Development of the dominant follicle: mechanisms of selection and maintenance of oocyte quality

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For a follicle to reach dominance, in mono-ovulatory species such as cattle, requires the integration of a number of processes involving both extra-ovarian signals and intra-follicular paracrine and autocrine regulators. Ovarian transplant studies in both cattle and sheep demonstrated that it takes approximately 4 months for primordial follicles to reach dominance. Gonadotrophins are not a prerequisite for the continued growth of pre-antral follicles, unlike antral follicles, but FSH does appear to stimulate development. Local growth factors, such as IGFs and BMPs, are expressed throughout follicle development and interact with gonadotrophins to stimulate development. As follicles become dominant, there is a transfer of dependency from FSH to LH. There are also differences in LH-responsiveness of theca and granulosa cells during follicular development, due to differential regulation and control by intricate local mechanisms altering LH receptor (LHR) mRNA expression. In addition, both the BMP and IGF systems can modulate the proliferative and differentiative responses of both granulosa and theca cells to gonadotrophins. There is a significant interaction between BMPs and the IGF system in regulating follicular development. A range of factors, including nutrition, will also determine the fate of the growing follicle and the quality of the oocyte. Nearly all follicles regress and apoptotic cell death throughout follicular development is an underlying mechanism of cell loss during follicular atresia. Several markers of follicular atresia have been identified including IGFBPs. There is a significant correlation between the presence of low molecular weight IGFBPs in bovine follicular fluid and caspase-3 activity of granulosa cells in individual follicles. In conclusion, it is the interaction between extra-ovarian and intra-ovarian factors that determine the fate of the follicle and the quality of the oocyte.

Introduction

Ovarian folliculogenesis is a lengthy and intricately regulated process marked by dramatic proliferation and precisely orchestrated differentiation of both the somatic and germ cell elements. Primordial follicles represent the source from which follicles will be recruited for growth throughout life, with paired ovaries of an individual containing around 100,000-250,000 of these follicles

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at birth (human: Gougeon 1996; sheep: Turnbull *et al.* 1977). Once follicles have been initiated to grow, the granulosa cells proliferate to form multilaminar structures (pre-antral follicles) which subsequently form a fluid filled space (antrum) and a well differentiated theca layer. Follicular development in sheep and cattle takes around 4-5 months with the majority of this time (3-4 months) being spent in the pre-antral stages of development (Cahill 1981; Gougeon 1996; Turnbull *et al.* 1977). When the follicle reaches a diameter of 200-400 μm, the antrum develops (Turnbull *et al.* 1977) and this is followed by widespread atresia (50-70% for follicles over 1 mm) so that the vast majority (>99%) of follicles fail to ovulate.

It is widely accepted that control of the terminal stages of folliculogenesis lies primarily with the pituitary gonadotrophins, FSH and LH, combined with the differential expression of somatic cell-derived growth factors that modulate the action of gonadotrophins at key points during the process of follicle development (Campbell *et al.* 1995; Webb *et al.* 1999). Communication between the oocyte and the surrounding somatic cells (Carabatsos *et al.* 2000), via a range of locally produced growth factors, is essential for the coordinated development of both cell types (Eppig 2001), making folliculogenesis a highly synchronised process. These local growth factor systems include the insulin-like growth factor (IGF) system (Webb *et al.* 1999), the inhibin/activin system (Knight & Glister 2001) and the bone morphogenetic (BMP) system (Shimasaki *et al.* 2003; Knight & Glister, 2006). In addition, recent studies also suggest that the oocyte, rather than being purely a passenger within the follicle, secretes numerous factors that modulate follicle development and ovarian function. Known oocyte-secreted factors include growth differentiation factor-9 (GDF-9) (Dong *et al.* 1996) and BMP-15 (Aaltonen *et al.* 1999; Dube *et al.* 1998) as well as factors in the germline alpha (FIG-α) (Huntriss *et al.* 2002; Soyal *et al.* 2000) and c-kit receptor (Driancourt *et al,* 2000; Reynaud *et al.* 2000; Reynaud *et al.* 2001; McNatty *et al.* 2007 this supplement). However the temporal pattern and quantity of secretion of these factors has yet to be determined.

Ovarian follicular growth is therefore a developmental process during which the follicle progressively acquires a number of properties at a specific time and sequence, each of which is an essential prerequisite for further development. The orderly expression of these somatic and oocyte-derived factors, or "intrafollicular cascade", is thought to be essential for the development of the follicle to an ovulatory size, the subsequent production of an ovulatory signal and the release of a fully developmentally competent oocyte in response to that signal. Although some components of this intrafollicular cascade have been elucidated, most have been studied in isolation and the relative importance and temporal relationships between known and novel regulatory factors at different stages of follicle development remain to be elucidated. In this review we will examine recent advances in this area with respect to follicle development in sheep and cattle, concentrating primarily on the factors regulating antral and dominant follicle development.

Pre-antral follicle development

Intra-ovarian factors

Mechanisms regulating the activation and subsequent growth of primordial follicles have been reviewed in detail, particularly in relation to sheep (McNatty *et al.* 2007 this supplement) and so will not be reviewed here. In a number of species, as well as for the initiation of primordial follicle growth, the continued growth of pre-antral follicles is dependent upon the secretion of a range of local factors including GDF-9, BMPs, activins, inhibins, basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (Knight & Glister 2001; Smitz & Cortvindt 2002; Webb *et al.* 2003; Hunter *et al.* 2004). As will be discussed, only studies identifying genetic mutations in sheep have been carried out (see Hanrahan *et al.* 2004; McNatty *et al.* 2007). However, Armstrong *et al.* (2002a) demonstrated that bovine pre-antral follicles express mRNAs encoding both IGFBP-2, -3 and type 1 IGF receptor. EGF, also stimulates pre-antral follicle growth (Gutierrez *et al.* 2000; Saha *et al.* 2000).

Studies in our laboratory have also examined fetal ovaries for evidence of local regulatory systems operating during oogenesis. BMP-6 and BMP receptors (BMPR) IA, IB and II have been shown to be present in bovine fetal ovaries between 2-9 months of gestation (Dugan *et al.* 2004; Fouladi-Nashta *et al.* 2005). The presence of both the ligand (BMP-6) and the receptors at this early stage illustrates the presence of a fully functional BMP system early in development. The expression of BMP-6 appears to be localised both in the oocyte, with the intensity of staining increasing with fetal age, and also in granulosa cells from 6 months of gestation. These results, together with results from post-natal bovine and ovine ovaries (Dugan *et al.* 2004), suggest that expression of both the ligand and the receptors is present right through gestation, parturition and into adulthood, since BMP-6 has been shown to be abundant in both the oocyte and granulosa cells of adult bovine follicles (Glister *et al.* 2004; Campbell *et al.* 2006). However, BMP-6, but not -2, -4 or -7 mRNA expression has been detected in ovine oocytes of pre-antral follicles (Juengel *et al.* 2006). Interestingly, Fatahei *et al.* (2005) was only able to immunolocalise BMP-2 and BMP-4 in bovine oocytes and theca cells of adult antral follicles, whereas BMPRII was observed in oocytes from the primordial stage. The functional significance of the expression patterns of components of the BMPs in domestic ruminants is unknown, but in the absence of knock-out models in this species, some inferences may be drawn from naturally occurring mutations.

