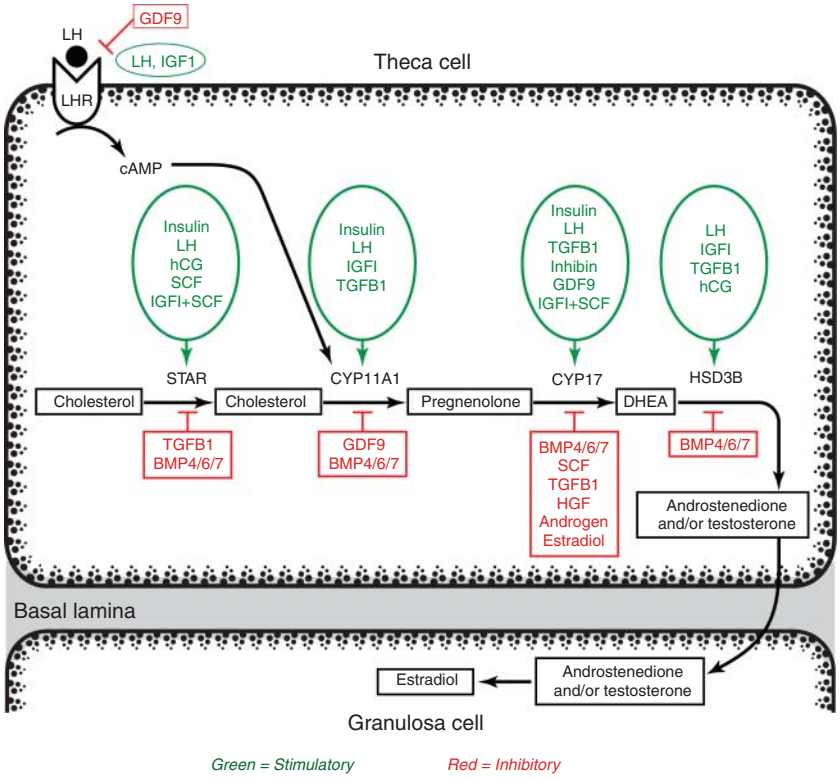

Figure 2 Modulation of steroidogenic enzymes. Expression levels of genes encoding steroidogenic enzymes are regulated by many different factors within the ovary. The process of androgen production requires the following enzymes: cholesterol side-chain cleavage cytochrome P450 (CYP11A1), 17α-hydroxylase (CYP17) and 3-β-hydroxysteroid dehydrogenase (HSD3B), as well as steroidogenic acute regulatory protein (STAR). Theca cells from sheep, humans, and primates principally produce androstenedione, whereas rodents produce testosterone as precursors for estradiol production in the neighboring granulosa cells. The modulating factors are depicted here, where in green circles the stimulatory factors are shown, and the inhibitory factors are listed in red boxes. BMP, bone morphogenetic protein; GDF9, growth differentiation factor 9; HGF, hepatocyte growth factor; hCG, human chorionic gonadotropin; IGF, insulin-like growth factor; SCF, stem cell factor/kit ligand; TGFB, transforming growth factor β.

Senger *et al.* 1993), and is highly expressed in granulosa cells and at lower levels in the thecal layer of follicles from the secondary stage onwards in primate ovaries (Taylor *et al.* 2004). In rodents, cows, and pigs, VEGF expression is weak in early follicle development but increases as the follicle progresses to ovulation (Maisonpierre *et al.* 1997, Barboni *et al.* 2000, Greenaway *et al.* 2005), although levels of VEGF decrease in granulosa cells in sheep and marmoset follicles just prior to ovulation (Ravindranath *et al.* 1992, Redmer *et al.* 2001, Taylor *et al.* 2004). In primates, mRNA encoding VEGF has been observed at the secondary stage in both the theca and granulosa cells (Taylor *et al.* 2004). VEGF was upregulated in rat ovaries during the primordial to primary transition thus preceding vascularization (Kezele *et al.* 2005). Certainly at early stages of follicle development, inhibition of VEGF prevents endothelial cell proliferation, and decreases thecal cell proliferation therefore hindering follicle development (Wulff *et al.* 2002, Fraser *et al.* 2005, Fraser & Duncan 2009). Suppression of gonadotropins using a GNRH antagonist results in reduced thecal and endothelial cell proliferation, and lower vascular density in antral follicles (Taylor *et al.* 2004), an effect probably due to reduced production of VEGF (Fraser & Duncan 2009).

While VEGF is critically involved in regulating follicle development in many species (Barboni *et al.* 2000, Mattioli *et al.* 2001, Wulff *et al.* 2001*a*, 2001*b*, 2002, Hunter *et al.* 2004, Martelli *et al.* 2008), many other factors also contribute and modulate angiogenesis and vasculogenesis in mammals, such as transforming growth factor β (TGFB) superfamily members and their antagonists, angiopoietins, fibroblast growth factor (FGF), and gonadotropins, but whether they directly affect theca cell function remains to be explored.

