Review

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Regulation of folliculogenesis and the determination of ovulation rate in ruminants

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Abstract. The paper presents an update of our 1993 model of ovarian follicular development in ruminants, based on knowledge gained from the past 15 years of research. The model addresses the sequence of events from follicular formation in fetal life, through the successive waves of follicular growth and atresia, culminating with the emergence of ovulatory follicles during reproductive cycles. The original concept of five developmental classes of follicles, defined primarily by their responses to gonadotrophins, is retained: primordial, committed, gonadotrophin-responsive, gonadotrophin-dependent and ovulatory follicles. The updated model has more extensive integration of the morphological, molecular and cellular events during folliculogenesis with systemic events in the whole animal. It also incorporates knowledge on factors that influence oocyte quality and the critical roles of the oocyte in regulating follicular development and ovulation rate. The original hypothetical mechanisms determining ovulation rate are retained but with some refinements; the enhanced viability of gonadotrophin-dependent follicles and increases in the number of gonadotrophin-responsive follicles by increases in the throughput of follicles to this stage of growth. Finally, we reexamine how these two mechanisms, which are thought not to be mutually exclusive, appear to account for most of the known genetic and environmental effects on ovulation rate.

Additional keywords: ewe, follicle, nutrition, oocyte, ovary.

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Dedication

We dedicate this review to the memory of a colleague and friend, Dr Hannah Peters (b 20/03/1911: d 04/03/2009), who made many significant and insightful contributions to our understanding of ovarian function. Our review addresses several basic events of ovarian function in ruminants including follicular formation and growth and the roles of intra-ovarian factors and gonadotrophins in these processes. These were topics investigated by Hannah Peters in the 1960s and 1970s. In 1975, at an ovarian workshop in Glasgow, she presented a memorable and insightful summary of her studies of ovarian biology in rodents and humans (Peters *et al.* 1975). The views expressed in this summary have stood the test of time and represent a remarkably accurate understanding of our current concepts of follicular physiology. In brief, Peters and her colleagues concluded that: (1) the initiation of follicular growth is continuous during infancy and adult life, irrespective of reproductive status and whether mammals ovulate or not; (2) follicles grow sequentially and continue to grow without rest until they die or ovulate; (3) the number of follicles that start to grow is dependent upon the size of the pool of small follicles (e.g. the larger the pool, the more follicles leave the pool per unit time) and inferred that intraovarian mechanisms via factors from growing follicles were likely to be involved; (4) the initiation of follicular growth is independent of gonadotrophins and involves intraovarian factors; (5) the continued growth of medium and large follicles is dependent upon gonadotrophins and the larger that follicles become, the more important gonadotrophins become; and (6) exogenous gonadotrophins reduce the incidence of atresia in large follicles. In addition, Hannah Peters provided the first classification system for the sequential stages of follicular growth and these have been utilised for our classifications in ruminants. Her published works include studies on duration of amenorrhea and age of menarche in Indian women, ovarian development during fetal life, radiation sensitivity and DNA synthesis in oocytes, the kinetics of follicular growth and the effects of breast cancer, Down's syndrome and leukaemia on ovarian function. In her retirement, she continued to mentor her colleagues and remained interested in whether we had learnt anything new about how follicles start to grow or how the environment (e.g. life-style, nutrition) affects reproductive function. She often expressed concern about the ethics of modern reproductive technologies and was puzzled as to why many of us have avoided researching the mechanisms as to how the ovary constantly remodels itself while disposing of degenerating follicles and luteal tissue. She remained fascinated by the dynamics of ovarian function and perhaps this can be summarised by a favourite quotation of hers from Plato 'nothing ever is, but is always becoming'.

Background

Most mammals precisely regulate their ovulation rate. In ruminants, this number seldom exceeds three and is an important determinant of litter size. In studies of the mechanisms responsible for follicular development and differentiation, sheep have proven to be an excellent experimental model because their ovulation rate is affected by genetic differences and by environmental factors such as photoperiod, diet and stress. Cattle have also been useful because the processes leading to ovulation in the cow are more tightly controlled, so multiple ovulations are rarer than in the ewe.

Progress in this field has been aided by a steady escalation of our knowledge of reproductive physiology leading to considerable depth in our understanding of the processes that control folliculogenesis. To summarise our knowledge of these processes and to stimulate international collaborative research, a workshop was held in 1991 when eleven researchers from laboratories around the world met at Terrigal, Australia. Particular attention was directed towards the mechanisms in sheep that produce different ovulation rates among breeds and among environmental or experimental conditions. The outcome was a review paper representing a consensus of the group (Scaramuzzi *et al.* 1993). It outlined models of follicular development and selection and of the determination of ovulation rate based on knowledge of the physiological, rather the than anatomical, changes during these processes. These models were therefore functional rather than descriptive and since then, they have been used to develop and test hypotheses on the regulation of folliculogenesis and ovulation rate.

The present paper is the result of a follow-up workshop held in July 2008 at Tours, France, that aimed for an updated consensus of the state of our knowledge. In the intervening 17 years, five of the original participants had left the field, but the remaining six agreed to hold an update-workshop. The scope was extended by inviting additional researchers to cover new developments, for example, the role of the oocyte in folliculogenesis. Some of the scientific changes have been profound, either because of major contributions from techniques in molecular biology or from dedicated effort in previously neglected areas. In particular, we have new perspectives on the development of ovarian follicles in the fetus, the role of the oocyte in the process of follicular development leading to ovulation and on the ways in which metabolic factors can directly affect follicular function.

The scope of the workshop, and thus this review, was limited to the events and processes in ovarian folliculogenesis from the formation of the follicle in the fetus to the final selection of the ovulatory follicle(s). We have omitted the processes of ovulation and luteinisation, not because we believe they are unimportant, but because of the necessity to limit the scope of the workshop. Again, there has been a strong focus on mechanisms at various levels of analysis from molecular and cellular to the whole organism. This paper reviews major conceptual advances since 1991 and re-addresses the functional model of folliculogenesis incorporating new information and exploring how this affects our interpretation of follicular responses to nutrition. The model for the control of ovulation rate is updated and, finally, primary foci for future research are considered.

Update on the mechanisms that control folliculogenesis

Primordial and committed follicles

Our original view (Scaramuzzi *et al.* 1993) was that primordial follicles were quiescent but we now know this to be incorrect because of significant new information from studies in sheep and, to a lesser extent, in cattle, concerning the ontogeny of

follicular formation, including the origins of the granulosa cells in primordial follicles (Sawyer *et al.* 2002). Moreover, as we shall see below, many of the genes expressed in follicles during their formation and the early stages of growth have now been identified. The process leading to the formation of follicles takes place during fetal life. In sheep, it begins shortly after sexual differentiation, ~35 days after conception. Initially, in the cortical region of the ovary, mitotically active oogonia containing c-kit interact with adjacent mesonephric cells that contain the kit ligand to form the ovigerous cords that subsequently isolate the oogonia from the interstitium, but not the cells of the surface epithelium, which also contain kit ligand. Once the ovigerous cords are formed, mitotically active oogonia can only recruit somatic pre-granulosa cells from the surface epithelium. In both sheep and cattle, the oogonia form tight junctions with the mesonephric or surface epithelial cells that subsequently become pre-granulosa cells (Sawyer *et al.* 2002). The oogonia then attach to pre-granulosa cells and enter meiosis, during which at least 80% of the germ cells, but not their pre-granulosa cells, undergo apoptosis. In sheep, the evidence suggests that the pre-granulosa cells then associate with nearby surviving oocytes and thus each oocyte recruits different numbers of pre-granulosa cells from different sources. After Day 75 of fetal life in sheep, and Day 90 in cattle, the first follicles separate themselves by producing a basement membrane at the base of the ovigerous cords (Sawyer *et al.* 2002; Garverick *et al.* 2010). In sheep, it can be calculated that 90% or more of the granulosa cells are recruited from the surface epithelium (Sawyer *et al.* 2002) and that primordial follicles contain a mean of 16 (range 3–52) granulosa cells (Lundy *et al.* 1999). In sheep and cattle, follicular growth is initiated before the last primordial follicles are formed and then continues throughout fetal, neonatal and adult life.

The delay between the appearance of the first primordial and the first primary follicles is relatively long at 25 days in sheep and 50 days in cattle (McNatty *et al.* 2007). In cattle, this delay is associated with progression through to meiotic prophase I and arrest at diplotene. *In vitro* studies suggest that the capacity of the first-formed bovine follicles to initiate growth is inhibited reversibly by oestradiol and is associated with inhibition of the progression to meiotic prophase (Yang and Fortune 2008). It remains to be established if this is also the case in sheep.

Ovine primordial follicles have been used to generate ~2500 expressed sequence tags and these have been matched to 500 mRNAs linked to cytoskeletal events, DNA repair, mRNA processing, ribosomal function, protein synthesis and ubiquitination, and signalling pathways (McNatty *et al.* 2007). Examples of genes expressed in oocytes, granulosa cells and theca cells of primordial, primary and preantral follicles are listed in Table 1. Collectively, this evidence indicates that the primordial follicle is expressing hundreds of genes that fulfil housekeeping and signalling functions. Moreover, given that the oocyte has receptors for oestradiol and several growth factors, it is either receptive or developing receptivity, to its environment.