McNatty *et al.* (2007) has reviewed the effect of null mutations of the oocyte secreted factors GDF-9 and BMP-15 in sheep. These studies show that these factors are essential for normal pre-antral follicle development. In contrast, the FecB mutation in the BMPRIB receptor, whilst inducing precocious maturation of ovulatory follicles (see later), has not been shown to have had a major effect on pre-antral follicle development, although some changes suggesting precocious development have been detected (McNatty *et al.* 1986). At present, the available evidence suggests that the FecB mutation results in a down-regulation in BMP signalling (Fabre *et al.* 2003) and therefore it appears likely that signalling through this receptor system is not of major importance during pre-antral follicle development, although it is possible that significant redundancy exists. The abundant expression of the BMPRIA receptor tends to support this possibility.

Research into the functional role of local regulatory systems during early folliculogenesis in ruminants and other mono-ovulatory species is severely hampered by the lack of physiological culture systems that will allow normal follicle and oocyte development from the primordial/primary stages of development through to antrum formation with high efficiency. Although significant advances have been made over recent years in this regard (Gutierrez *et al.* 2000; Fortune *et al.* 2000; McCaffery *et al.* 2000, Picton *et al.* 2003), existing systems remain sub-optimal and it is a research priority to develop *in vitro* model systems for these species.

Extra-ovarian factors

It is generally agreed that gonadotrophins are not involved in the initiation of follicle growth from the primordial follicle pool. FSH receptor (FSHr) mRNA is detected in follicles with only one or two layers of granulosa cells (Bao & Garverick 1998) and both *in vivo* (Campbell *et al.* 2000) and *in vitro* (Gutierrez *et al.* 2000) studies have demonstrated that FSH can accelerate the rate of pre-antral follicle development. Expression of LH receptor (LHr) mRNA is first detected when the theca interna forms around the granulosa cells (Bao & Garverick 1998), presumably stimulating androgen precursor production. This is supported by a range of steroidogenic enzymes, these include cytochrome P450 side chain cleavage (P450scc), cytochrome P450 17α-hydroxylase (P450c17), and 3ß-hydroxysteroid dehydrogenase (3ß-HSD) mRNAs which are first expressed soon after formation of the theca interna (Bao & Garverick 1998), with cytochrome P450 aromatase (P450arom) being localised solely to granulosa cells. Steriod enzyme protein information is very limited, although mRNA expression patterns agree with recent results showing that both small (KJ

Dugan, M Lopez-Bejar, DG Armstrong and R Webb, unpublished observations) and larger (Thomas *et al.* 2001) pre-antral follicles are capable of producing oestradiol early in development.

Additional functional investigation into the role of gonadotrophins and their interaction with other extra-ovarian factors *in vivo* have utilised ovarian autografts treated with bovine somatotrophin (BST). In this model system, early follicle development is synchronised through the loss of growing follicles during graft re-vascularisation. In addition these studies in both sheep and cattle, in which gonadotrophin and insulin/IGF concentrations were modulated, showed that BST had a marked effect on the growing follicle population and that there was a clear interaction with gonadotrophic status (Campbell BK, Armstrong DG, Telfer EE and Webb R, unpublished observations). Thus, under normogonadotrophic conditions in hemi-ovariectomised sheep (FSH: 1.1 ± 0.1 ng/ml), BST treatment had a negative effect on the relative proportion of secondary/tertiary, pre-antral and antral follicles, whereas under hypergonadotrophic conditions (ovariectomised; FSH: 5.3 ± 0.6 ng/ml) there were less secondary and tertiary follicles, but more late pre-antral and antral follicles in BST-treated animals (Campbell BK, Armstrong D & Telfer E, unpublished observations). Whilst confirming our previous data (Campbell *et al.* 2000) that gonadotrophin levels influence the rate of pre-antral follicle development, these findings also support the hypothesis that the IGF system represents a key determinant of successful follicle and oocyte development during stages of pre-antral follicle development. Our current view is that exposure of the developing follicle and oocyte to the potent proliferative and differentiative actions of the IGFs is modulated through the abundant local expression of IGF-BP2 during the pre-antral stages of development (Webb *et al.* 2003; 2004) and findings from follicle culture studies support this hypothesis (Walters *et al.* 2006). These findings therefore demonstrate possible interactions between extra-ovarian hormones and intra-ovarian growth factors in the control of these early stages of follicle development.

Antral follicle development

Extra-ovarian factors

The formation of a fluid filled cavity or antrum occurs at a diameter of ~ 200-400μm in sheep and cattle (Turnbull *et al.* 1977). In hypophysectomised sheep antral follicle development continues to a diameter of 2-4 mm. This stage of follicle development is commonly referred to as the gonadotrophin-responsive phase in recognition of the fact that while FSH can accelerate the rate at which these follicles grow, the presence of FSH is not an essential requirement (Webb *et al.* 1999), certainly for sheep. In contrast, antral follicle growth from 2-4 mm in diameter is under gonadotrophic control (Campbell *et al.* 1995), with each wave of follicular growth being preceded by a transient increase in FSH secretion (Adams 1999; Souza *et al.* 1997). The antral follicle stage marks the transition point from a proliferative to a differentiative phenotype for the follicular somatic cells and is accompanied by a decrease in mitotic index, a marked increase in the rate of follicular atresia (Turnbull *et al.* 1977) and marked changes in gene expression (Webb *et al.* 2003). Changes in the expression patterns of mRNAs for both gonadotrophin receptors (FSHr and LHr) and steroidogenic enzymes, including P450scc, P450c17 and P450arom, and 3ß-HSD (Bao *et al.* 1997; Webb *et al.* 1999) occur at this stage of development. An increase in follicular diameter to around ~ 5mm in diameter is characterized by induction of mRNA expression for P450scc and P450arom in granulosa cells. As follicles grow further there is increased expression of mRNA for P450scc and P450arom in granulosa cells and P450c17 in theca cells.

FSH infusion in cattle, in which pituitary gonadotrophin secretion had been significantly reduced by GnRH agonist (GnRHa) or GnRH immunisation, has been shown to stimulate follicle growth up to 8.5 mm in diameter (Crowe *et al.* 2001; Garverick *et al.* 2002). Also infusion of FSH in cattle can induce an increase in mRNA expression for P450scc and P450arom in granulosa cells

in small (1-4mm) follicles (Garverick *et al.* 2002). Interestingly, although there is a seven-fold variation between cattle in the maximal number of follicles >3mm in diameter during follicular waves, there appears to be high repeatability of numbers of follicles >3mm in diameter during follicular waves within individual dairy cattle (Burns *et al.* 2005). This supports further the presence of a tightly regulated compensatory mechanism that regulates follicle growth as well as ovulation rate in sheep and cattle.