Life span of theca cells

Folliculogenesis is a process that spans many weeks where the majority of follicles undergo atresia and only a few become dominant and go on to ovulate successfully (reviewed by Scaramuzzi *et al.* (1993)). During atresia, cell death is not confined to a specific cell type, but the entire follicle is degraded during this process. There are various ways that ovarian cells have been reported to die including apoptosis, autophagy, necrosis, and cornification (Jolly *et al.* 1994, Van Wezel *et al.* 1999, D'Haeseleer *et al.* 2006). In bovine follicles, oocytes of preantral follicles are the first component to die, whereas in later stages of development, the granulosa cells die first, although in particular types of atresia, thecal cells also die very early (Rodgers & Irving-Rodgers 2009; see Fig. 3). In the cow, atretic antral follicles have been classified into two types; antral atresia and basal atresia. Basal atresia occurred only in small antral follicles (<5 mm in bovine ovaries) where the theca cell layer becomes disrupted, having high levels of collagen, early death of endothelial and thecal cells, reduced

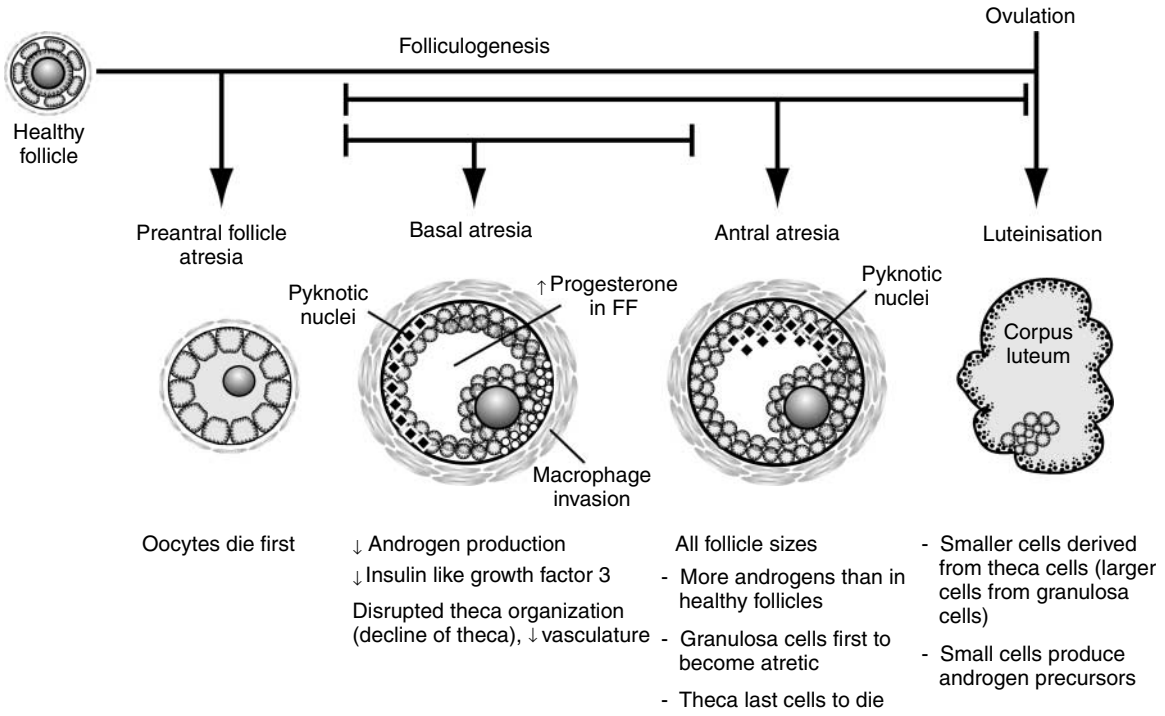

Figure 3 Theca cell fate. Atresia during follicle development is a more common fate of thecal cells than progression through to luteinization. In preantral follicles, the oocytes die first, and as follicles progress through development, follicles undergo basal or antral atresia. Thecal cells appear to be more susceptible to cell death early in follicular development, whereas in basally atretic follicles, the thecal layers are disrupted and are less vascular. Throughout all stages of development, antral granulosa cells are commonly the first cells to become atretic; hence, thecal cells are often the last to die during follicle atresia and the entire follicle is degraded during this process. FF, follicular fluid.

insulin-like factor 3 expression, and reduced androgen production associated with higher levels of progesterone in the follicular fluid in these follicles (Irving-Rodgers *et al*. 2003, Clark *et al*. 2004). These observations suggest that the theca is more susceptible to cell death early in follicular development compared to at later stages (Irving-Rodgers *et al*. 2001). In contrast, in atretic antral follicles where pyknotic nuclei were first observed in antral granulosa cells, thecal cells were the last to be disrupted (see Fig. 3). Thus, thecal cells from follicles undergoing atresia appear to respond differently depending on the stage of follicle development.