In ruminants, all current evidence is consistent with follicular formation being completed during fetal life (Garverick *et al.* 2010) and with a decline in the number of remaining follicles throughout life. In sheep, counts of primordial follicles suggest that 25–30 begin growing each day (McNatty *et al.* 2007). Many

Table 1. The temporal patterns of expression and location of selected genes expressed during the primordial, primary and preantral stages of folliculogenesis

All genes were localised by *in situ* hybridisation (Juengel *et al.* 2007; McNatty *et al.* 2007). For definitions of follicular Types 1–4 see Lundy *et al.* (1999). O, oocyte; G, granulosa cells; T, theca cells

Primordial (Type 1)	Primary (Type 2)	Preantral (Type 3)	Preantral (Type 4)
ALK3^{O,G}	ActRIIB^{G}	ActRIIB^{T}	AR^{G,T}
ALK5^{O}	ALK6^{G}	ALK3^{T}	3βHSD^{T}
ALK6^{O}	AMH^{G}	ALK5^{G,T}	ERα^{G}
Betaglycan^{O}	AMHRII^{G}	ALK6$^{T?}$	ERβ^{T}
3βHSD^{G}	βB activin^{G}	Betaglycan^{G,T}	IGF2^{T}
BMP6^{O}	BMP15^{O}	BMPRII^{T}	α Inhibin^{G}
BMPRII^{O,G}	Connexin 43^{G}	Follistatin^{G}	LHR^{T}
c-kit^{O}	FIGα^{O}	FSRP^{G,T}	PR^{T}
Connexin 37^{O}	FSH-R^{G}	IGFR1^{T}	SF1^{T}
ERβ^{O,G}	IGFRI^{G}	TGF-β1, 2^{T}	StAR^{T}
GDF9^{O}		TGF-βR11^{T}	17αOH lyase^{T}
Kit ligand^{G}			
StAR^{G}			
WT1^{G}			

of the proteins in primordial follicles are associated with cell maintenance and preparation for growth (Table 1). In primordial follicles of rodents, the kit-ligand–c-kit system and components of the phosphoinositol (PI_3) signalling system are present as well as phosphatase and tensin homologue (PTEN), which acts as a negative regulator of PI_3 signalling (Reddy *et al.* 2008). The kit-ligand–c-kit system is present in sheep primordial follicles (Tisdall *et al.* 1999); however, unlike in rodents it seems that PTEN is not present (Froment *et al.* 2005). Nevertheless, given that primordial and committed follicles have numerous receptors and ligands for growth factors of the transforming growth factor (TGF)-β super family and oestradiol (Table 1), it is likely that several growth-promoting factors are involved, both negatively and positively, in the initiation of follicular growth. *In vitro* studies with cortical fractions of bovine and human ovaries show that the majority of primordial follicles are activated within a few days of isolation from the ovary, suggesting that inhibitory factors may be important for controlled exit from the pool of primordial follicles and there is evidence that anti-Müllerian hormone (AMH) inhibits follicle activation and early follicular growth in bovine ovaries (Fortune 2003). Thus, although we have learnt much about the characteristics of primordial follicles, the fact remains that, as reported by Scaramuzzi *et al.* (1993), the processes that control the departure of primordial follicles from their pool remain to be determined.

Once follicles have left the pool of primordial follicles they are 'committed' to gonadotrophin-independent growth. As a follicle grows to the primary stage, the granulosa cells increase in number and change shape, becoming uniformly cuboidal. In the ewe, the number of granulosa cells increases to a mean of 128 (range 30–520; Fig. 1). The oocyte also enlarges, with 3- to 10-fold increases in the volume of smooth endoplasmic reticulum, mitochondria, ribosomes and lipid droplets, and the zona

pellucida, absent in primordial follicles, is deposited (Lundy *et al.* 1999). Analysis of expressed sequence tags from primary follicles shows that several hundred genes not found in primordial follicles are activated during this stage of growth, including some involved in mitochondrial function, cell signalling and communication, and the synthesis of the zona pellucida (Table 1).

Formation of large preantral follicles

As ovine and bovine follicles develop to form large preantral follicles (Lundy *et al.* 1999), they gain multiple layers of granulosa cells, a zona pellucida and a theca interna that contains luteinising hormone receptor (LH-R) (Table 1). The acquisition of the enzymes required for thecal androgen production is essentially complete before antrum formation (McNatty *et al.* 2007). The collective evidence suggests that the rate of follicular atresia (i.e. apoptosis) is very low during the preantral stages of growth. In sheep, a large preantral follicle contains 1000–3500 granulosa cells and the oocyte is 70–120 μm in diameter (Fig. 1). Thus, the granulosa cells undergo about seven to eight doublings in population before the formation of an antrum and the emergence of the distinct phenotypes of the

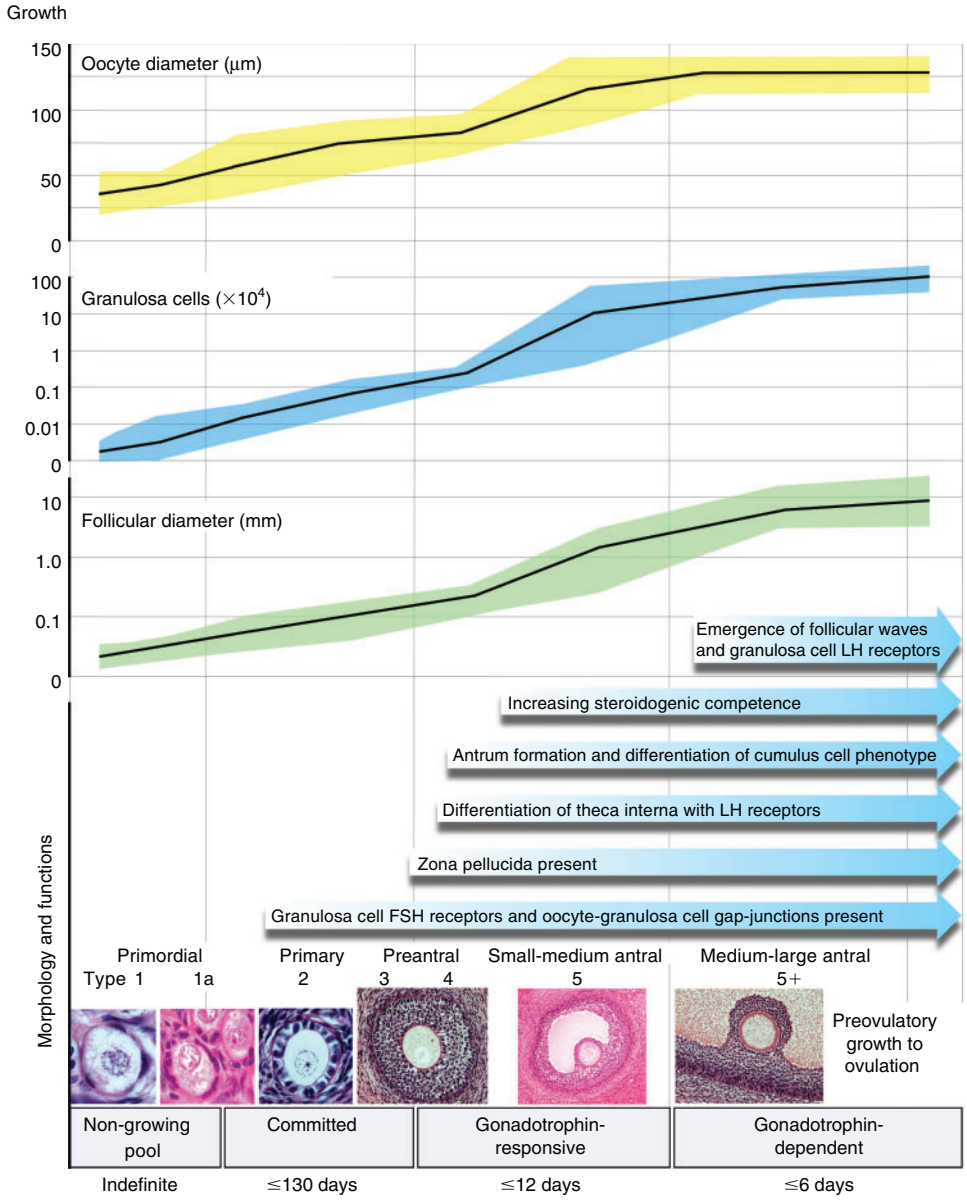

Fig. 1. A summary of folliculogenesis in the ewe, developed with data from several sources (Lundy *et al.* 1999; K. P. McNatty, unpubl. data). The upper panel shows the mean (central line) and range (shaded band) time lines of growth of the follicle and oocyte and the number of granulosa cells, from primordial to ovulatory stages. The lower panel shows the progressive emergence of several critical functional and morphological characteristics of follicles as they develop. The stages of development have been defined by two different systems – one based on morphology and the other on functional characteristics of follicles.

cumulus and mural cells (McNatty *et al.* 2007). Very similar changes in the number of granulosa cells and in the diameter of the oocyte take place in cattle. In both species, the transition to an antral follicle is accompanied by a significant increase in the rate of atresia, suggesting that it is physiologically challenging to remodel the avascular granulosa to form an antrum and to maintain the granulosa–oocyte syncytium.

Follicle–oocyte interactions

It has long been known that oogenesis and folliculogenesis are inextricably linked with the oocyte growing and developing in an intimate and mutually dependent relationship with the somatic cells of the follicle (Thibault 1977). During the course of folliculogenesis, oocytes first acquire meiotic competence and then gradually acquire developmental competence – a biochemical and molecular state that allows a mature oocyte to undergo normal fertilisation, support normal preimplantation embryo development and subsequent healthy growth of the implanted embryo to term (Hyttel *et al.* 1997). Support for the acquisition of meiotic competence is the most important function of the follicle, but we are still striving to understanding the molecular processes involved (Sirard *et al.* 2003).

The traditional perspective on the relationship between oocyte and follicle has been that the oocyte is passive and that its growth and development are dictated by the endocrine system and follicular somatic cells. However, we now know that the oocyte is not simply a passenger in the process of folliculogenesis. In sheep, for example, it produces at least two growth factors that regulate follicular development from its earliest stages (McNatty *et al.* 2006*a*). Their role in determining ovulation rate was unexpected because an equivalent effect was not evident from studies in mice, perhaps because rodents already have a high ovulation rate, in contrast to the typically low ovulation rate of sheep. In this section, we review both aspects of the active bi-directional relationship between oocyte and follicle, and develop perspectives on how these interactions can affect the outcomes of folliculogenesis, including ovulation rate.

The follicle as a regulator of oocyte development

In primordial and committed follicles (i.e. gonadotrophin-independent), the oocyte is meiotically and developmentally incompetent. During the gonadotrophin-responsive phase, oocytes actively synthesise RNA as evidenced by dispersed chromatin configurations and transcriptionally active nucleoli (de Smedt *et al.* 1994). The initiation of these processes in the oocyte probably involves local paracrine mechanisms. Towards the end of the gonadotrophin-independent period, and after formation of the follicular antrum, oocytes from livestock species complete their growth phase and acquire the capacity to resume, but not complete, meiosis. During the early stages of antral follicle growth, the oocyte acquires the capacity to complete meiosis. Oocytes from mid-sized antral follicles possess low developmental competence but as follicles progress towards pre-ovulatory size, the oocyte acquires the full complement of cytoplasmic machinery necessary to support complete embryo and fetal development (Sirard *et al.* 2006). This latter phase of oocyte development has been termed 'oocyte capacitation'.