The development of methods to detect and increase the number of the gonadotrophin-responsive and gonadotrophin-dependent antral follicles is of immense practical significance for the application of assisted reproduction technologies in domestic species and in humans. The number and health of the antral follicle population represents the so-called "ovarian reserve" of antral follicles that will respond to ovarian stimulation protocols employing exogenous gonadotrophins (Macklon & Fauser 2005). Conceptually, all ovarian stimulation protocols work on the principle of artificially increasing circulating FSH concentrations for a protracted period of time by administering gonadotrophin preparations that contain varying concentrations of FSH and LH. This treatment recruits and stimulates a variable proportion of the gonadotrophin-responsive follicles present in the ovaries to an ovulatory stage. The oocytes within these follicles can be retrieved by ultra-sound guided ovum pick-up (OPU) prior to ovulation or flushed from the oviducts following ovulation. An exogenous ovulatory stimulus such as hCG (in humans) or GnRH (in cattle) is usually delivered to induce the final maturational changes in the follicles and oocytes prior to OPU. Irrespective of species, aggressive and unphysiological ovarian stimulation regimes produce oocytes of variable developmental competence (Kanitz *et al.* 2002; Macklon *et al.* 2006). In human ART, indicators of ovarian reserve such as FSH concentrations, antral follicle counts, inhibin B and anti-mullerian hormone (AMH) concentrations are used increasingly to try and predict ovarian response to treatment and design suitable ovarian stimulation regimes (Macklon & Fauser 2005; Visser *et al.* 2006; Yong *et al.* 2003). In domestic ruminants, whilst such individual treatment is impractical, there have been an increasing number of reports in the literature of the use of GnRH-analogues to enhance the response to ovarian stimulation (Berlinguer *et al.* 2006; Gonzalez-Bulnes *et al.* 2004; Lopez-Alonso *et al.* 2005). These enhanced responses seem to occur due to an accumulation in the number of follicles at the gonadotrophin-responsive to gonadotrophin-dependent threshold in animals rendered hypogonadotrophic by GnRH-analogue treatment. However, whilst oocyte or embryo number may be enhanced by such GnRH-analogue treatment, some authors have reported poor embryo quality with these types of stimulation regimes (Berlinguer *et al.* 2006; Gonzalez-Anover *et al.* 2004; Lopez-Alonso *et al.* 2005). It is not known whether these effects on embryo quality reflect high rates of atresia in this pool of arrested gonadotrophin-responsive follicles or inadequacies in the gonadotrophin-stimulation regimes in these studies.

The development of follicles does not depend solely on gonadotrophins, but also the interaction with a range of local and circulating growth factors. For example, we have previously reported that treatment with BST, through increasing circulating insulin and IGF concentrations, can increase both the number of gonadotrophin-responsive follicles and their quality (Gong *et al.* 1991; 1993; 1997) and that embryo yield can be enhanced by stimulation of these follicles to the ovulatory stage using exogenous BST prior to FSH treatment (Gong *et al.* 2002).

Intra-ovarian factors

Utilising *in vitro* culture systems, a wide range of local factors, including members of the TGFß superfamily, FGFs and EGF/TGFα have been shown to be involved in the regulation of early antral follicle growth (Webb *et al.* 2003; 2004; Buratini *et al.* 2005). For example, BMP localisation studies have been carried out in adult sheep (Souza *et al.* 2002) and cattle (Glister *et al.* 2004). In

line with rodent species (Shimasaki *et al.* 2004), and as discussed previously, there appears to be a functional BMP system within the ovary, with several BMPs being implicated as paracrine/autocrine regulators of ovarian follicle development. In cattle, BMP-4 and -7 have been immunolocalised to theca cells and BMP-6 has been immunolocalised to oocytes and granulosa cells (Glister *et al.* 2004) and BMPRIB and II to granulosa cells, theca cells and denuded oocytes from bovine antral follicles (Glister *et al.* 2004; Fatahei *et al.* 2005). As discussed for pre-antral follicles, in sheep, BMPRIA, IB and II receptors have been localised to the granulosa and theca cells of growing follicles (Souza *et al.* 2002) and BMP-6 is strongly expressed in the oocyte and granulosa cells, with weak expression in the theca cell layer of antral follicles (Campbell *et al.* 2006). The action of BMPs also appear to be modulated by a range of binding proteins e.g. follistatins; (see Lin *et al.* 2006; Knight & Glister 2006), although more detailed study is required.

Functionally, BMP-2, -4, and -6 have been shown to augment FSH-stimulate oestradiol and inhibin A production by cultured granulosa cells in sheep (Souza *et al.* 2002; Campbell *et al.* 2006). Similarly BMP -4, -6 and -7 have stimulated oestradiol, inhibin –A, activin –A and follistatin, but without FSH, in bovine granulosa cells (Glister *et al.* 2004). Conversely, BMPs in both cattle (BMP-4, -6 and -7: Glister *et al.* 2005) and sheep (BMP-2, -4 and -6: Campbell *et al.* 2006) are potent inhibitors of thecal androgen production. However, in sheep these BMPs have also been shown to stimulate thecal cell proliferation *in vitro* so that at very low doses total androgen production is actually increased by BMPs. Overall, these data suggest that BMPs are acting as both autocrine and paracrine factors to enhance ovarian steroidogenesis.

Recent studies utilising *in situ* hybridisation in sheep have confirmed that oocytes express mRNA for BMP-6, but failed to show mRNA expression for BMP-2, -4 and -7 in any cells of non-atretic ovarian follicles (Juengel *et al.* 2006). These data therefore suggest that BMP-6, like BMP-15 and GDF-9, is an oocyte secreted factor that is the primary mediator of paracrine interactions with ovarian somatic cells. These results, however, conflict with those of Souza *et al.* (2002) who reported mRNA expression for BMP-2, -4, -6 and -7 in whole ovary from this species following Northern analysis. Further studies are therefore required in both sheep and cattle to confirm the tissue specific expression of potential ligands for the BMP receptors.

As an augmenter of somatic cell differentiation, the BMPs have a similar role to the IGF-system (Webb *et al.* 2003; 2004). Glister *et al.* (2004) demonstrated that BMP-4, -6 and -7 enhanced IGF-induced secretion of oestradiol, inhibin-A, activin-A and follistatin by bovine granulosa cells, but did not examine the interaction with the level of FSH stimulation. In contrast, recent data from sheep shows clearly that the BMPs are ineffective in stimulating granulosa cell differentiation in the absence of FSH, but do reveal a clear interaction between the level of IGF and BMP exposure in terms of the induction of aromatase activity (Campbell *et al.* 2006; Fig. 1). Thus, in this species both BMP and IGF act to augment FSH-stimulated cellular differentiation. Furthermore, bovine granulosa cell culture studies have recently suggested that FSH and oestradiol can down-regulate the expression of BMPRII (Jayawardana *et al.* 2006) and hence are possibly involved in the selection of bovine follicles. Moreover utilising ovarian tissue from ewes with the FecB mutation of BMPRIB showed that the mutation resulted in an increased response of both granulosa and theca cells to BMPs, gonadotrophins and IGF-I stimulation (Campbell *et al.* 2006). The increased responsiveness of ovarian somatic cells to these factors could account for the precocious maturation of antral follicles in FecB mutants, which is characterised by the development of aromatase activity and LH receptors by granulosa cells of antral follicles at markedly smaller diameters than in wild-type ewes (Driancourt *et al.* 1985; McNatty *et al.* 1985; 1987). These *in vitro* observations are therefore consistent with the profound effect of the FecB mutation in inducing precocious maturation of ovarian follicles (Webb *et al.* 1999; 2003) and hence deregulating the normal follicle selection mechanisms operating in this species.