Since the blood supply is vital for follicle survival and transport of endocrine factors, vascularization is important in determining the fate of the follicle, and maintaining the blood supply is necessary for follicular health. The effect of atresia in the vasculature depends on the stage of follicle development when atresia is occurring. Small atretic follicles have reduced the numbers of capillaries in the thecal layers; the endothelium begins to degrade, and thecal capillaries become blocked by degrading material (O'Shea *et al*. 1977). Extensive hypertrophy of theca cells is observed during the early stages of atresia in human, rat, and rabbit, but not in sheep or bovine follicles (Himelstein-Braw *et al*. 1976, O'Shea *et al*. 1978, Erickson *et al*. 1985, Clark *et al*. 2004), which, together with the loss of granulosa cell function as they undergo apoptosis, results in androgens being the predominant steroids secreted by atretic antral follicles (Moor *et al*. 1978). In larger bovine antral follicles, the vasculature is well established in non-atretic follicles (Shimizu *et al*. 2003), while the thecal layers of atretic follicles show signs of apoptosis from the outer layers progressing internally, and throughout the vascular network (Jiang *et al*. 2003). Whether the changes in the vasculature observed during follicle atresia are the cause or the effect of the atretic process itself is yet to be established.

Follicle fate is regulated by apoptotic factors such as nodal, which is produced by the thecal cells and acts to promote apoptosis in the neighboring granulosa cells (Wang *et al*. 2006, Craig *et al*. 2007). The oncogene *Skil* (also known as SnoN), involved in regulating TGFB superfamily signal transduction, has been mapped in the mouse ovary recently and is expressed in the theca throughout development and during atresia (Xu *et al*. 2009). This factor can modulate differentiation, proliferation, and apoptosis of several cell types (reviewed by Deheuninck & Luo (2009)), and was shown to have a specific manner of expression relating to follicle atresia and luteinization, suggesting that SKIL may play roles in these processes (Xu *et al*. 2009). VEGF (Redmer *et al*. 2001, Fraser *et al*. 2005), FGF (Shimizu *et al*. 2002, 2003), and TGFB superfamily members (Tomic *et al*. 2002) have also been linked to follicle atresia.

Major factors influencing theca cell differentiation

The current evidence suggests that an activated follicle produces factors that induce thecal cell differentiation from stroma, but the exact identity and combination of the proteins remain unknown. Not unexpectedly, it appears that no single factor appears to be responsible, but complex networks of signals function synergistically to result in a fully functional steroidogenic thecal layer surrounding a developing follicle.

A recent *in vitro* study using bovine ovarian tissue showed that ovarian stromal cells cultured in the presence of granulosa cells from small antral follicles transformed into putative thecal cells with increased lipid droplets and androstenedione production (Orisaka *et al*. 2006). Studies suggest that granulosa cells, but not activated oocytes, are involved in the functional differentiation and acquisition of LH responsiveness in stromal cells, and the cellular origin of the stroma determines whether or not granulosa cells influence thecal cell differentiation and functionality such as expression of necessary steroidogenic enzymes and LHRs (Orisaka *et al*. 2006). It appears that stromal cells from the cortical region may be preprogrammed differently to medullary stromal cells, and able to respond to granulosa cell communication in a manner that medullary stromal cells are not.

Using neonatal mouse ovaries, putative thecal stem cells were purified and induced to differentiate *in vitro* (Honda *et al*. 2007). When these cells were treated with LH, insulin-like growth factor 1 (IGF1), stem cell factor (SCF, also known as kit ligand), or granulosa cell-conditioned media, the cells differentiated into thecal cells, and showed signs of lipid droplet accumulation, formation of smooth endoplasmic reticulum, mitochondria with tubular cristae, and produced androstenedione at later stages of culture. When these putative thecal cells were injected into ovaries of live mice, they were found to surround growing follicles akin to natural thecal cells *in vivo*.

Various factors have been studied *in vitro* for their effects on promoting thecal steroidogenesis. Selected molecules are discussed in the following sections.

Insulin-like growth factor

The ovary has a complete repertoire of IGF system components. IGF receptors are found on human thecal cells (Poretsky *et al*. 1985), and IGF1, the synthesis of which is regulated by FSH in the granulosa cells (Adashi *et al*. 1985, Hammond *et al*. 1985, Hernandez *et al*. 1989, Oliver *et al*. 1989), increases thecal cell proliferation *in vitro* (Hillier *et al*. 1991*a*, Stewart *et al*. 1995, Spicer & Chamberlain 1998, Huang *et al*. 2001, Mazerbourg & Hsueh 2003, Campbell *et al*. 2006, Kwintkiewicz & Giudice 2009). IGF1 alone stimulated the expression of LHRs (Magoffin & Weitsman 1994) and steroidogenic enzymes, CYP11A1 and HSD3B but not CYP17, and acts synergistically with LH to increase expression of these enzymes (Magoffin & Weitsman 1993*a*, 1993*b*, 1993*c*), and hence androgen synthesis in thecal cells *in vitro* (Hillier *et al*. 1991*b*; see Fig. 2). Interestingly, human stromal tissue cultured *in vitro* synthesized androgens when stimulated by insulin and IGF (Barbieri *et al*. 1983, 1984) giving additional evidence of IGF involvement in promoting thecal differentiation (see Fig. 4). IGF2 can stimulate bovine thecal steroidogenesis and acts through the type 1 IGF receptors (Spicer *et al*. 2004), so it may also play important roles in theca functionality.