At about the time of antrum formation, granulosa cells differentiate into two anatomically and functionally distinct lineages; the mural granulosa cells that line the wall of the follicle and that have principally, a steroidogenic role and the cumulus cells, that form an intimate life-support association with the oocyte. As we shall see below, this process of differentiation of granulosa cells is driven by the oocyte and the outcome is a follicular microenvironment that is critical to the final acquisition of developmental competence by the oocyte. However, the oocyte–cumulus cell communication system is both complex and dynamic and we have a relatively poor understanding of the nature and the diversity of molecules that transfer from the cumulus cells to the oocyte during follicular growth or of how they contribute to developmental competence (Gilchrist and Thompson 2007). Unravelling the intricate relationships between the follicle and its oocyte will likely generate new insights into fundamental mechanisms regulating folliculogenesis and oocyte development.

The oocyte as a regulator of follicular development

One of the most exciting new concepts to emerge since our 1993 review is the concept that the oocyte has an essential, very active role in folliculogenesis. The concept was confirmed when Matzuk and colleagues identified candidate factors using a knockout mouse model (Dong *et al.* 1996). It is now clear that the oocyte secretes growth factors that play a central role in the regulation of folliculogenesis, granulosa and cumulus cell differentiation, ovulation rate and litter size (Gilchrist *et al.* 2004; McNatty *et al.* 2004). The functional consequences of the processes driven by oocyte-secreted factors have been revealed through two lines of experimentation: (1) bioassays of granulosa or cumulus cells treated with oocyte-secreted factors and recombination-derived candidate molecules generated from transfected cell-lines; and (2) animals deficient in candidate molecules through natural genetic mutation or immunisation. The outcome is a powerful new perspective on the differentiation and functional control of granulosa cells in committed follicles during their transition to the gonadotrophin-responsive and gonadotrophin-dependent stages.

Oocyte regulation of granulosa cell differentiation

Oocyte-secreted factors regulate several important functions of granulosa and cumulus cells; including regulation of cellular growth, enhancement of cell survival, modulation of steroidogenesis, regulation of the expansion of cumulus cells and the metabolism of cumulus cells (Eppig 2001; Gilchrist *et al.* 2004). Interestingly, the oocyte has the greatest capacity to regulate many of these functions during the gonadotrophin-dependent phase of folliculogenesis, after the oocyte has completed its growth phase (Gilchrist *et al.* 2008). During pre-ovulatory maturation, FSH and LH drive the differentiation of mural granulosa cells towards a luteinised phenotype. However, in cumulus cells, this process is actively inhibited by the oocyte (Eppig *et al.* 1997), which secretes paracrine factors that establish concentration gradients that effectively become

morphogenic gradients (Hussein *et al.* 2005). Hence, through the actions of oocyte-secreted factors, the oocyte actively directs the lineage of its neighbouring granulosa cells towards the cumulus cell phenotype (Eppig *et al.* 1997; Li *et al.* 2000).

We suggest that the oocyte, with its unique nutritional and hormonal requirements for its development, must regulate its microenvironment to keep it distinct from the rest of the follicle (Eppig *et al.* 1997; Hussein *et al.* 2005). How does the oocyte do this? We believe that the oocyte maintains a population of cumulus cells that it then directs to regulate the microenvironment of the oocyte, a function for which it has a poor capacity (Sutton *et al.* 2003*a*, 2003*b*; Sugiura *et al.* 2005, 2007). The ability of the oocyte to control cumulus cell development and to maintain a regulatory loop with them is likely to be a critical developmental function for oocytes in general (Gilchrist *et al.* 2008). This is supported by the observation that adding exogenous oocyte-secreted factors to oocytes maturing *in vitro* improves the pre- and post-implantation development of their subsequent embryos (Hussein *et al.* 2006; Yeo *et al.* 2008).

Details of the molecular nature of the paracrine communication among oocytes, granulosa cells and cumulus cells are still emerging as specific oocyte-secreted factors are identified and characterised and their signalling pathways in granulosa cells and cumulus cells are elucidated. At this time it is already clear that two growth factors expressed primarily in oocytes have important functions: growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) otherwise known as GDF9b (Dong *et al.* 1996; Galloway *et al.* 2000). They are both closely related members of the TGF-β superfamily and they are produced as pre-pro-proteins consisting of a signal peptide, a large pro-region and a mature region (Shimasaki *et al.* 2004). Most unusually for this superfamily, both factors lack the otherwise conserved fourth cysteine residue that is required for the formation of disulfide bridges between the subunits, so their dimers are non-covalent. They signal to granulosa and cumulus cells through the TGF-β superfamily receptors (Juengel and McNatty 2005). Granulosa and cumulus cells express a large complement of cell-specific receptors, co-receptors and SMADs of the TGF-β superfamily. Bone morphogenetic protein receptor II (BMP-RII) is a critical receptor for GDF9 and BMP15. When GDF9 binds to it, activin-like kinase 5 (ALK5) is activated and phosphorylates SMAD2/3. Thereafter, SMAD2/3 associates with the common SMAD4 and this complex translocates to the nucleus where it interacts with specific DNA motifs and transcriptional regulators leading to the transcription of target genes. When BMP15 binds to BMP-RII, it causes recruitment and activation of ALK6, leading to signalling through the alternative BMP pathway mediated by SMADs 1, 5 and 8 (Juengel and McNatty 2005).

Oocyte-derived factors that affect folliculogenesis

Sheep are proving to be an excellent model for investigating the roles of the TGF-β superfamily in the control of ovulation rate and infertility in mono-ovular species. In sheep, over 12 different, naturally-occurring, point mutations in the BMP15, GDF9 and ALK6 genes that affect ovulation rate have been discovered. In humans, several point mutations have been identified in the pro- or mature regions of the BMP15 gene of infertile women. In 2001, three laboratories independently identified a point mutation ($FecB^B$) in the ALK6 gene in ewes carrying the Booroola mutation (Mulsant *et al.* 2001; Souza *et al.* 2001; Wilson *et al.* 2001). This point mutation has now been identified in many breeds including the Merino, Javanese Thin-Tail, Garole, Hu and Han breeds. Animals heterozygous or homozygous for this mutation have ovulation rates that are 1–4 and 4–9 times higher, respectively, than wild-type type ewes of the same breed. Since 1991, studies of inherited patterns of ovulation rate in sheep have revealed at least six other point mutations in either the pro- or mature regions of the BMP15 gene and at least two point mutations in the mature region of the GDF9 gene. Animals homozygous for mutations to BMP15 have streak ovaries and do not ovulate whereas those that are heterozygous, surprisingly, have higher ovulation rates than wild-type ewes (McNatty *et al.* 2006*a*; Bodin *et al.* 2007; Martinez-Royo *et al.* 2008; Monteagudo *et al.* 2009). For one of the GDF9 point mutations, ($FecG^H$), the homozygous and heterozygous phenotypes are similar to those reported for the BMP15 mutations (Hanrahan *et al.* 2004). Of particular interest is the finding that animals heterozygous for $FecG^H$ and $FecX^B$ (another mutation of BMP15) have additive effects on ovulation rate, suggesting that GDF9 and BMP15 cooperate with respect to regulating ovulation rate (Hanrahan *et al.* 2004); Research is continuing on several other breeds of sheep that have genetic mutations affecting ovulation rate (e.g. Lacaune, FecL, Thoka, and Woodlands $FecX^{2W}$). The FecL, Thoka and Woodlands $FecX^{2W}$ mutations have been mapped to sheep Chromosomes 11, 5 and X respectively and work is in progress to identify these genes. The ovarian phenotype in Thoka ewes is similar to that of GDF9 mutants so it has been suggested that the Thoka mutation is in the GDF9 gene (Nicol *et al.* 2009). Most, if not all, of these phenotypes can be reproduced by immunisation of ewes with BMP15 or GDF9 peptide constructs. Animals with high antibody titres leading to total immuno-neutralisation of BMP15 or GDF9 activity have inhibited follicular growth from the primary and primordial stages. In contrast, partial immuno-neutralisation against either GDF9 or BMP15, or both, results in higher than normal ovulation rates and increased numbers of lambs (McNatty *et al.* 2006*a*). Immunisation of cattle against GDF9 or BMP15, or both, has also been shown to affect ovulation rate (Hudson *et al.* 2007).

In the ovary of the ewe and cow, GDF9 is produced exclusively by the oocyte (Bodensteiner *et al.* 1999). Moreover, GDF9 and its mRNA have been identified in oocytes at the time of follicular formation and throughout all stages of follicular development (McNatty *et al.* 2006*a*). In contrast, BMP15 and its mRNA were first identified in sheep, at the primary stage of follicular growth and at all stages thereafter. Both BMP15 and GDF9 are thought to be important during pre-ovulatory maturation because passive immunisation of ewes during the follicular phase of the oestrous cycle inhibited either ovulation or the normal function of the corpus luteum (Juengel *et al.* 2002). Evidence from *in vitro* studies suggests that BMP15 and GDF9 cooperate as a complex to influence proliferation and steroidogenesis in granulosa cells (McNatty *et al.* 2005). Given that both BMP15 and GDF9 are present in follicular fluid and that immunisation interferes with their actions, the current view is

that these oocyte-secreted factors act in a concentration-dependent paracrine manner on adjacent cumulus and granulosa cells. Moreover, reduced concentrations of BMP15 and GDF9 alter only slightly the responsiveness of granulosa cells to either FSH or LH while the absence of BMP15 or GDF9 prevents normal follicular growth beyond the primordial or primary stages.

Within the ovary, ALK6 and its mRNA have been localised to oocytes from the primordial stage and in granulosa cells from the primary stage of growth and thereafter in both cell types throughout follicular development. It is evident from morphological studies that follicles in homozygous ALK6 ($FecB^B$) mutants mature and ovulate at significantly smaller diameters and with fewer granulosa cells than in the wild-type. Despite the homozygous mutant ovulating 5–9 follicles compared with 1–2 in the wild-type, the overall secretion rates of steroids and inhibin are similar among these genotypes. Another important and relatively subtle difference is that oocytes in homozygous $FecB^B$ ewes reach their mature diameter of 130 μm during the preantral stage of development whereas the oocytes in the wild-type do not reach this diameter until after antrum formation. Thus, the effects of the ALK6 mutation can be detected both morphologically and functionally during preantral development in committed follicles.