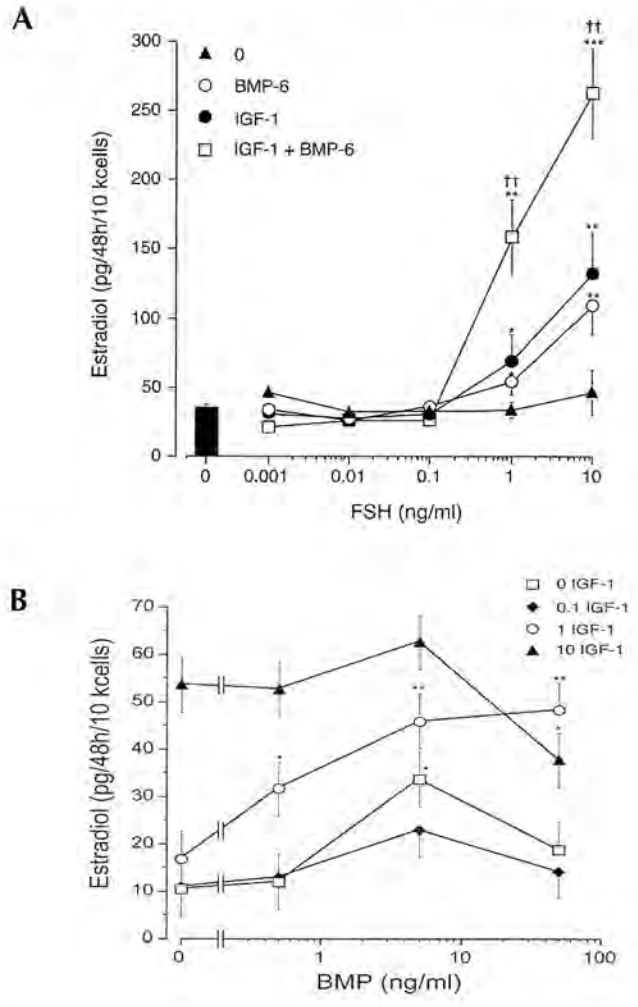

Fig. 1 A. Effect of increasing doses of oFSH in the presence of either 10 ng/ml insulin (full triangle), insulin and 5 ng/ml BMP-6 (open circle), insulin and 10 ng/ml IGF-1 LR3 (closed circle) and insulin, BMP-6 and IGF-1 LR3 combined (open squares) on ovine granulosa cell *in vitro* oestradiol production. The column represents overall mean production in the presence of all factors, but in the absence of FSH. Asterix indicate significant difference from zero dose of FSH with $*P<0.05$, $**P<0.01$ and $***P<0.001$. † indicates significant difference between BMP and IGF supplementation alone compared to the combined treatment at a given dose of FSH with BMP-6 and IGF-I LR3 $P<0.01$.

B. *In vitro* oestradiol production by ovine granulosa with increasing doses of BMP in the presence of 10 ng/ml insulin and FSH (open square), insulin, FSH and 0.1 ng/ml IGF-1 LR3 (full diamond), insulin, FSH and 1 ng/ml IGF-1 LR3 (open circle) and insulin, FSH and 10 ng/ml IGF-1 LR3 (closed triangle). Note that the *in vitro* oestradiol production data are pooled from cultures utilising BMP-2, -4 and -6 due to similar responses to the three BMPs. A marked interaction between dose of BMP and IGF-1 is clearly evident from the data ($P<0.05$), with a flattening of the BMP dose response curve with increasing dose of IGF-1. Asterix indicate significant difference from zero dose of BMP within each dose of LR3 IGF-1 with $*P<0.05$, $**P<0.01$ and $***P<0.001$. After Campbell *et al.* 2006.

As well as for the BMPs, the IGFs are also involved in local control mechanisms. It is around the time of antrum formation that IGF-II mRNA is first detected in thecal tissue in cattle. Type 1 IGF receptor and a range of IGFBPs (IGFBP-2, -3 and -4) have also been detected at this stage of development (Armstrong *et al.* 1998; 2000). Despite contradictory evidence on the production of IGF-1 by ovine and bovine granulosa cells (Webb *et al.* 2003; 2004), there is general agreement that IGF-II, produced by theca cells, is the major intrafollicular IGF ligand regulating the growth of bovine antral follicles (Yuan *et al.* 1998; Armstrong *et al.* 2000; Webb *et al.* 1999), acting through the type 1 IGF receptor (Lucy 2000). Despite the major site of IGF-II production being the thecal cells, Spicer *et al.* (2004) demonstrated that the stimulatory effects of IGF-II on thecal cell steroidogenesis, from large bovine follicles (>7.9mm), is mediated via IGF type 1 receptors.

The ovarian IGF system also appears to interact directly with the oocyte. Small follicles from cattle offered high energy diets had significantly reduced expression levels of mRNA encoding IGFBP-2 and -4 (Armstrong *et al.* 2001), potentially regulating the bioavailability of IGF and hence influencing oocyte developmental potential. Concentrations of IGF-I that are optimal for follicle growth *in vitro* were seen to be detrimental to oocyte maturation (McCaffery *et al.* 2000). Hence, over-stimulation by IGFs, and possibly insulin, may be detrimental to oocyte quality (Armstrong *et al.* 2001). It appears that nutritionally induced changes in both circulating concentrations of insulin and IGF-I and the ovarian IGF system are important for follicle recruitment. However, these changes may also be detrimental to the quality of the oocyte within the growing follicle (Adamiak *et al.* 2005).

In conclusion, it is clear that the antral follicle stage is a transitional phase during which the follicle becomes increasingly dependent on the pituitary gonadotrophins as the rate of somatic cell proliferation declines and the cells differentiate and develop the ability to secrete increasing amounts of ovarian steroids and peptides. The control of this phase of development therefore involves complex interactions between local factors, many of which we now know are derived from the oocyte, other extra-ovarian factors circulating in the blood and the pituitary gonadotrophins. An outstanding characteristic of this follicle class is their heterogeneity and this reflects the fact that antral follicles each form their own micro-environment that will transduce gonadotrophic signals differentially. A positive response to a given level of gonadotrophic input will therefore ensure progression of a small number of individual follicles to the next large antral and/or dominant stage. The majority of follicles that reach this stage of development, however, will be unable to elicit a positive response to gonadotrophins and will be lost through the process of atresia.

Dominant follicle

Extra-ovarian factors

The precise mechanism for the selection of dominant follicles remains to be fully elucidated, but does involve the action of gonadotrophins as well as locally produced factors. As discussed, both FSH and LH exert their effects on follicular somatic cells via specific membrane bound receptors that exhibit alternate patterns of expression. It is well established that the granulosa cells of large oestrogenic (dominant) antral follicles in sheep and cattle develop LH receptors, (Webb & England 1982; Ireland & Roche 1982; Bao & Garverick 1998; Webb *et al.* 1999; 2003) and it is now generally accepted that this event is critical to the process of follicle selection in mono-ovulatory species. It has been suggested that after the emergence of a follicular wave it is oestradiol and inhibin, produced by the growing and selected follicles that *acts to suppress* the secretion of FSH (Webb *et al.* 1999; 2003) resulting in the rapid deviation in the size of the future dominant follicle

and the largest subordinate follicle (Kulick *et al.* 1999). Thus the presence of LH receptors (LHR) on granulosa cells is thought to allow a follicle to switch its gonadotrophic dependence from FSH to LH and attain dominance over a follicular cohort which remains FSH-dependent. Indeed Hampton *et al.* (2004) demonstrated that while FSH can support bovine follicular growth to > 10mm, LH increases androgen production and expression of P450c17. Interestingly, changes in expression of mRNA for LHR in granulosa cells was not associated with changes in LH pulsatility and it was P450c17 mRNA expression, rather than aromatase activity, that was the most sensitive indicator of androgen production by thecal cells and oestrogen production by granulosa cells.