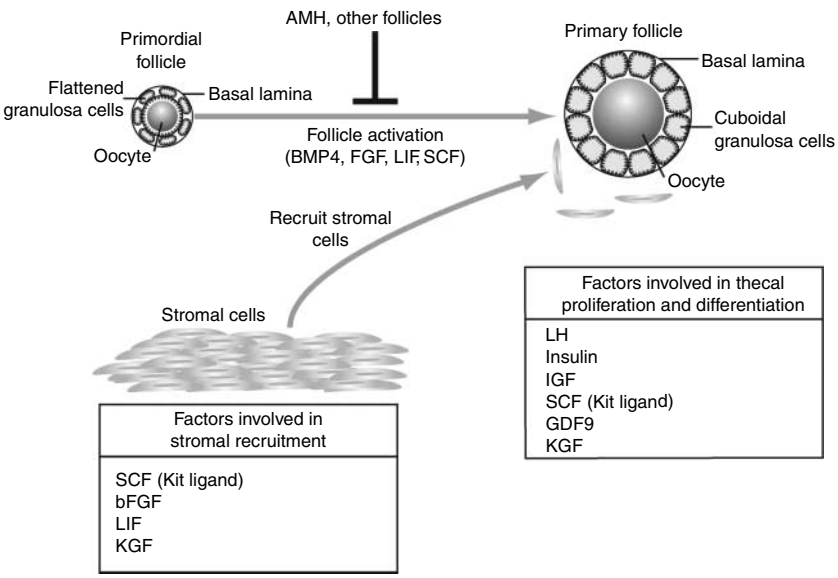

Figure 4 Thecal cell recruitment and differentiation. Evidence suggests that thecal stem cells reside in the ovarian stroma and are recruited by factors released from follicles after they are activated. Individual factors may be responsible for the recruitment of these cells, whereas others may promote differentiation and proliferation. The origin of these factors is not currently clear, but it appears that a complex and tightly controlled set of signals from multiple factors is required for thecal recruitment and differentiation. AMH, anti-Müllerian hormone; bFGF, basic fibroblast growth factor; BMP, bone morphogenetic protein; GDF9, growth differentiation factor 9; IGF, insulin-like growth factor; KGF, keratinocyte growth factor; LIF, leukemia inhibitory factor; SCF, stem cell factor (kit ligand).

Stem cell factor (SCF/kit ligand)

SCF, also known as kit ligand, is a growth factor that acts through the c-kit tyrosine kinase receptor (Zsebo *et al.* 1990, Besmer 1991), is synthesized by granulosa cells, and acts on differentiated thecal cells as well as undifferentiated stromal cells and oocytes where the receptor c-kit is located (Manova *et al.* 1990, 1993, Motro *et al.* 1991, Horie & Broxmeyer 1993, Motro & Bernstein 1993, Parrott & Skinner 1997). The addition of neutralizing antibodies to SCF and IGF1 together to follicle-conditioned media reduced the stimulatory effects on theca cell differentiation by more than 90% *in vitro* (Huang *et al.* 2001). In rat theca, neither SCF nor IGF1 alone stimulated androstenedione production, whereas, in combination, these factors dose dependently induced androgen production, but to a lesser extent than that induced by LH. The effects of adding these factors together with LH showed that IGF1 acted synergistically with LH to increase androgen production, whereas SCF had no added effect when in combination with LH. SCF alone decreased CYP17 and had no effect on the expression of CYP11A, HSD3B, or LHR expression (see Fig. 2). IGF1 alone had no effect on the expression of STAR and CYP17, but increased mRNA levels of LHR, CYP11A, and HSD3B. However, the combination of IGF1 and SCF increased the expression of STAR, CYP11A, CYP17, HSD3B, and LHR, giving strong evidence that these factors may act synergistically to regulate thecal cell differentiation into steroid-producing cells, at least in the rat.

SCF stimulated the growth of bovine primary cell cultures of theca and stromal cells under sub-confluent conditions *in vitro*, but when the cells were grown to confluence, SCF stimulated androstenedione production (Parrott & Skinner 1997). In contrast, SCF did not affect human chorionic gonadotropin-induced androgen production by bovine stromal cells (Parrott & Skinner 2000) suggesting that, in contrast to the rat, SCF alone affects bovine thecal cells once they have been differentiated.