In ewes carrying the $FecB^B$ mutation, the oocyte reaches full size earlier with fewer granulosa cells which, in turn, undergo fewer doublings to reach their ovulatory size compared with wild-type ewes. *In vitro* evidence suggests that granulosa cells from $FecB^B$ mutants develop earlier responsiveness to FSH (with respect to cyclic AMP synthesis) and to LH than granulosa cells from wild-type ewes. This earlier responsiveness to gonadotrophins is further illustrated by superovulation treatments where the ovulation rates in ewes carrying the $FecB^B$ mutation are significantly higher than in wild-type ewes (McNatty *et al.* 2006*b*). This is in contrast to ewes carrying the BMP15 or GDF9 mutations. It is not known how the $FecB^B$ mutation has such profound effects on folliculogenesis and ovulation rate. However, *in vitro* studies have shown that the mutation in the ALK6 receptor causes a loss of function with respect to the action of BMP ligands (Fabre *et al.* 2003) and perhaps this explains the higher responsiveness to FSH observed in related studies (Fabre *et al.* 2006). Potential molecular mechanisms and signalling pathways including the Type I and Type II receptors and their second-messenger pathways are reviewed elsewhere (Kaivo-oja *et al.* 2006).

Regulation of folliculogenesis by the insulin-like growth factor (IGF) system

The concept of local modulation of gonadotrophin-stimulated folliculogenesis was new in 1991, although it was becoming clear to us and others that 'FSH action is modulated at the ovary by the actions of metabolic hormones, growth factors, other peptides and steroids' (Scaramuzzi and Campbell 1990). This concept is now widely accepted, although precisely how endocrine and metabolic signals to the follicle interact with paracrine and autocrine modulators is not fully established. Recent years have seen very substantial increases in our knowledge of the intra-ovarian regulation of both gonadotrophin-responsive and gonadotrophin-dependent follicles. In addition to those already discussed above, the follicle contains several other paracrine and autocrine systems and, of these, the IGF system appears to be particularly significant for folliculogenesis. It has been defined in detail and its role in folliculogenesis has been firmly established in several species, including some ruminant species (Silva *et al.* 2009).

The role of IGF in folliculogenesis

Studies using several *in vivo* animal models (e.g. overexpression of IGF-I in transgenic mice, targeted deletion of IGF-I and IGF-deficient animals) have shown that IGF-I is not required for ovarian organogenesis, the recruitment of primordial follicles or growth of gonadotrophin-independent follicles. Rather, they show that IGF-I increases the sensitivity of small follicles (diameter 200 μm in the mouse, 2 mm in the sheep, 5 mm in cattle and humans) to gonadotrophin stimulation and simulates their transition from the gonadotrophin-responsive to the gonadotrophin-dependent stages (Mazerbourg *et al.* 2003).

In ovarian tissue *in vitro*, IGF-I stimulated steroidogenesis by thecal cells and both proliferation and differentiation of granulosa cells (Mazerbourg *et al.* 2003). In sheep, IGF-I stimulated proliferation of granulosa cells in small follicles (1–3 mm diameter), and the secretion of progesterone by granulosa cells of large (>5 mm diameter) but not small follicles (Monniaux and Pisselet 1992). The secretion of oestradiol by granulosa cells in culture was also stimulated by IGF-I (Campbell *et al.* 1996) and in the ewe, *in vivo* data have shown that follicular oestradiol and inhibin are stimulated by IGF-I during the follicular phase of the oestrous cycle (Campbell 1988). Therefore, it seems that IGF-I can stimulate either the proliferation or the differentiation and differentiated functions of granulosa cells, depending on the stage of development of the follicle.

The IGF binding proteins (IGFBPs) as modulators of IGF activity in the follicle

It is clear IGF-I can exert profound effects on the later stages of folliculogenesis and steroidogenesis, and both high and low levels of IGF-I activity are probably deleterious to the follicle and its oocyte. Consequently, IGF activity within the follicle needs to be regulated within narrow limits. The problem is that, in follicular fluid, there is no correlation between the concentration of total IGF-I and follicle size (Monget and Monniaux 1995) because most IGF-I in follicular fluid is derived from blood (Mazerbourg *et al.* 2003). Critically, follicular fluid concentrations of total IGF-I are always within the range that will stimulate proliferation and steroidogenesis of granulosa cells in culture.

The supply of IGF-I to the follicle is outside the control of the reproductive axis, therefore intra-follicular IGF activity is regulated locally by intra-ovarian factors, primarily the IGF binding proteins (IGFBPs). The low-molecular weight IGFBPs (BP-2, -4 and -5) are inhibitory to IGF actions because they bind IGF, preventing it from binding to its receptor. Thus, it is the bioavailability of IGF-I, rather than its total concentration, that changes during folliculogenesis. In the ewe, sow, cow and mare the intrafollicular concentrations of IGFBP-2 and IGFBP-4 decrease as follicles grow from 1–2 mm to pre-ovulatory size

(Mazerbourg *et al.* 2003), and in ruminants the intrafollicular concentrations of these IGFBPs and of IGFBP-5 increase in follicles as they become atretic. For IGFBP-3, intrafollicular concentrations do not change during folliculogenesis, except in the ewe, where they decrease in small follicles that become atretic. The removal of the smaller (<40 kDa) IGFBP-2, -4 and -5 from pre-ovulatory follicles and their accumulation in atretic follicles suggests that there are major changes in the local bioavailability of IGF-I (Monget *et al.* 1993).

The role of follicular pregnancy-associated plasma protein-A (PAPP-A) in the regulation of the IGF system

The role of pregnancy-associated plasma protein-A (PAPP-A) in ovulatory follicles is highly conserved across mammalian species. The low concentrations of IGFBPs in healthy growing follicles are caused by increased rates of proteolytic degradation of IGFBP-2, -4 and -5 by PAPP-A and by low rates of gene transcription for IGFBP-2 (Mazerbourg *et al.* 2003). In bovine granulosa cells the expression of the mRNA for PAPP-A is greatest in ovulatory follicles and is positively correlated with aromatase and LH receptor (Mazerbourg *et al.* 2001). In rodents, pregnant mare serum gonadotrophin (PMSG) stimulates PAPP-A mRNA in granulosa (Hourvitz *et al.* 2002) and FSH induces the degradation of both IGFBP-4 and -5 (Resnick *et al.* 1998). These observations suggest that gonadotrophin-stimulated PAPP-A gene expression is associated with selection of the dominant follicle. However, the regulation of PAPP-A expression by FSH and intra-ovarian factors is not verified for ruminants, although in the rodent BMP15 appears to be involved in the induction of PAPP-A (Matsui *et al.* 2004).

In cattle PAPP-A activity was higher in dominant than in subordinate follicles on Days 2 and 3 of the first follicular wave of the oestrous cycle and the levels of activity correlated positively with oestradiol and negatively with low-molecular weight IGFBPs in follicular fluid (Rivera *et al.* 2001). When cattle were treated with low doses of recombinant bovine FSH for 2 days shortly after wave emergence, two co-dominant follicles were selected, both with higher PAPP-A activities and oestradiol concentrations and lower amounts of IGFBP-4 in their follicular fluid than subordinate follicles (Rivera and Fortune 2001). These results suggest that the action of FSH on dominant follicles is to increase PAPP-A activity. Careful analyses of various characteristics of follicles just before and after follicle emergence showed that an increase in PAPP-A activity in one follicle of the wave was detected before any detectable difference in diameter or in the concentrations of oestradiol or IGFBP-4 or -5 in follicular fluid (Rivera and Fortune 2003). These results point to changes in the IGF system as an integral component of selection for dominance.

In conclusion, it is clear that the IGF system is essential for follicular growth and development. However, a successful outcome requires fine-scale local modulation of the bioactivity of externally derived IGF-I because of the profound effects it can exert on the differentiation and function of follicular cells. This modulation is controlled by the IGF binding proteins and PAPP-A. Their importance is illustrated by situations in which there is a breakdown in the regulation of IGF-I production by the liver, such as the high-producing dairy cow (Lucy 2008). Indeed, the relationships among reproduction, nutrition and metabolism, probably depend to a great degree on IGF-I and on other metabolic factors that directly affect the follicle. This topic is the focus of the next section of this review.

Nutrition and folliculogenesis

Nutritional influences, either directly through dietary nutrients or through metabolic intermediates, affect folliculogenesis at multiple levels in the hypothalamo–pituitary–ovarian axis (Scaramuzzi *et al.* 2006). At a hypothalamic level, conditions resulting in hypoglycaemia and negative energy balance inhibit LH pulsatility in sheep, cattle and humans (Jorritsma *et al.* 2005), most probably by inhibiting GnRH secretion resulting in a failure of the LH surge mechanism and anovulation. Severe negative energy balance can also have deleterious influences on folliculogenesis and the oocyte; these have been most clearly shown using induced ovulation in postpartum dairy cows. Increasing the supply of energy or improving body condition to produce a state of positive energy balance appears to have little effect on LH pulsatility (Rhind *et al.* 1989*a*, 1989*b*), perhaps because negative feedback rapidly counters any stimulatory effect on the follicle unless there are concomitant direct ovarian or peripheral effects that modify negative feedback efficiency. Thus, there appears to be an energy threshold for GnRH and increasing the energy supply above the threshold has no apparent effect on ovulation (Loucks and Thuma 2003).

At an ovarian level, the situation is reversed and increased energy supply stimulates folliculogenesis in sheep and cattle (Webb *et al.* 2004; Viñoles *et al.* 2005; Letelier *et al.* 2008). There is now good evidence showing that the stimulatory effects of nutrition on folliculogenesis are mediated directly at an ovarian level (Scaramuzzi *et al.* 2006) and glucose, fatty acids and several metabolic hormones have all been shown to have direct actions on the follicle. Since 1991, research into the nutritional regulation of folliculogenesis has explored in considerable detail and two general themes have emerged. They are the identification of the nutrients and metabolic signals that mediate nutritional effects in the follicle and the elaboration of the local intra-follicular nutrient-sensing and integrative pathways and mechanisms that modulate gonadotrophin-stimulated folliculogenesis.