Study of the LHR in sheep and cattle has shown that LHR mRNA is highly polymorphic and a number of splice variants have been described. These isoforms vary due to deletion of all or part of two variable coding regions: one located between exon 3 and exon 7 and the other between exon 9 and exon 11, incorporating the first 266 bases of exon 11 (see Fig. 2A). To avoid confusion we have designated these 5´ and 3´ variable deletion sites (VDS) respectively. Several studies concentrating on the 3´VDS have described four mRNA isoforms, which are expressed in the ovary of large domestic ruminants and several other species and these have been designated 'A, B, F, and G' isoforms in sheep, (Bacich *et al.* 1999), but appear to have corresponding isoforms in a number of other species including cattle, pig, rat and humans (Kawate & Okuda 1998; Loosfelt *et al.* 1989; Reinholz *et al.* 2000). Full LHR functionality, which includes ligand specificity and nuclear signalling capacity, can only be conferred by the translation of the undeleted mRNA splice variant member of the 'A' form family. This form encodes an LH/hCG specific ectodomain (exons 1 to 10), and a membrane-bound nuclear signalling endodomain (exon 11); . Members of the 'B' and 'G' variant family mRNAs are truncated due to an early stop codon being included when part of exon 11 is spliced out resulting in an open reading frame shift. Putative proteins would therefore not incorporate any of the endodomain and would thus be soluble. The 'F' variant family loses exon 10 only and do not undergo frame shift. Therefore putative 'F' form LHR proteins should retain ligand specificity, and incorporate transmembrane and intracellular signalling regions. However to date no role has been suggested for either this soluble form or any of the putative proteins encoded by the various LHR mRNA isoforms and studies in our laboratory have shown no consistent change in the ratio between these isoforms during somatic cell differentiation in sheep either *in vivo* or *in vitro* (Marsters *et al.* 2007). This suggests that alternative splicing of the common precursor LHR primary RNA is regulated so as to produce a constant molar ratio between the mature transcripts. Thus the regulation of LHR expression in ovarian somatic cells requires further work to determine the biological role of the different splice variants.

To date several surveys of LHR mRNA in follicular somatic cells during the antral phase have been published, and provide valuable insights into the complexities of antral follicle development. The results of classic LH-binding (Carson *et al.* 1979; Webb and England 1982; Ireland & Roche 1982; Peng *et al.* 1991) and *in situ* hybridisation studies (Xu *et al.* 1995), demonstrated that granulosa cells from large pre-ovulatory follicles have LH-binding capacity. Furthermore LHR mRNA expression has been detected by RT-PCR in both granulosa and theca cells throughout the antral phase of follicle development in sheep (Abdennebi *et al.* 2002) and cattle (Robert *et al.* 2003) and is markedly upregulated in granulosa cells from large, highly oestrogenic pre-ovulatory follicles (Bao *et al.* 1997). Moreover, recent findings suggest that post- transcriptional regulation of LHR may involve LHR mRNA-binding proteins (LRBPs) that induce rapid degradation (Kash & Menon 1999). These workers have identified an LRBP binding site adjacent to the transcription start site and demonstrated that LRBP/LHR mRNA complexes are more rapidly degraded than unencumbered LHR mRNA thereby limiting time for translation. More work is therefore required to examine the mechanisms regulating the transcription and translation of LHR mRNA in the somatic cells of ovarian follicles in domestic ruminants.

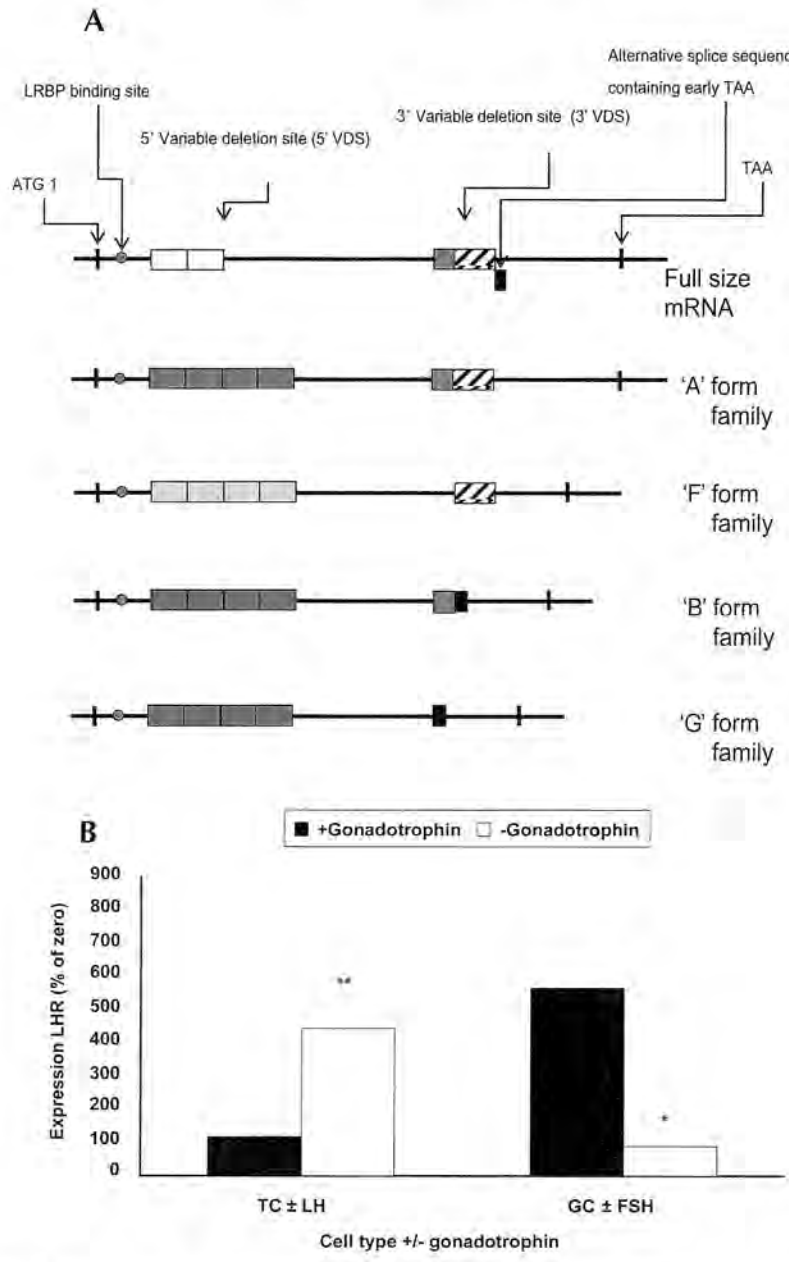

Fig. 2. A. Schematic representation of the LH receptor full size mRNA showing the positions of the 5´ and 3´ variable deletion sites (VDSs) and other important loci. Also shown the alternative splicing of the 3´ VDS which determines the variant families (A, F, B and G).

B. Semi-quantitative comparison of total LH receptor mRNA expression in theca cells (TC) and granulosa cells (GC) taken from small bovine antral follicles (3-5mm) and cultured for 96 hrs in serum-free media ± gonadotrophins (LH and FSH respectively). Expression was determined by RT-PCR. Asterisks denote individual statistical significances (*, $P<0.05$; **, $P<0.01$) between the ± gonadotrophin treatments. After Marsters *et al.* 2007.

Recent studies using somatic cells induced to differentiate *in vitro* have revealed important differences in the way that follicle stimulating hormone receptors (FSHR) and LHR expression are regulated. Granulosa cell FSHR expression appears to be constitutive from the primary stage of follicle development through to the dominant follicle. We have previously reported that FSHR expression in cultured granulosa cells declined rapidly in the period after plating and, in the presence of optimum doses of FSH and IGF-1, increase rapidly prior to induction of aromatase activity and gene expression (Marsters *et al.* 2003). Removal of FSH, but not IGF from culture media, however, results in a marked attenuation in this recovery in FSHR expression and the absence of aromatase indicating that FSH positively regulates expression of its own receptor in granulosa cells (Marsters *et al.* 2007). Further, expression of LHR in granulosa cells follows a similar pattern that parallels aromatase expression, confirming that induction of LHR in granulosa cells is an FSH dependent phenomenon that occurs concurrently with acquisition of the ability to secrete oestradiol. Conversely, LHR expression in cultured theca cells exhibits a similar profile, but is markedly up-regulated in the absence of LH in the media, indicating that thecal LHR expression is negatively regulated by LH in this species (Marsters *et al.* 2007; Fig. 2b).