The expression of SCF can also be modulated by leukemia inhibitory factor (LIF), keratinocyte growth factor (KGF), and hepatocyte growth factor (HGF; Parrott *et al.* 1994, Parrott & Skinner 1998, Nilsson *et al.* 2002). Thecal cells produce KGF and HGF (Parrott *et al.* 1994), which act on granulosa cells to produce SCF, which then signals back to the theca to produce KGF and HGF in a positive feedback mechanism (Parrott & Skinner 1998; see Fig. 5). Therefore, SCF may potentially act as a final common factor involved in thecal cell differentiation and activation.

Basic bFGF

Basic FGF (bFGF) has been shown to affect somatic cell mitosis, steroid synthesis, differentiation, and apoptosis (Tilly *et al.* 1992, Lavranos *et al.* 1994, Vernon & Spicer 1994). bFGF is expressed by primordial and primary oocytes, and granulosa cells of larger preantral follicles, and in the theca of rodent, bovine, and human follicles (van Wezel *et al.* 1995, Yamamoto *et al.* 1997, Berisha *et al.* 2000, Nilsson *et al.* 2001). The receptors for bFGF

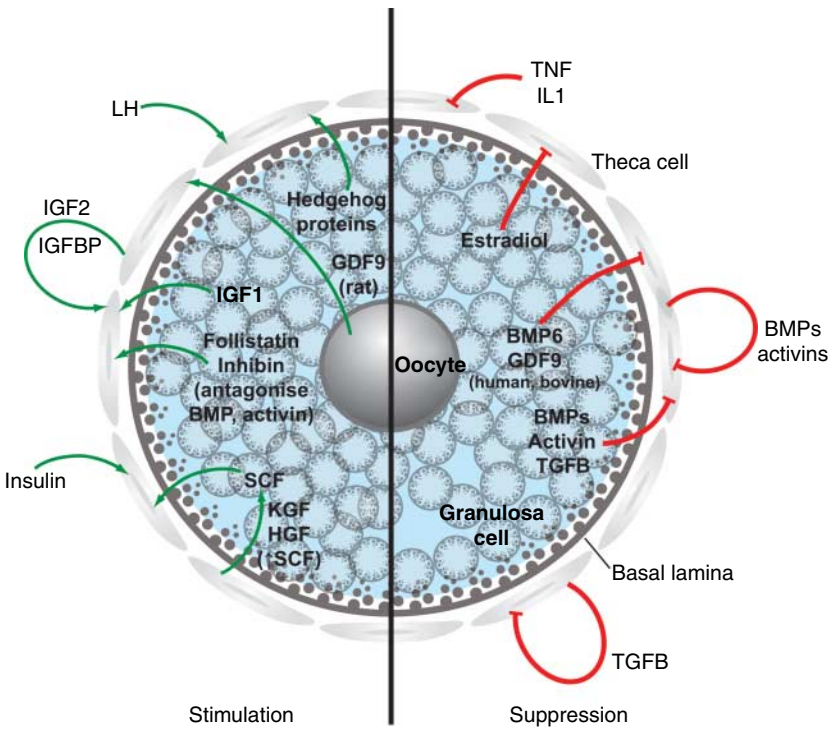

Figure 5 Androgen production from theca cells. Factors released by cells comprising the follicle (granulosa cells, thecal cells, and oocytes) can modulate androgen production in thecal cells in addition to external influences such as gonadotropins and insulin. These molecules can stimulate (green) or inhibit (red) thecal androgen production both directly and/or indirectly. Specific molecules have been observed to act in opposing manners, indicating species-specific differences. bFGF, basic fibroblast growth factor; BMP, bone morphogenetic protein; GDF9, growth differentiation factor 9; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; IGFBP, IGF-binding protein; IL1, interleukin-1; KGF, keratinocyte growth factor; LIF, leukemia inhibitory factor; SCF, stem cell factor/kit ligand; TGFB, transforming growth factor β; TNF, tumor necrosis factor α.

have been found in granulosa and theca cells (Shikone *et al*. 1992, Wandji *et al*. 1992, Shimizu *et al*. 2002, Schams *et al*. 2009). In rat follicle cultures, bFGF activated primordial follicle development to the same extent as SCF (Nilsson *et al*. 2001) and also promoted cell growth in bovine thecal and stromal cells, and is therefore thought to act in a similar fashion to SCF by regulating somatic cell growth and development (Nilsson *et al*. 2001). While bFGF may also influence thecal development indirectly through stimulating SCF expression (Nilsson & Skinner 2004), bFGF, at least in the mouse, is not essential for folliculogenesis since the bFGF null mouse is fertile (Ortega *et al*. 1998).

TGFB superfamily members

TGFB superfamily members are now well established as having vital roles in controlling follicular growth and development (Shimasaki *et al*. 2004, Knight & Glister 2006, Xia & Schneyer 2009). The superfamily consists of a large group of proteins that include the bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), anti-Müllerian hormone (AMH; also known as Müllerian inhibiting substance (MIS)), activins, and inhibin, all of which are expressed in the ovary. These molecules bind to the BMP/TGFB/activin receptors to initiate phosphorylation cascades to influence gene expression in the cell nucleus. BMPs act through the SMAD1/5/8 pathway, whereas TGFB/activin/GDFs act through SMAD2/3. Evidence shows that specific members are involved in thecal cell recruitment, proliferation, and differentiation, which may or may not interact with the gonadotropins. Specific proteins will be discussed in this section, outlining the relevant research in this area and how each factor may contribute to theca growth and function.