When discussing nutritional influences on folliculogenesis, there are some important generalities that should be borne in mind: (1) the energy requirement for folliculogenesis when considered in relation to whole body energy turnover is insignificant. Thus, the effect of energy on folliculogenesis is most likely regulatory rather than providing metabolic fuel; (2) the stimulatory effect of nutritional energy on folliculogenesis is relatively small and its increase induces minor alterations in folliculogenesis. More importantly, these effects can be overridden by other physiological mechanisms and by many common experimental models. Thus, in sheep where short-term energy supplements stimulate folliculogenesis, only 30–50% of treated ewes will convert increases in follicle number to increased ovulation rate. In cattle, energy supplements also stimulate folliculogenesis but this is rarely converted into increased ovulation rate. Presumably in the cow a more

powerful mechanism of follicle selection completely overrides the stimulatory effect of nutrition; and (3) nutritional effects on folliculogenesis have been linked to absolute bodyweight (i.e. adiposity), the rate of gain in bodyweight and short-term nutrient fluxes that have no effect on bodyweight (Scaramuzzi *et al.* 2006). In each of these three situations the nutrient and metabolic profiles will be different as will metabolic signals that reach the follicle. Thus, it is unlikely that there is a single mechanism or a single metabolic mediator of nutritional influences on folliculogenesis, although it may be possible that mechanisms converge in the follicle.

Identification of metabolic and nutritional mediators of folliculogenesis

The list of metabolites and nutrients that have direct effects on follicular function is extensive. The most studied of the intrafollicular mediators of nutritional influences on folliculogenesis are leptin, glucose–insulin, growth hormone (GH) and IGF. The blood concentrations of leptin, glucose and insulin are all elevated when animals are in positive energy balance as well as immediately following feeding and can convey metabolic information to the follicle. In contrast, GH is elevated during negative energy balance (Downing *et al.* 1995) when its principal function is to mobilise the body's energy reserves to counter negative energy balance. Thus, during negative energy balance GH can convey negative metabolic information to the follicle via follicular GH receptors (Eckery *et al.* 1997). The signalling role of GH is complicated because GH stimulates the production of hepatic IGF-I and prolonged exposure to high concentrations of circulating GH can induce insulin resistance.

The IGF system is also usually listed among the potential mediators of nutritional effects on folliculogenesis. The blood concentrations of IGF-I were reduced during severe negative energy balance (Lucy 2008) and thus the IGF system can convey metabolic information to the follicle. The action of IGF-I in the follicle is modulated by complex interactions with its receptor, the various IGF binding proteins and IGF-II, as discussed earlier in this paper. IGF-I, a potent stimulator of follicular growth and follicular oestradiol secretion (Scaramuzzi *et al.* 1999), is almost certainly essential for normal folliculogenesis and follicle selection (Mihm and Evans *et al.* 2008). When used exogenously in the sheep auto-transplant model it induced severe hyperstimulation of both folliculogenesis and follicular oestradiol and inhibin secretion (Campbell 1988; Scaramuzzi *et al.* 1999). Thus, under normal physiological conditions the activity of IGF-I in the follicle is maintained within precise limits by systems in the follicle whose critical function is to prevent over-activity of intra-follicular IGF-I. During negative energy balance low IGF-I inhibits folliculogenesis, but during positive energy balance elevated IGF-I is likely to have only small effects on the follicle because of its intra-follicular inhibition by the IGFBPs and sequestration of the IGF-I receptor by IGF-II.

Nutritional influences on the oocyte

There is now good evidence that diet and body condition can affect the quality of oocytes. Thus, depending on composition, some diets are beneficial to oocyte quality whereas others are detrimental irrespective of their effect on folliculogenesis. In cattle there is a link between nutrient intake and the developmental competence of oocytes and it was shown that high-fat diets enhanced the developmental competence of oocytes (Fouladi-Nashta *et al.* 2007; Garnsworthy *et al.* 2009). Cows were fed a diet formulated to stimulate insulin in order to restore ovarian cyclicity and were then switched to a diet that lowered insulin and increased fatty acids during the mating period (Garnsworthy *et al.* 2009). The results showed that this two-diet strategy increased the pregnancy rate from 27% to 60%. Thus, it appears that optimal dietary strategies can enhance oocyte quality. However, it is clear that further research is required to fully elucidate the mechanisms and to determine if they are mediated by follicular somatic cells or if they involve direct actions on the oocyte.

Metabolic-sensing systems in the follicle

It is now widely accepted that there are direct nutritional effects on folliculogenesis, suggesting that there are specific nutrient-sensing mechanisms in the follicle. Also emerging is an understanding of how these sensing mechanisms affect folliculogenesis.

The leptin system in follicles

All of the components of a functional leptin system are found in follicles. In ruminants, leptin protein and its mRNA have been detected in theca cells and the oocyte (Ryan *et al.* 2002; Muñoz-Gutiérrez *et al.* 2005; Pisani *et al.* 2008). In granulosa cells, however, leptin is only weakly expressed and attempts to identify its mRNA have been unsuccessful (Muñoz-Gutiérrez *et al.* 2005; Pisani *et al.* 2008). Both mRNA and the protein of the functional long form of the leptin receptor have also been detected in granulosa cells, theca cells and the oocyte (Muñoz-Gutiérrez *et al.* 2005; Pisani *et al.* 2008). These findings suggest that leptin may also have paracrine and autocrine functions in the follicle in addition to its endocrine functions.

Much of the *in vivo* research dealing with the reproductive effects of leptin have used the mutant obese (ob/ob) mouse or have been descriptive in nature; that is, measuring leptin in blood and follicular fluid or describing the presence and distribution of leptin and the various forms of its receptor in reproductive tissues. The few physiological studies suggest that exogenous leptin stimulates folliculogenesis in sheep (Kendall *et al.* 2004; Muñoz-Gutiérrez *et al.* 2005). Some *in vitro* and *in vivo* studies have also provided data on the effects of leptin on follicular steroidogenesis. For example, passive immunisation against leptin increases ovarian oestradiol secretion in ewes and, conversely, infusion of leptin directly into the ovarian artery reduces ovarian oestradiol secretion (Kendall *et al.* 2004). The intrafollicular actions of leptin have been studied *in vitro* using cultured granulosa cells and the published data suggest that leptin inhibits hormonally stimulated oestradiol production by granulosa cells *in vitro* (Zachow and Magoffin 1997; Spicer 2001) and by the follicle *in vivo* (Kendall *et al.* 2004). The inhibitory influence of leptin on oestradiol secretion appears to involve the IGF system; immuno-neutralisation of IGF-I reverses the inhibitory effect of leptin on oestradiol secretion (Sirotkin *et al.* 2005) and leptin inhibits the effect of IGF-I on

FSH-stimulated oestradiol production by granulosa cells *in vitro* (Zachow and Magoffin 1997). In cultured bovine granulosa cells, leptin attenuates the effect of IGF-I on FSH-stimulated oestradiol production (Spicer *et al.* 2000). Similarly, it inhibits the effect of IGF-I on LH-stimulated androstenedione production in theca cells (Spicer *et al.* 2000).

The glucose–insulin system in follicles

The mammalian follicle also has a fully functional glucose–insulin system. The follicle has insulin receptors (Poretsky *et al.* 1999) and insulin has been shown to act primarily through its own receptor and not the closely related IGF-I receptor (Willis and Franks 1995). The effects of insulin on cellular function are mediated by a complex array of intracellular pathways (Taniguchi *et al.* 2006) with a multitude of phosphatases, intermediary and terminal kinases as well as numerous scaffold and docking proteins that impart hormone- and tissue-specificity on the cellular responses to insulin. The insulin receptor substrate proteins and the downstream kinases Akt, PI3K and ERK and the phosphatase PTEN have all been identified in the follicle. In a rodent study, it was shown that selective deletion of the gene for IRS-2 was associated with impaired folliculogenesis (Neganova *et al.* 2007). Insulin-dependent glucose transport proteins have also been reported in granulosa and theca cells in ovarian follicles from sheep (Williams *et al.* 2001; Nishimoto *et al.* 2006). The presence of GLUT4 in the follicle suggests a role for insulin-mediated uptake of glucose in the follicle.

Overall, there is no doubt that the follicle contains a functional glucose–insulin system, but we are left with a critical unanswered question – what is its role in folliculogenesis and oocyte development? There are at least two possibilities. The first, and simplest, is that the glucose–insulin system, or insulin itself, has only non-specific functions in the maintenance of cellular health and integrity, as it does for all cells and tissues. Alternatively, the glucose–insulin system may also have specific functions that affect granulosa and theca cells. This second possibility is supported by an overwhelming body of evidence, derived principally from *in vitro* studies.

Adenosine monophosphate-activated kinase (AMPK)

Adenosine monophosphate-activated kinase (AMPK) is a serine/threonine kinase that is activated by an increase in the ratio of AMP to ATP that is associated with a depletion of ATP in response to nutritional and environmental stress (Hardie 2004). Adenosine phosphate-activated kinase has been identified in theca cells, granulosa cells and oocytes (Bilodeau-Goeseels *et al.* 2007; Tosca *et al.* 2007). Adenosine phosphate-activated kinase is often viewed as a metabolic master molecule and in the ovary it may regulate metabolic influences on folliculogenesis and oocyte maturation. Thus, the AMPK signalling pathway is a potential modulator of interactions between energy balance and folliculogenesis.

The hexosamine pathway

The hexosamine nutrient sensing system has been reported in muscle and adipose tissue (Marshall *et al.* 1991). Hexosamine biosynthesis is active in bovine follicles and is particularly important for the production of hyaluronic acid during cumulus expansion (Thompson *et al.* 2007). Perturbations in glucose flux (hyperglycaemia) through this pathway may have substantial negative effects on oocyte competence (Thompson *et al.* 2007). It has been suggested that this nutrient-sensing pathway may also mediate nutritionally stimulated folliculogenesis (Muñoz-Gutiérrez *et al.* 2004).

Local integrative pathways and crosstalk between insulin and gonadotrophin signalling

A concept not widely appreciated in 1991, but now generally accepted, is that a cascade of divergent intracellular signalling pathways are activated when gonadotrophins stimulate follicular cells (Richards *et al.* 2002; Hunzicker-Dunn and Maizels 2006). By replacing the linear models of hormone signalling with functional interactions among signalling pathways (often referred to as cross-talk) and networks in the follicle (Taniguchi *et al.* 2006), we can begin to understand how short-term nutritional stimulation might modify gonadotrophin-stimulated follicular function. In a recent experiment a short-term infusion of glucose at 10 mM per hour increased the total number of follicles >1 mm in diameter but decreased the amount of phosphorylated Akt and AMPK as well as aromatase in granulosa cell lysates (Gallet *et al.* 2009). The molecular details of how and when these pathways interact (cross-talk) in granulosa cells and their implications for folliculogenesis remain to be explored.