At a more functional level, infusion studies in cattle have demonstrated that FSH alone, or in combination with LH, can stimulate follicles to develop to the preovulatory stage and these preovulatory follicles are capable of ovulating in response to hCG (Webb *et al.* 2003). Furthermore, adequate pulsatile LH support appears to be required to maintain the ovulatory competence of large follicles (>9 mm in diameter) when FSH concentrations are decreased. These studies also indicated that gonadotrophins are significantly involved in the control of ovulation rate. This is supported by the finding that cattle with three dominant follicles had higher FSH concentrations than cattle with two dominant follicles, with cattle with a single dominant follicle having the lowest mean FSH concentration (Lopez *et al.* 2005). These data in cattle are comparable with those generated using a similar model in sheep and agree with our current understanding of the role of declining FSH and subsequent LH support in selection of the dominant follicle. The results of recent experiments utilising a GnRH-antagonist suppression model confirm that LH is an essential requirement for normal ovulatory follicle development and subsequent luteal function, but show that a pulsatile mode of LH stimulation is not required by the ovulatory follicle for normal dominant follicle development and luteal function (Campbell *et al.* 2007; Fig. 3). These data suggest therefore that as well as an FSH threshold being necessary for the support of gonadotrophin-dependent follicles, a threshold concentration of LH is required for normal steroidogenesis and development of the dominant follicle. In addition to these direct effects of LH on dominant follicle development, LH, by modulating both oestradiol and inhibin A secretion by the ovulatory follicle, can also indirectly control the level of pituitary FSH release and hence the fate of FSH-dependent follicles.

Dominant follicles, even in the first follicular wave of the oestrous cycle, are mature enough to ovulate and corpora lutea (CL) can be generated experimentally by treatment with a number of factors including hCG (Price & Webb 1989), GnRH (Mee *et al.* 1991; 1993) and GnRH analogues (Twagiramungu *et al.* 1995). However the CL appears to be impaired with reduced progesterone production (Webb *et al.* 1992). In addition to impaired CL function, Perry *et al.* (2005) demonstrated that administration of GnRH to induce ovulation likely initiates a preovulatory gonadotrophin surge before some dominant follicles attain physiological maturity. Also GnRH-induced ovulation of follicles that are physiologically immature had a negative impact on pregnancy rates and late embryonic/fetal survival. It was suggested (Perry *et al.* 2005) that these observations in cattle may have implications for human assisted reproductive procedures. However there appears to be an optimum duration of ovulatory follicle development since an

increased duration from the time of emergence (or dominance) to oestrus is associated with reduced pregnancy rates following AI in dairy cows undergoing spontaneous oestrous cycles (Bleach *et al.* 2004).

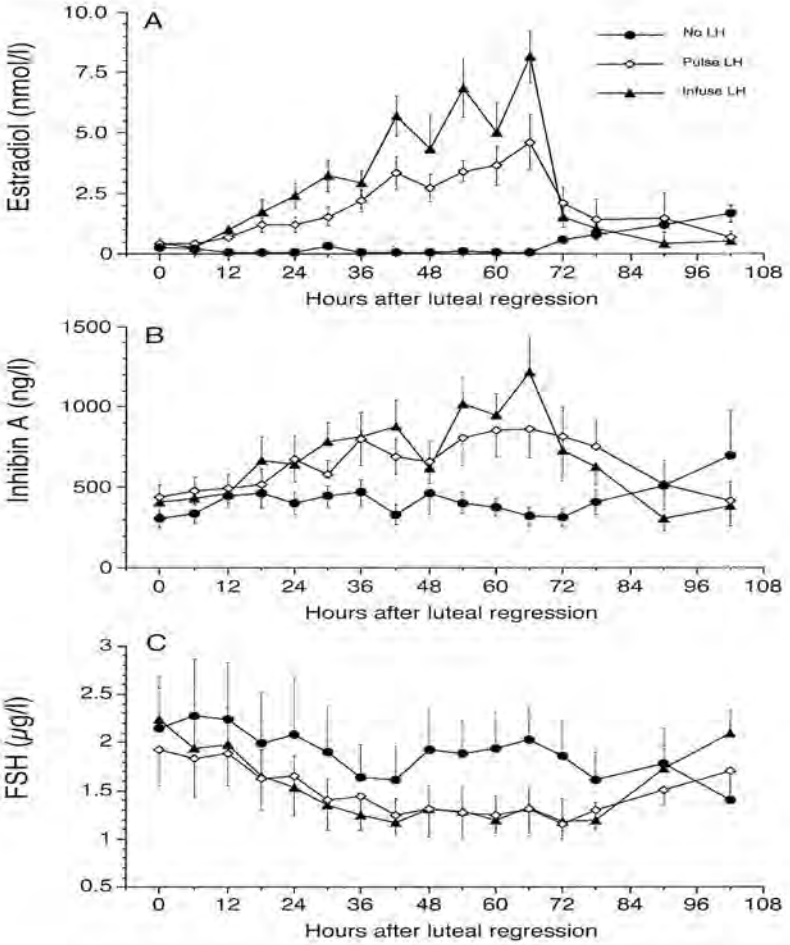

Fig. 3 Ovarian venous oestradiol (A) and inhibin A (B) and jugular venous FSH (C) concentrations in GnRH agonist suppressed ewes which received either no LH (n = 8; closed circles), pulsed LH (n = 8; open diamonds) or constant LH (n = 8; closed triangles) for 60 h after induction of luteal regression followed by an ovulatory stimulus at that time. The results demonstrate a key LH requirement for final maturation. However, these results also demonstrate that both the oestradiol and inhibin A responses occurred whether LH was given as a constant infusion or as pulses. After Campbell *et al.* 2007.

Other extra-ovarian factors are also involved in dominant follicle function. A large number of *in vitro* studies have demonstrated the direct action of metabolic factors on granulosa and theca cells (Webb *et al.* 1999; 2003; 2004; Armstrong *et al.* 2003). Bovine granulosa cells also appear to be dependent on the presence of physiological concentrations of insulin (Gutierrez *et al.* 1997) and furthermore, infusion of insulin into beef heifers increased the diameter of the dominant follicle (Simpson *et al.* 1994). Dietary-induced increases in circulating concentrations of insulin have also been correlated with increased oestradiol production in cultured

granulosa cells from small antral (1-4mm) follicles (Armstrong *et al.* 2002b), demonstrating a direct action of metabolic hormones throughout the later stages of follicle development. This is supported by results of a study in post-partum dairy cows where insulin infusion increased oestradiol secretion after 30h by the dominant follicle of the first postpartum follicular wave. Interestingly these changes appeared not to be mediated through changes in pulsatile LH release (Butler *et al.* 2004), suggesting a direct effect of insulin on the follicle. However circulating free IGF-1 was also raised, which as discussed, could have increased the response of the follicle to peripheral gonadotrophins resulting in increased aromatase activity by the dominant follicle. Hence there appears to be an optimum concentration of insulin for follicle health, since it has recently been demonstrated that hyperinsulinemia can occur in cattle and that this condition is associated with impaired oocyte quality (Adamiak *et al.* 2005; 2006). Interestingly in this study, there was an interaction between body condition and level of feeding, which were cumulative, with both a positive and negative influence on oocyte quality as measured by the ability of oocytes to develop to blastocysts, with high level of feeding having a beneficial effect in animals of low body condition, but a detrimental effect in cows with a moderately high body condition.