Activin and inhibin

Activin and inhibin influence follicle activation, hormone synthesis, and luteolysis within the ovary (reviewed by Knight & Glister (2001)). Activin promoted the development of preantral follicles in sheep (Thomas *et al*. 2003) and human (Telfer *et al*. 2008) ovarian strips in culture, and this was also observed in rat studies (Li *et al*. 1995, Zhao *et al*. 2001), while in the mouse, activin from secondary follicles inhibited activation of small follicles (Mizunuma *et al*. 1999).

Studies on isolated human thecal cells cultured *in vitro* showed that activin suppressed androgen and progesterone production (Hillier *et al*. 1991*a*, Shukovski *et al*. 1993), while the activin antagonist inhibin enhanced LH-mediated androgen production (Hillier *et al*. 1991*b*). It has been suggested that granulosa cells secrete inhibin to control the amount of androgen synthesized by theca cells as substrates for estrogen synthesis in granulosa cells (Hillier *et al*. 1991*b*). Thus, activins have direct effects on thecal cell function, and are regulated by the extracellular antagonists inhibin and follistatin (Findlay 1993, Welt *et al*. 2002).

In some species, an indirect effect of activins may occur through stimulation of granulosa cell proliferation or preantral follicle development (Li *et al*. 1995, Choi *et al*. 2008), which may be enhanced by additional IGF1 (Li *et al*. 1998). Activin can act by upregulating FSH receptors and aromatase gene expression in granulosa cells, and is involved in promoting estradiol production (Nakamura *et al*. 1993, El-Hefnawy & Zeleznik 2001, Ogawa *et al*. 2003, Park *et al*. 2005). Furthermore, since estradiol may suppress activin expression, this forms a possible interaction between activin and estrogen signaling during folliculogenesis (Kipp *et al*. 2007). Increased estradiol and inhibin production by the putative preovulatory follicle(s) would act to suppress activin production through estradiol, and block activin action at the theca through inhibin, thus enhancing thecal androgen production.

Inhibin is a critical factor in the control of steroid production and for control of gonadotropin secretion (McNeilly 2001, Padmanabhan & McNeilly 2001, McNeilly *et al*. 2003), but the effects of inhibin on thecal cell recruitment and differentiation are not known. Inhibin alone increased androgen production from human thecal cells in culture and also blocked the inhibitory effect of added activins (Hillier *et al*. 1991*b*). Inhibin is thought to modulate hormone production through antagonizing activin and BMPs, rather than through a signal cascade of its own (Wiater & Vale 2003, Farnworth *et al*. 2006).

Bone morphogenetic proteins

During folliculogenesis, BMPs are released at specific time points and act in either an autocrine or paracrine manner to modulate growth, differentiation, and function of follicular cells. BMP expression patterns have been investigated in rodents, ruminants, and primates, and observations suggest that species-specific differences occur for some molecules (reviewed by Shimasaki *et al*. (2004)).

BMP4 is expressed in the stromal cells surrounding primordial follicles, and BMP4/7 are expressed in the theca layer of antral follicles (Shimasaki *et al*. 1999, Nilsson & Skinner 2003, Lee *et al*. 2004). In neonatal rat ovaries, BMP4/7 increased the formation of primary follicles (Nilsson & Skinner 2003) and the percentage of growing follicles in the adult rat ovary (Nilsson & Skinner 2003, Lee *et al*. 2004).

BMP6 is produced by oocytes, but its function appears to differ between rodents and ruminants (Otsuka *et al*. 2001, Glister *et al*. 2004, Shi *et al*. 2009*b*). In sheep (Souza *et al*. 2002) and bovine (Kayani *et al*. 2009) ovaries, full complements of BMP/activin receptors were observed in granulosa, theca, and luteal tissues.

Human theca-derived tumor cells show reduced androstenedione production *in vitro* when treated with BMP4, and this effect is enhanced in the presence of cAMP agonists (Dooley *et al*. 2000). BMPs 2/4/6/7 all significantly decreased androstenedione secretion from ovine and bovine thecal cells *in vitro* while moderately increasing progesterone production and cell numbers (Glister *et al*. 2005, Campbell *et al*. 2006), and reducing *CYP17* gene expression, and to a lesser effect for *STAR*, *CYP11A1*, and *HSD3B* (Glister *et al*. 2005; see Fig. 2). The BMP antagonist, chordin, reversed the inhibitory effects of BMP7 on androgen production in bovine theca cells (Glister *et al*. 2005), while gremlin selectively reversed the effects of BMP4 but not BMP6 or 7. The BMP antagonist follistatin did not affect BMP-inhibited androgen production (Glister *et al*. 2005). Taken together, observations indicate that BMPs may act directly on theca to inhibit androgen production and also function to regulate the expression of factors from granulosa cells that act in a paracrine manner on thecal steroidogenesis. There are many BMP mutations that lead to aberrant fertility (McNatty *et al*. 2005), and these proteins may play roles in theca recruitment.