Systemic endocrinology

Endocrinology of the oestrous cycle, follicle dynamics and follicle waves

The temporal patterns of secretion for the major reproductive hormones during the oestrous cycle had been well described by 1991 (fig. 1 in Scaramuzzi *et al.* 1993). The secretion of GnRH is inhibited by ovarian feedback exerted through a synergistic interaction between oestradiol and progesterone acting on centres in the hypothalamus. This process explains the induction of the follicular phase and the sequence of events leading to the LH surge and ovulation. For the hormonal control of the terminal stages of folliculogenesis and the determination of ovulation rate, the secretion of FSH is important and FSH secretion is controlled primarily by negative feedback at the pituitary level through a synergistic interaction between inhibin and oestradiol. In the 17 years since our initial workshop, we have seen the emergence of more precise descriptions of the patterns of secretion of inhibin A and B and the close association between the wave-like patterns of terminal follicle growth and regression (described in the next section) and the patterns of secretion of reproductive hormones, particularly FSH, oestradiol and inhibin.

Inhibins and activins

It was clear in 1991 that inhibin suppressed FSH secretion both *in vivo* and *in vitro*, acting with oestradiol to suppress secretion of FSH by a direct inhibitory action on the expression of the mRNA for FSHβ. However, in 1991 the only available assays for inhibin detected its α sub-unit and thus were unable to

discriminate between the biologically inactive free α sub-unit and the intact and biologically active inhibin α–βA and α–βB dimers. An increase in inhibin concentrations was seen in the follicular phase and inhibin secretion was stimulated by FSH, but the adrenal cortex also secreted inhibin α sub-unit (McNeilly *et al.* 1994), so interpretation of these data was problematic. All of the inhibin subunits, as well as activin receptors and follistatin, have been detected in ovarian follicles from sheep (Engelhardt *et al.* 1993). In addition, betaglycan, the putative inhibin receptor (Gray *et al.* 2001) has been identified in sheep ovarian follicles (McNatty *et al.* 2007). Multiple isoforms of the inhibin dimers have been identified in bovine follicular fluid (Glister *et al.* 2006) but nothing is known of the dimers' presence in sheep follicles or of their relative patterns of secretion and physiological functions in either species.

The successful development of two-site enzyme linked immunosorbent assays (ELISA) that were able to differentiate the two biologically active forms of dimeric inhibin namely, inhibin A and inhibin B (Groome *et al.* 1994, 1996), was a major technical advance in the field of inhibin physiology. These assays showed that sheep produced only inhibin A (McNeilly *et al.* 2002). In follicles from sheep, much less of the βB subunit of inhibin B was expressed compared with the βA subunit of inhibin A. In the ewe, the plasma concentrations of inhibin A increased during the follicular phase up to the point of ovulation (Souza *et al.* 1997).

While early studies concentrated on investigating inhibin as an endocrine regulator of FSH secretion, it is now clear in sheep (Campbell and Baird 2001) as well as other species (Knight and Glister 2006) that inhibin also acts locally within the follicle to augment LH-stimulated production of androgen by theca cells and FSH-induced aromatase activity in granulosa cells. The activins appear to inhibit these effects. The only identified inhibin receptor, betaglycan, does not appear to mediate a signalling response to inhibin (Gray *et al.* 2001), so inhibin probably acts to block the action of activin and possibly some BMPs. This remains to be confirmed. Activin A enhanced the growth of follicles in rodents and humans (Knight and Glister 2006) and improved the competence of bovine oocytes (Silva *et al.* 2003) as well as the *in vitro* development of ovine oocytes and preantral follicles (Thomas *et al.* 2003). However, activin A had no effect on the development of bovine primordial and primary follicles *in vitro* (Fortune *et al.* 2000) and inhibin A reduced the competence of bovine oocytes *in vitro* (Silva *et al.* 2003). How these effects are modulated *in vivo* is unclear because little is known about the control of activin secretion due to the lack of assays sufficiently sensitive to detect activin A, activin AB, and particularly activin B, in sheep. Furthermore, both inhibin and follistatin can modulate the action of activin, suggesting that the physiological effects of activin are tightly regulated within individual follicles.

Technical issues – the measurement of inhibin and FSH

The sensitivity and specificity of hormone assays, particularly for peripheral inhibin and FSH remain a problem. There is an almost total lack of data on the effects of nutrition on inhibin and the role of ovarian feedback in mediating nutritional effects on folliculogenesis cannot be resolved until we can measure peripheral inhibin concentrations. There is now good evidence showing that nutrition suppresses follicular oestradiol secretion while increasing the number of small and medium follicles. So why does FSH not increase? Perhaps it is because nutrition stimulates inhibin secretion! This would be consistent with the stimulation of folliculogenesis in small and medium-sized follicles. The combined effect of lower oestradiol and increased inhibin could, in effect, counter each other so that there is no alteration in FSH secretion. One report showed that ewes in moderate body condition had higher concentrations of circulating inhibin-α than ewes in low body condition (Rhind and Schanbacher 1991). Clearly this is a critical area where knowledge is deficient.

A further complexity is that the plasma concentrations of FSH measured using current homologous radioimmunoassays show that the mean concentrations of FSH are similar within ewes across several oestrous cycles and during anoestrus (A.S. McNeilly, unpubl. data). This suggests that the FSH threshold concentration for the emergence of gonadotrophin-dependent follicles varies among individuals according to age, season and other as-yet unidentified variables as was shown in one study (Picton and McNeilly 1991). The large variation in ovulation rates after PMSG or FSH suggests that differing FSH thresholds are a significant factor in maintaining breed-specific ovulation rates. However, the nature of this threshold and unravelling how it affects selection of the ovulatory follicle remains to be determined.

Conceptual issues – the feedback–ovulation rate paradox

The hypothalamo–hypophyseal–ovarian axis, with its feed-forward and feedback endocrine links, is essentially a homeostatic system that will tend to neutralise the effects of external factors (e.g. a nutritional supplement) on folliculogenesis. Any stimulus that leads to the emergence of more ovulatory follicles would induce higher rates of secretion of follicular hormones, leading to greater inhibitory feedback, and thus a reduction in gonadotrophin secretion, thereby reducing follicular growth and re-establishing the *status quo*. Thus, based on first principles, we have two mutually incompatible *a priori* hypotheses: (1) an increase in the secretion of FSH is needed to stimulate folliculogenesis; and (2) increased folliculogenesis leads to lower concentrations of FSH. This conflict probably explains the variety of experimental observations in the literature, with studies supporting both hypotheses and others showing no significant change in FSH concentrations. Arguably, lack of change is the correct and normal outcome. This is only feasible if there are changes in the balance of the feedback loop such as the ratio of oestradiol to inhibin, or there is another variable, other than concentration, involved in the response to the hormones comprising the feed-forward and feedback loops (e.g. variable time lags).

With respect to the balance of the feedback equilibrium, two options can be considered: (1) there is variation in the responsiveness of follicles to FSH, for both proliferation and hormone production; and (2) there is a change in clearance of the feedback hormones, so that concentrations reaching the

hypothalamo–hypophyseal axis do not directly reflect secretion rates. These are not mutually exclusive and there is evidence for both. For example, when ewes are fed a supplement, more ovarian follicles develop but the amount of oestradiol produced per follicle is reduced (Viñoles *et al.* 2005; Somchit 2008). In addition, diet affects the rate of metabolic clearance of oestradiol (Adams *et al.* 1994) so the overall effect is less inhibition of FSH secretion than might be expected for the number of follicles that are developing.

Dynamic patterns in the growth of gonadotrophin-responsive, gonadotrophin-dependent and ovulatory follicles

The last 17 years have seen a rapid expansion in the application to farm animals of ultrasound imaging for monitoring follicle development, a technique that had been widely used in clinical medicine for monitoring of ovarian structures in women (Kerin *et al.* 1981). It was initially adapted for use in cattle (Pierson and Ginther 1984) and then in small ruminants. Repeated, precise measurement of individually identified ovarian follicles allowed a clear description of the wave-like patterns of terminal follicular growth and has led to considerable refinement of hypotheses and experiments.

In small ruminants, ultrasound imaging of ovarian follicles is technically challenging because of the small size of the animals, the position of the reproductive tract, and the size of the ovaries and its structures. For follicles larger than 2 mm in diameter, the technique is accurate but, below this limit, the resolution is insufficient to provide quality data (Viñoles *et al.* 2004). Some of these limitations can be overcome by coupling ultrasound with ovarian autotransplantation, in which the left ovary and its vascular pedicle are relocated to a subcutaneous site in the neck (Souza *et al.* 1997, 1998). This allows transdermal imaging in two planes and, when it is allied with contemporaneous sampling of ovarian venous blood, the physiological and morphological changes within the follicle population can be followed over considerable periods of time. The transplant model has highlighted a primary limitation of conventional ultrasound by showing that large antral follicles can remain morphologically normal, as assessed by ultrasound, for several days after losing hormonal function (Souza *et al.* 1997). The value of the technology is that it allows us to study the processes of transformation that follicles undergo as they progress through these functional stages of development. Ultrasound-based studies have been particularly useful for studying follicle selection and follicle dominance, critical aspects of the very final stages of folliculogenesis that determine the outcome for ovulation and the ovulation rate.