Intra-ovarian factors

As the selected follicle reaches dominance there are also changes in the expression patterns of locally produced factors. For example, in healthy bovine follicles up to 9 mm in diameter, IGFBP-2 and -4 mRNA expression are restricted to granulosa and theca tissue, respectively (Webb *et al.* 2003; 2004). Indeed the conversion of a subordinate follicle to a future dominant follicle has been associated with a transient increase in follicular fluid activin A and oestradiol, but a decrease in IGFBP-2 (Armstrong *et al.* 1998; Ginther *et al.* 2002; Kojima *et al.* 2003). The reduction in follicular fluid IGFBP-2 and -4 concentrations has been coupled to the increase in oestradiol concentrations, in dominant follicles in cattle (Mihm *et al.* 2000). Hence, lower amounts of IGFBP-2 and increased LH receptors in granulosa cells appear to be associated with the establishment of the dominant follicle (Webb *et al.* 2003; 2004).

This reduction in IGFBPs has been associated with increased proteolytic activity (Mazerbourg *et al.* 2000). The protease that degrades IGFBP-4 and –5 has been shown to be the pregnancy-associated plasma protein –A (PAPP-A) (Monget *et al.* 2003). PAPP-A mRNA expression is also more abundant in growing dominant bovine follicles than in non-selected small follicles (Fayad *et al.* 2004). Furthermore it has been shown that PAPP-A is responsible for IGF-dependent degradation of IGFBP-2 probably leading to increased IGF bioavailability (Monget *et al.* 2003). Post-translational modification of IGFBPs are also known to occur and it has been demonstrated that at least 51 isoforms of IGFBP are present in bovine follicular fluid (Nicholas *et al.* 2002), but the physiological role of these isoforms has yet to be clarified.

Other locally produced growth factors include members of TGFß superfamily of ligands, operating through Smad signalling pathways (Knight & Glister 2006). Certainly a range of BMPs and associated factors are involved in follicular maturation as indicated by the marked increase in ovulation rate in sheep with a range of mutations (McNatty *et al.* 2007 this supplement). As with other stages of follicle development there is now evidence for a functional role of BMPs in the dominant follicle, acting in concert with other locally produced factors and gonadotrophins. However, the exact mechanisms through which all these growth factors operate and the degree of redundancy need to be elucidated.

In addition to known local factors, recent data derived from genetic array approaches (Mihm *et al.* 2006) have revealed a large number of factors that may be associated with dominance in

mono-ovulatory species. Some of these effects, such as the association of dominance with the development of LH receptors on granulosa cells (Evans *et al.* 2004; Mihm *et al.* 2006) are well established (Webb & England 1982; Xu *et al.* 1995), whereas many others have unknown actions which may or may not be causally related to the attainment of dominance. Furthermore, other factors of unknown identity have been described through the more conventional means of the observation of a biological effect. One of the most intriguing of these activities, given the increased understanding of the importance of oocyte secreted factors in controlling follicular development, is the observation that oocytes from a number of species release a potent activity that is capable of inhibiting gonadotrophin-induced differentiation of granulosa cells *in vitro* without markedly affecting cellular proliferation (pig: Brankin *et al.* 2003; cow: Glister *et al.* 2003; sheep: Sfontouris 2004; Table 1). Comparison of the effect of culturing ruminant granulosa cells with a single oocyte on cell number and oestradiol production, with the known effects of candidate factors for this activity such as GDF-9, BMP-15, BMP-6 and TGFα/EGF (see Table 1), reveal that GDF-9/BMP-15 are possible candidates for this activity. This inhibitory action is consistent with the sheep models in which ovulation rates are increased in heterozygote carriers of null mutations (Hanrahan *et al.* 2004) and following immuno-neutralisation (Juengel *et al.* 2004). The role of GDF-9/BMP-15 in the control of dominance is therefore worthy of future investigation with a primary question being the mechanism whereby this putative inhibitory moiety is controlled during selection.

Table 1: Comparison of the effects of co-culture of oocytes with granulosa cells from sheep and cattle on cellular proliferation and oestradiol production under optimal conditions for induction of cellular differentiation (i.e. in the presence of IGF-1 and FSH) with other known oocyte-secreted factors. After Glister *et al.* 2003; Campbell *et al.* 2005.

Factor	Proliferation	Oestradiol production	Reference
Oocyte co-culture (+FSH/IGF)	None	Negative	Glister *et al.* 2003 Sfontouris 2004
BMP-6 (+FSH/IGF)	None	Positive	Glister *et al.* 2004 Campbell *et al.* 2006
TGFα/EGF (+FSH/IGF)	Positive	Negative	Glister *et al.* 2003 Campbell *et al.* 1996
GDF-9/BMP-15 (+FSH/IGF)	None	Negative	Campbell *et al.* 2005

Follicular atresia

As discussed, atresia occurs throughout follicular development and apoptotic cell death is an underlying mechanism of cell loss during follicular atresia (Tilly *et al.* 1991). Granulosa cell apoptosis may occur early in the process of atresia in rodents, before other morphological or biochemical changes are detected (Tilly *et al.* 1992), although in cattle a decline in intrafollicular oestradiol production has been shown to precede apoptosis of granulosa cells (Austin *et al.* 2001). The theca interna is also susceptible to cell death, with a reduced number of P450SCC- positive cells in atretic follicles (Clarke *et al.* 2004). Indeed theses authors suggested that the theca interna could be a site of initiation of atresia.

Atresia, along with follicular growth differentiation and ovulation are dependant on cyclical remodelling of the extra cellular matrix (ECM). For example plasminogen activators and inhibitors, including protease nexin-1 (PN-1) have been associated with the process of atresia in non-ovulatory dominant bovine follicles (Cao *et al.* 2006). In addition, gelatinolytic and caseinolytic matrix metalloproteinase's (MMPs) which degrade the proteinaceous components of the ECM are temporally and spatially regulated within the thecal and granulosa compart-

ments of bovine follicles (Smith *et al.* 2005). Indeed, gelatinase A (MMP-2) activity was increased in response to physiological concentrations of LH. The controlled degradation of ECM proteins by MMPs and their inhibitors (TIMPs) may be essential for preserving a microenvironment conducive to follicular function.

A number of markers of atresia have been used to assess follicular status including caspases (Fenwick & Hurst 2002), Fas and Fas-L (Quirk *et al.* 2000), TUNEL staining of granulosa cells (Zeuner *et al.* 2003) and IGFBP-5 expression (Devine *et al.* 2000). Whilst several of these markers have been used to identify atretic follicles, very little evidence of their subsequent use as markers of oocyte quality exists (Nicholas *et al.* 2005). Unlike progression of apoptosis in oocytes, that relies on caspase-2, apoptosis in granulosa cells from pre-antral to preovulatory follicles is dependent upon the activity of caspases -3 and -7 (Matikainen *et al.* 2001). In cattle, it has been demonstrated that follicular atresia is accompanied by a considerable increase in the lower molecular weight IGFBPs (Nicholas *et al.* 2005). Importantly, IGFBP-5 appears to be a particularly good marker of atresia, since other IGFBPs are expressed at different stages of follicular development, whereas IGFBP-5 is exclusive to atresia (Monget *et al.* 1998). Indeed, it has recently been demonstrated that the IGFBP expression profile of follicular fluid can be used to better predict oocyte developmental competence (Nicholas *et al.* 2005). A distinctive IGFBP profile in follicular fluid of ovarian follicles has been demonstrated, with the dominant bovine follicle containing only IGFBP-3 together with a high oestradiol content, whereas the two largest subordinate follicles contain increased levels of IGFBP-2, -4 and the 29-31kDa (IGFBP-5) band, concomitant with reduced oestradiol content (Fig. 4). Furthermore, an extremely significant correlation between 29-31kDa, (IGFBP-5) expression in follicular fluid and caspase-3 activity in the granulosa cells, measured in the same follicles, has been shown (Nicholas *et al.* 2005; Fig. 4), demonstrating that IGFBP expression patterns can be used to group follicles into healthy, early atretic and late atretic follicles. Furthermore, a higher proportion of oocytes derived from follicles in early atresia progressed to the blastocyst stage after IVF (Fig. 4).