Growth differentiation factor 9

GDF9 is primarily produced specifically by the oocyte (McNatty *et al*. 2004), although it may also be produced in human (Shi *et al*. 2009*a*) and porcine (Paradis *et al*. 2009) granulosa and porcine theca cells (Paradis *et al*. 2009). Mutations of the *GDF9* gene lead to arrested folliculogenesis at the primary stage in mice, sheep, and humans (Shimasaki *et al*. 2004, Laissue *et al*. 2006, Kovanci *et al*. 2007, Nicol *et al*. 2009), suggesting that GDF9 is not vital for follicle activation but it is vital for onward primary growth. Follicles from GDF9 null mice and sheep (Thoka) lack supporting thecal cells indicating a role for GDF9 in the regulation of thecal recruitment, differentiation, and proliferation, but this appears to be dependent on the stage of follicle development (Dong *et al*. 1996, Elvin *et al*. 1999*a*, 1999*b*, Nicol *et al*. 2009). GDF9 alone, and in combination with IGF1, stimulated bovine thecal cell proliferation, but was found to inhibit IGF1- and LH-induced progesterone and androgen production, as well as decreasing LHR, LH-induced cAMP, and CYP11A1 expression levels without altering IGF1 receptor, STAR, or CYP17 levels (Spicer *et al*. 2008; see Fig. 2). The level of proliferation appeared higher in theca cells from small follicles compared to those from large follicles, and this may be related to higher levels of the putative GDF9 receptor, ALK5, in theca cells from small follicles. Thus, in small follicles, GDF9 could enhance proliferation, yet have no effect on promoting differentiation of thecal cells. GDF9 increased androgen production in rat thecal cells (Solovyeva *et al*. 2000), but reduced androgen production from human thecal cells indicating some important species-specific differences (Yamamoto *et al*. 2002). Alternatively, the difference may be due to luteinization of the cells in culture.

GDF9 may also act in an indirect manner to modulate theca cell function, perhaps through regulating SCF expression (Dong *et al*. 1996, Joyce *et al*. 2000, Nilsson & Skinner 2002, Wang & Roy 2004). GDF9 also stimulated inhibin production (Hayashi *et al*. 1999, Kaivo-Oja *et al*. 2003, Roh *et al*. 2003), and the GDF9–inhibin-α double knockout mouse model is observed to have morphological thecal cells surrounding preantral follicles (Wu *et al*. 2004). However, these cells do not appear to have been differentiated into thecal cells since the expression of theca cell markers such as CYP17A1, LHR, or KIT are absent. These findings indicate that recruitment of putative theca-like cells can occur in the absence of GDF9, but differentiation of these cells does not appear to occur. GDF9 appears to have an indirect function on thecal cells, perhaps by promoting granulosa cell proliferation.

Transforming growth factor β

Both TGFB1 and TGFB2 are present in theca cells from follicles at the small preantral stage of development onwards and in stromal tissue and vascular systems in sheep ovaries, but not in granulosa cells or oocytes (Juengel *et al*. 2004). The receptors, TGFBR1 and TGFBR2, had variable expression, and R1 was found in stromal and vascular cells, whereas R2 mRNA was found in thecal cells from the preantral stage onwards through development, as well as in the surface epithelium and some stromal cells. Furthermore, latent TGFB-binding proteins, which affect the bioavailability of TGFBs in tissues, are localized to the ovarian cortical stroma and theca externa of bovine antral follicles (Prodoehl *et al*. 2009). TGFB1 has been reported to suppress androgen synthesis from human and rat thecal cells (Fournet *et al*. 1996, Attia *et al*. 2000; see Fig. 5), while in the mouse, TGFB1 increased *Cyp11a*, *Cyp17*, and *Hsd3b* gene expression at various times during the culture period (Fournet *et al*. 1996), but inhibited STAR expression in a human thecal-like tumor cell line (Attia *et al*. 2000; see Fig. 2). A study using whole rat ovarian dispersed cell cultures suggested that TGFB1 blocks steroidogenesis at the level of CYP17 (Hernandez *et al*. 1990). These results are not clear-cut, but it appears that in the human at least, TGFB has a similar role to activin and BMPs in suppressing androgen production.