Dominance and waves of development in gonadotrophin-dependent follicles

The development of the pool of gonadotrophin-responsive follicles can be considered hierarchical with most, if not all, being at a different stage of growth. Subsequently, the most advanced of these gonadotrophin-responsive follicles (i.e. during the antral growth stage) are those that emerge concomitantly with the increases in FSH to form what is commonly referred to as the cohort of gonadotrophin-dependent follicles. Gonadotrophin-dependent follicles grow and regress in a regular sequential pattern of waves, a phenomenon first described in cattle in 1960 and now detailed by ultrasound-based observations. A wave begins under the influence of a small rise in FSH concentrations that is caused by the momentary disappearance of inhibitory feedback (oestradiol and inhibin) when a dominant follicle ovulates or when a potentially ovulatory follicle undergoes atresia. The rise in FSH induces a cohort of gonadotrophin-dependent follicles to emerge from a pool of gonadotrophin-responsive follicles (Souza *et al.* 1997, 1998; Viñoles *et al.* 1999). About three days after emergence, one (or a few) of these follicles each achieve potentially ovulatory status (diameter 5–8 mm in the ewe, 15–18 mm in the cow) by developing LH receptors on their granulosa cells and becoming independent of FSH; they, in fact, switch their absolute dependence on gonadotrophins from FSH to LH. They also secrete large amounts of oestradiol, androstenedione and inhibin A, reducing FSH concentrations to below the threshold needed to sustain the other gonadotrophin-dependent follicles (Fig. 2). Thus, dominance by the potentially ovulatory follicle(s) causes atresia in any remaining members of the cohort that have failed to switch their absolute dependence on gonadotrophins from FSH to LH and thus cannot continue to function in the presence of low concentrations of FSH (Campbell *et al.* 1999). If the potentially ovulatory follicle(s) achieve their status soon after luteolysis, they will continue to develop and ovulate; otherwise, they will stop producing oestradiol, even if provided with normally adequate gonadotrophic support (Dobson *et al.* 1997), although they will persist and be identifiable by ultrasonography for several days thereafter (Souza *et al.* 1997; Viñoles *et al.* 1999). Ultimately, they also undergo structural and functional atresia, thus initiating the next follicular wave in the sequence.

During the oestrous cycle, the ewe has two to four follicle waves each of 4–8 days. The reason for this variation is unknown, but body condition has been suggested (Murphy *et al.* 1991). The variation in waves per cycle might explain much of the variability that is encountered in experiments designed to study the effects of short-term nutritional supplements on follicular development. In a randomly-selected group of ewes, during any random period of the oestrous cycle, there will be a mix of animals going through any of the final stages of follicular development, from gonadotrophin-responsive to gonadotrophin-dependent to atretic, despite treatments used to synchronise oestrous cycles. Unless we can control this variation, it will be difficult to obtain robust data in detailed physiological studies of ovulation rate. In concluding this section, we need to point out that there is debate over whether follicular dominance actually exists in the ewe (Duggavathi *et al.* 2005). In the ewe, there is ample evidence suggesting that in each follicle wave, a potentially ovulatory follicle is present and that in the mono-ovulatory ewe, one follicle becomes larger and more highly oestrogenic (or deviates). However, the oestrogenic follicle is not always the largest follicle and the ewe can rapidly promote replacement follicles into the ovulatory role so that further recruitment and selection can occur during the follicular phase (Souza *et al.* 1997). This debate thus concerns the mechanisms responsible for the wave pattern and not the existence of the wave pattern itself.

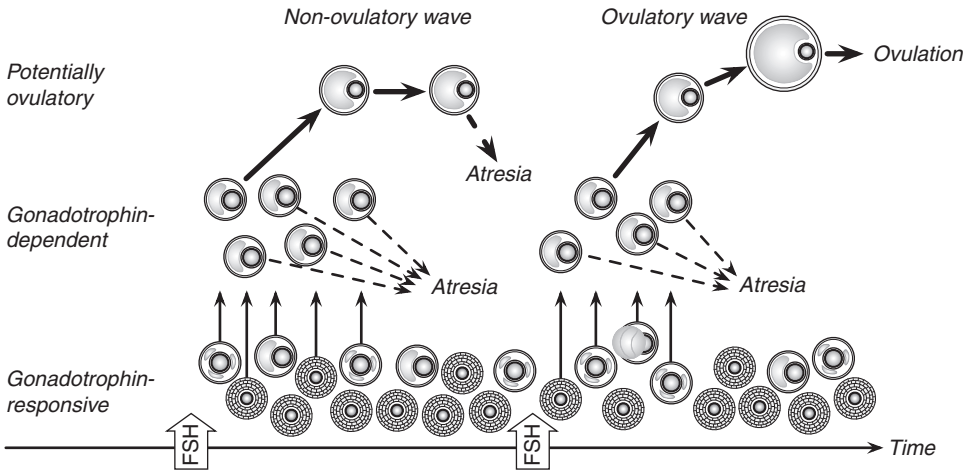

Fig. 2. The wave-like pattern in the terminal stages of follicle growth in ruminants. Growth to the gonadotrophin-responsive stage is approximately linear ensuring a continuous supply of gonadotrophin-responsive follicles. Under the influence of FSH, gonadotrophin-responsive follicles become gonadotrophin-dependent in a process that reflects the dynamics of the negative feedback loop between oestradiol and inhibin and pituitary FSH. A peak of FSH (indicated by the arrows) leads to the emergence of a group of gonadotrophin-dependent follicles, visible by ultrasound, with a higher threshold for FSH than gonadotrophin-responsive follicles. A potentially ovulatory follicle or follicles emerge from this group and, while present and fully functional, it is dominant, secreting sufficient oestradiol and inhibin to suppress FSH, promote atresia in the remaining gonadotrophin-dependent follicles and preventing the emergence of a new cohort of gonadotrophin-responsive follicles. In the presence of a corpus luteum (a non-ovulatory wave), the dominant follicle(s) becomes atretic after 4–5 days, secreting less oestradiol and inhibin so that FSH can increase and start a new wave. If the corpus luteum has regressed, an ovulatory wave results and the dominant follicle(s) ovulate(s).

AMH and the transition from gonadotrophin-responsive to gonadotrophin-dependent follicles

In rodents, the granulosa cells of committed and gonadotrophin-responsive follicles produce AMH (Themmen 2005). First detected in granulosa cells of committed follicles, AMH reaches its highest level in granulosa cells of gonadotrophin-responsive follicles and then gradually diminishes until no longer detectable in gonadotrophin-dependent follicles. Similarly, in the cow the concentrations of AMH are elevated in follicular fluid from healthy gonadotrophin-responsive follicles and decrease during terminal follicular development (Rico *et al.* 2009) and AMH is not present in atretic follicles (Rico *et al.* 2009). Furthermore, in rat and pig granulosa cells, AMH was able to reduce FSH-stimulated aromatase and the expression of LH receptors (di Clemente *et al.* 1994). These data suggest that AMH may regulate the responsiveness of follicles to FSH. Overall, the evidence suggests that AMH modulates both early and terminal stages of follicle development in rodents. However, in ruminants more research is required to clarify the patterns and levels of expression as well as the bioactivity of AMH isoforms during follicle development and in particular the role of gonadotrophins in the control of AMH.

Update on models of folliculogenesis, ovulation rate and nutritional influences on folliculogenesis

Models of folliculogenesis

With respect to the general mechanistic model proposed in 1991 (fig. 3 in Scaramuzzi *et al.* 1993), the basic model still holds, although significant new information has been added and additional mechanisms proposed to explain the progressive transformation of a follicle from one stage of development to the next. However, there is information that is not easily incorporated into a general model. In particular, *in vitro* culture systems using oocytes and follicular somatic cells have generated detail on the molecular and cellular mechanisms responsible for local control systems. The problem is that, at this time, it is difficult to decide the relative physiological importance of many of these newly described intrafollicular systems in comparison with, say, the gonadotrophins or IGF-I. Therefore, to provide a durable model, we have retained a general model (Fig. 3) based on that put forward in 1991 and included a molecular-based model that describes events between the oocyte, cumulus and granulosa cells (Fig. 4). Here we have sifted information, omitting some possibilities from the model. Clearly, this is a relatively subjective process. The most important additions to the models are:

(1) The interactions between the oocyte and the granulosa cells including the differentiation of the granulosa cells into mural and cumulus phenotypes.
(2) The initiation of growth and development in the primordial follicle, a process that was essentially a mystery in 1991, has been updated along with an updated understanding of the development of committed follicles.
(3) Advances in our understanding of the physiology of antral follicle growth and differentiation of gonadotrophin-responsive follicles into gonadotrophin-dependent and ovulatory follicles. Here progress has been relatively modest compared with the above two areas.
(4) Confirmation of the concept that metabolic factors can directly influence follicular development and thus ovulation rate.