Gross morphological assessment of cumulus oocyte complexes is not an accurate method of determining whether oocytes have been derived from healthy or atretic follicles (Nicholas *et al.* 2005). Hence, selecting oocytes from follicles of defined quality using non-invasive markers, for example, IGFBP expression patterns, which can be performed rapidly enough to preselect the oocytes prior to IVF would be a major advantage. Selection of good quality oocytes is also highly desirable for procedures that require extensive manipulations such as pronuclear microinjection, intra-cytoplasmic sperm injection (ICSI) and nuclear transplantation, which need to ensure a high developmental competence after embryo transfer. These results may enable an improvement in IVF pregnancy rates from the current success rate of ~30% by pre-selection of oocytes on a more biochemically sound basis than by simple morphological evaluation, which is currently used, and is a rather non-empirical method of assessment.

Conclusions

The final stages of follicle development are driven by an absolute requirement for gonadotrophic support (see Fig. 5). In addition, this requirement is developmentally regulated with dominant follicles changing their reliance from FSH to LH support. The response of these follicles to gonadotrophins is modulated by other extra-ovarian factors, such as insulin and IGF-1, which also influence follicle development, oocyte quality and a panoply of locally produced growth factors. This review has centred on those that have been studied in most detail, namely the IGF and BMP systems. Limited, but rapidly increasing, information to date has already demonstrated an interaction between these two important local regulatory systems (see Fig. 5). Fur-

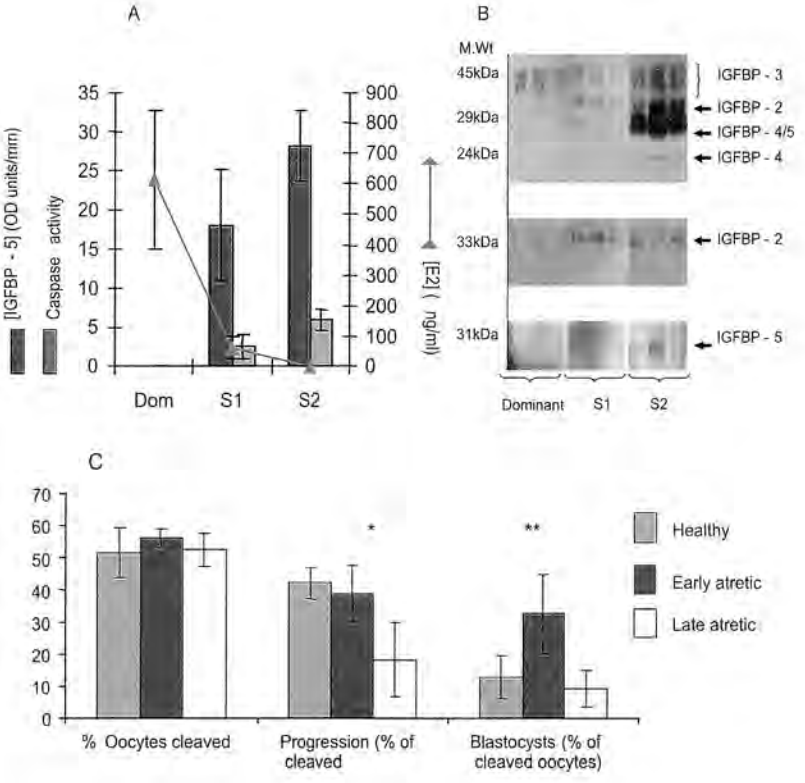

Fig. 4 Bovine granulosa cell caspase-3 activity, oestradiol 17ß follicular fluid IGFBP-5 and concentrations (A), and (B) IGFBP content, visualised by Western ligand blot using biotinylated IGF-II, in follicular fluid from ovarian follicles from synchronised heifers on day 5 after oestrus and (C) the outcome of *in-vitro* fertilization on pre-selected oocytes from non-synchronised animals. Adapted from Nicholas *et al.* 2005.

thermore, gene expression profiling approaches will identify additional novel and differentially expressed genes within follicles. The challenge for the future, following identification of these factors, will be the determination of their physiological role, in particular how they interact with extra-ovarian factors. An increased understanding of these complex systems controlling follicular development will result in the *in vivo* and *in vitro* production of better quality oocytes resulting in enhanced embryo survival and pregnancy rates.

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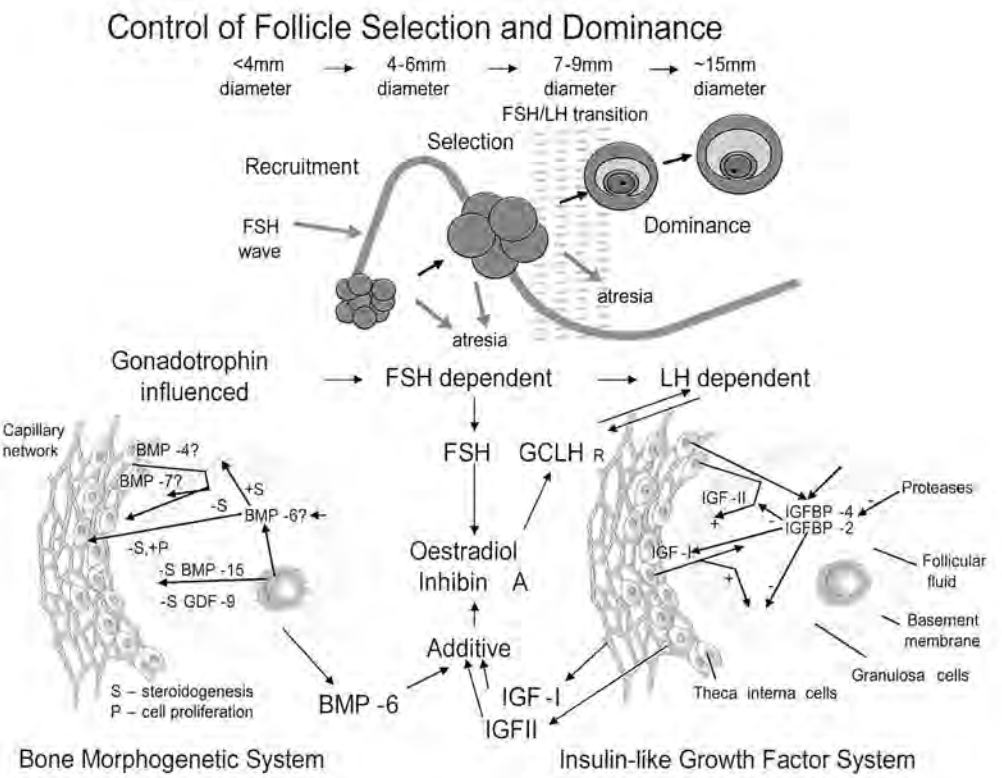

Fig. 5 Diagram showing the role of gonadotrophins in antral follicle development and the interaction with the follicular IGF and BMP systems. Note the additive effect of the IGF and BMP systems on FSH stimulated follicular development. Adapted from Webb *et al.* 2003; Campbell *et al.* 2006.

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