Hedgehog proteins

The hedgehog pathway has recently been shown to intersect with pathways involved with FGF receptor 2, BMPs, and other regulatory networks (Katoh 2009). Theca cells appear to be modulated by hedgehog signaling with the expression of hedgehog target genes

Ptch1, *Ptch2*, *Hip1*, and *Gli1* all present within theca cells (Wijgerde *et al*. 2005). Hedgehog proteins are expressed in the granulosa cells, but oocytes are unable to respond since they do not contain the necessary receptors. Hedgehog target genes are expressed in the pre-thecal cell compartment, and are therefore possible markers of pre-thecal cells and potentially involved in inducing theca cell differentiation. In cultured bovine thecal cells, sonic hedgehog-induced cell proliferation and androstenedione production (see Fig. 4), and hedgehog genes were shown to activate *Gli1* transcription factor in thecal cells (Spicer *et al*. 2009). In a transgenic mouse model, the hedgehog pathway was dominantly activated, and these mice displayed defective thecal development with reduced or absent smooth muscle actin normally seen in the thecal layer of growing follicles (Ren *et al*. 2009). The dominant activation of the hedgehog pathway therefore appears to block the differentiation of precursor cells into muscle cells that are normally located in the outer thecal layers, and are perhaps required for ovulation.

Synergism

A common trend appears to be emerging where the TGFB superfamily members are involved in fine tuning the modulation of androgen biosynthesis and steroidogenic enzyme gene expression. TGFBs, activin, and GDF9 all signal through SMAD2/3, whereas BMPs signal through the SMAD1/5/8 signaling pathway (Shimasaki *et al*. 2004). It makes sense that these two independent pathways influence the expression of completely separate groups of genes; otherwise, they would simply utilize the same pathway for the same effects. If the two pathways do in fact activate separate sets of genes, then these genes appear to be having similar effects on steroidogenesis. BMP4/6/7 suppress STAR, CYP11A1, CYP17, and HSD3B (Glister *et al*. 2005), and TGFB acts similarly by suppressing CYP17. GDF9 conversely appears to function in a more complicated manner, where it increases CYP17 expression while suppressing CYP11A1 levels. These contrasting results in different species coincide with the observed effects on androgen production, where in rat theca cells, GDF9 enhanced androgen synthesis (Solovyeva *et al*. 2000), but in bovine theca cells, GDF9 acted in an inhibitory manner (Spicer *et al*. 2008). There may be important species-specific differences in the function of GDF9 in particular, and this may also be the case with other TGFB superfamily members and therefore requires closer investigation.

There are also important species differences between rodents and humans with regard to stem cells. Mouse ES cells require LIF (Smith *et al*. 1992) and BMPs (Ying *et al*. 2003) to maintain pluripotency, whereas human counterparts rely on activin/nodal (Vallier *et al*. 2004, 2005, James *et al*. 2005) and FGFs (Xu *et al*. 2005). However, studies have shown that the same genes; *POU5F1* (*OCT4*), *SOX2*, and *NANOG*, are required for pluripotency in both species. Activin/nodal signaling controls expression of the key pluripotency factor NANOG. NANOG prevents differentiation induced by FGF signaling and limits transcriptional activity of SMAD2/3 (Vallier *et al*. 2009). By studying each factor in isolation, one is able to infer a specific function and role in ovarian folliculogenesis. However, we know that *in vivo* this is clearly not the case, and many different factors at varying, tightly controlled, concentrations all work synergistically to control the very delicate balance between the life and death of an ovarian follicle. The level and pattern of gene expression are vital factors; moreover, crosstalk between pathways and the presence of antagonists are additional levels of control for folliculogenesis and are yet to be fully elucidated.

Concluding remarks

Classically, it was thought that the oocyte was passively carried along the developmental process, and its maturation was controlled entirely by the production of endocrine hormones and surrounding somatic cell factors influencing the follicle as a whole. The latest concept in reproductive biology is that the oocyte itself is actively involved in regulating the surrounding somatic cells in order to provide an environment suitable for its own maturation. With this new and exciting theory in mind, it is possible that the oocyte itself is responsible for sending the signal for primordial follicle activation and thecal cell recruitment. However, since oocytes produce only limited factors, it is most likely that the interaction and communication between the oocyte and its somatic cells control the follicle development as a whole, and when one component fails, the entire process is halted. Nevertheless, thecal cells are vital for folliculogenesis in the ovary. They are specialized cells that are recruited to surround an activated follicle and provide structural support at first, and then by proliferating and differentiating, and acquiring a capillary network, they have become essential components of developing follicles. Their primary function is to synthesize androgens which act as substrates for estrogen production in granulosa cells, which is crucial for the pituitary–gonadal axis and endocrine control of reproduction. Androgen production is largely under the control of LH produced by the pituitary and transported to thecal cells via the blood stream. However, it is now clear that many other factors play an essential and important role in the modulation of theca function, including IGFs, insulin, FGF, SCF, TGFB superfamily members, and their related pathways and regulators. Theca cells have been somewhat forgotten more recently, where topical research has focused on granulosa cells and oocytes, but these specialized cells have a highly significant role in follicular function and are crucial for normal follicular development.

Declaration of interest

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

Funding

This work was funded by MRC (G7.002.0007.01) to A S McNeilly, which supports J M Young.

Acknowledgements

We would like to thank Ted Pinner for preparation of the figures, and Bruce Campbell for useful discussions.

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Received 16 February 2010
First decision 31 March 2010
Revised manuscript received 6 July 2010
Accepted 13 July 2010