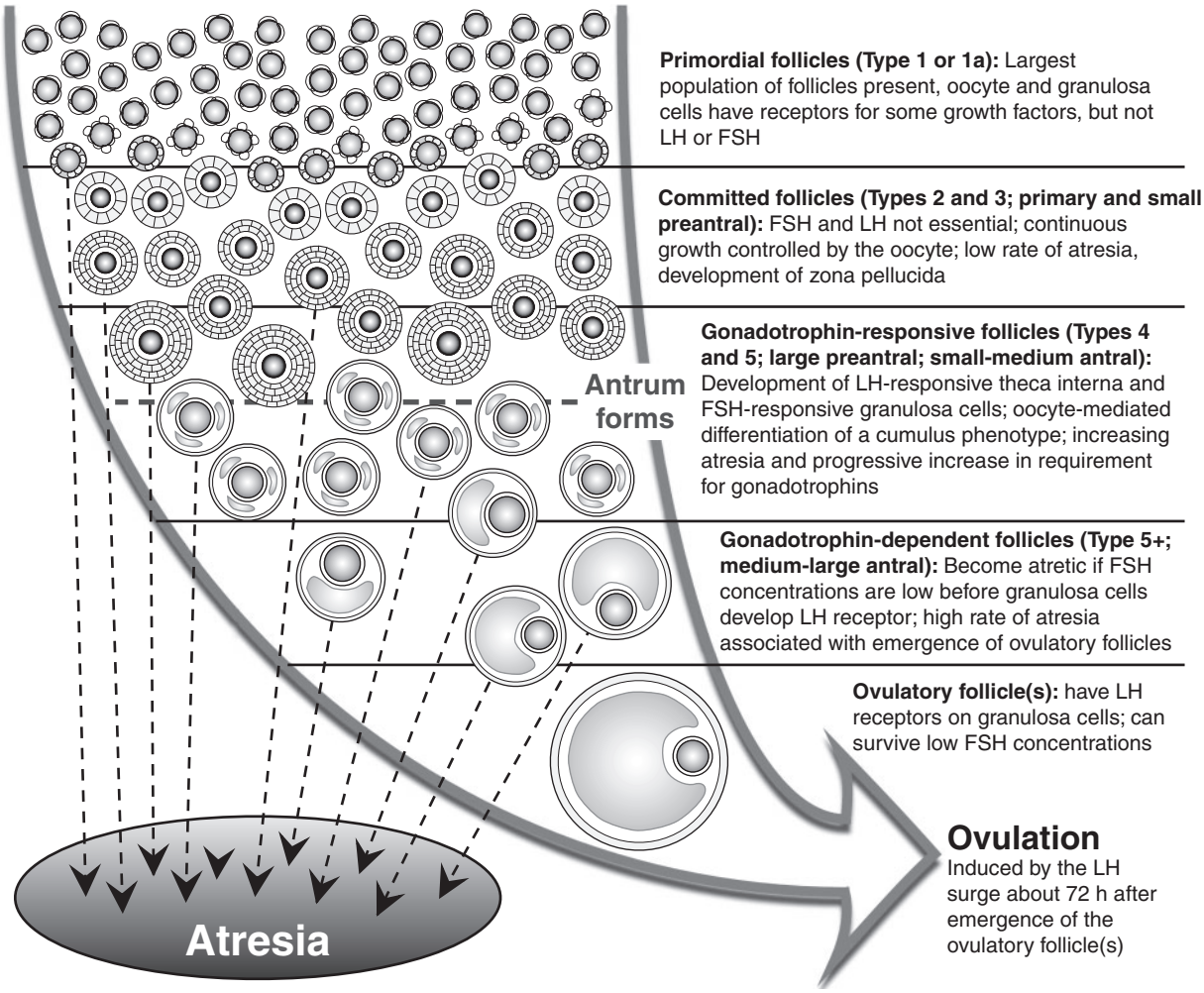

Fig. 3. A model for folliculogenesis in the ewe based on that developed by Scaramuzzi *et al.* (1993). Folliculogenesis is illustrated as a cascade of development during which follicles emerge from a pool of primordial follicles to enter a process of growth and development that is continuous and ends in either atresia or ovulation. In most mammals this process is approximately linear to the gonadotrophin-responsive stage and, especially in ruminants, becomes wave-like in the gonadotrophin-dependent stage, as shown in Fig. 2.

The models portray the developmental path of a single follicle from the primordial stage to ovulation. Ultimately, we anticipate a model encompassing the entire follicle population functioning in a physiological context to produce a cooperative outcome: the ovulatory quota.

An updated model for folliculogenesis

An updated general model for folliculogenesis is presented in Fig. 3. This model retains the functional classification system of the 1991 model. This classification system is based on the interrelationship of the follicle with the gonadotrophins. Primordial and committed follicles are largely independent of gonadotrophins and even though they are affected by gonadotrophins their essential feature is that they do not require gonadotrophins for their survival and continued development. They are sometimes called gonadotrophin-independent follicles; however, we have preferred the term committed follicle to describe this class of follicle as they are preantral and have a consistent morphology. The continued orderly development of these follicles (Lundy *et al.* 1999) appears to be a linear process controlled primarily by the oocyte. As follicle development progresses follicles gradually become more and more reliant on gonadotrophins, first as gonadotrophin-responsive follicles and then as gonadotrophin-dependent follicles. The antrum forms under the influence of FSH and thus gonadotrophin-responsive follicles have a more variable morphology; they can be preantral, early antral (with antral gaps yet to coalesce into a complete antrum) or fully antral. In gonadotrophin-responsive follicles and then as gonadotrophin-dependent follicles inadequate support from gonadotrophins leads to atresia. It is the developing dependence on gonadotrophins that transforms folliculogenesis from a linear process in the preantral and early antral stages of development into a wave-like process during the terminal stages of folliculogenesis. The final class of follicle is the potentially ovulatory follicle. We have chosen to use the term 'potentially ovulatory' to describe this class of follicle rather than alternatives such as 'selected', 'dominant' or

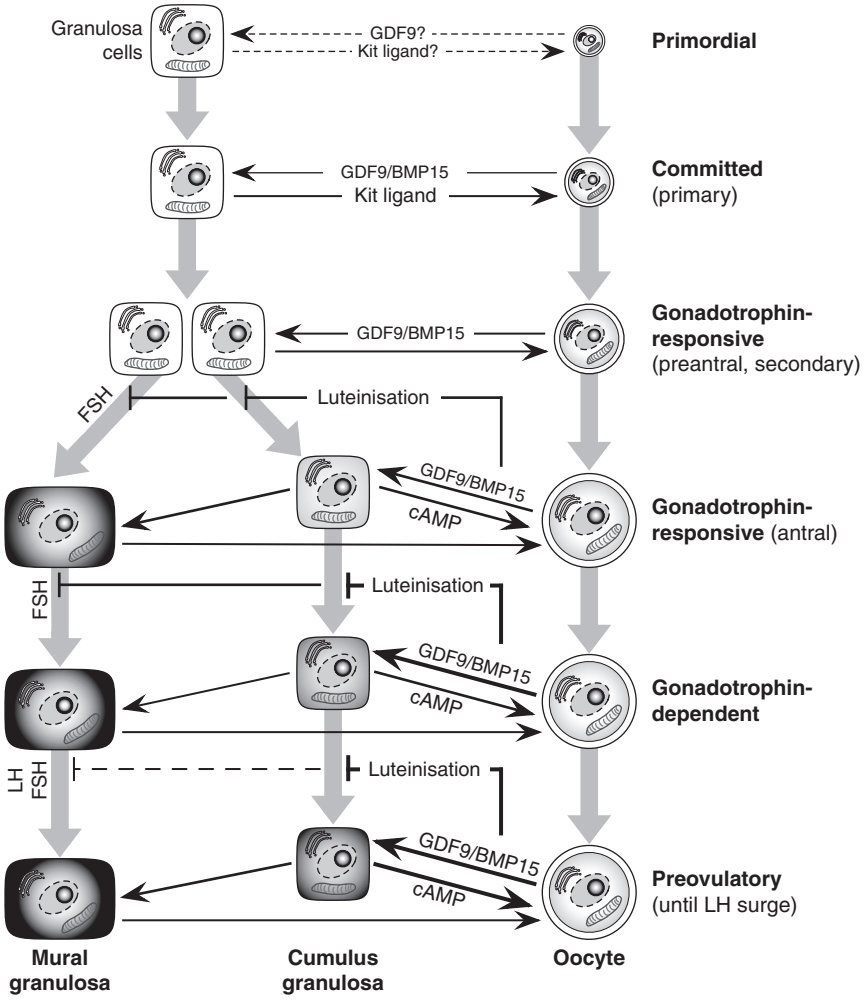

Fig. 4. A scheme illustrating the complex and highly integrated nature of folliculogenesis involving interactions between the oocyte and granulosa cells of developing follicles and indicating pathways of granulosa cell differentiation (into cumulus and mural granulosa cells) and function, oocyte growth and the acquisition of meiotic and developmental competence. Up to antrum formation, oocytes and granulosa cells are intimately associated and signal to each other via gap-junctions and with paracrine growth factors such as GDF9, BMP15 and kit-ligand. From antrum formation onwards, a critical role of oocyte-secreted factors (principally GDF9 and BMP15) is to prevent luteinisation of the oocyte's neighbouring granulosa cells, thereby establishing and maintaining the distinctive cumulus cell phenotype. During the antral phase, oocyte-secreted factors also continue to act on mural granulosa cells to promote mitogenic activity and simultaneously inhibit luteinisation. In turn, both granulosa cell types, but in particular the cumulus cells, provide the oocyte with paracrine factors and multiple metabolites and regulatory molecules via gap-junctions (e.g. cyclic AMP), required for the oocyte to acquire developmental competence in the follicle. Broken arrows indicate pathways for which the evidence is limited. Increasingly heavy arrows indicate stronger effects. The figure provides a model for future research.

'oestrogenic'. Ovulatory follicles are those that are capable of ovulation given the correct endocrine milieu of high oestradiol, low progesterone and high LH pulse frequency. Selected and oestrogenic follicles are indeed the physiological equivalent of ovulatory follicles and they can ovulate if challenged exogenously with an LH surge (Webb *et al.* 1992). The dominant follicle is a morphological description and refers to the largest follicle. However, the concept of the dominant follicle has doubtful physiological relevance in small ruminants because the largest follicle is not necessarily the ovulatory follicle and may even be atretic (Souza *et al.* 1997; Viñoles *et al.* 1999).

Molecular model of folliculogenesis and interactions between the oocyte and somatic cells

This is an area of conceptual revolution since 1991. The oocyte has been confirmed as a major regulator of preantral and early antral follicular growth, affecting ovulation rate and the differentiation and metabolism of the cumulus oophorus, including its capacity to expand and the oocyte's acquisition of developmental competence. The oocyte secretes two important growth factors, GDF9 and BMP15, and other potentially important oocyte secreted factors including BMP6, the fibroblast growth factor (FGF), TGF-α and α2-macroglobulin (ovostatin).

Known and suspected interactions between the oocyte and follicular somatic cells during the different stages of folliculogenesis are shown in Fig. 4.

Models for the regulation of ovulation rate

The model for the regulation of ovulation rate proposes a metaphorical wall with a gate that regulates the number of gonadotrophin-dependent follicles that pass through it and avoid atresia to become ovulatory follicles (Baird 1987). The gate represents the period of time during a follicle wave when the concentration of FSH remains above the minimum or threshold value required by gonadotrophin-dependent follicles to avoid atresia. A narrow gate only allows a single follicle to avoid atresia and if the timing is right, to go on and ovulate (Fig. 5*a*). A wider gate allows for multiple ovulations (Fig. 5*b*). Alternatively, for a gate of fixed width, there may be a larger pool of gonadotrophin-dependent follicles undergoing development and statistically more will be able to pass through to become ovulatory (Fig. 5*c*). The number of gonadotrophin-dependent follicles at the gate depends on two factors: (1) a genetically determined constant that does not change; and (2) non-genetic environmental influences that are not constant and may change even from follicle wave to follicle wave.

There are two ways of increasing the number of ovulatory follicles (Baird and Campbell 1998):

(1) Widening the gate by decreasing the sensitivity of the hypothalamo–pituitary–ovarian axis to negative feedback to maintain the concentration of FSH above the threshold level for longer, thus allowing more time for follicles to pass through the widened gate (Fig. 5*b*). This is the case in the Romanov and Finnish Landrace breeds of sheep. The administration of oestradiol antiserum to sheep or anti-oestrogens to women has the same effect.
(2) Increasing the number of potentially ovulatory follicles available to pass through the gate before it is closed by suppression of FSH below its threshold level (Fig. 5*c*). In this case the levels of FSH and the feedback mechanisms are the same in ewes with single and multiple ovulations but follicles are more sensitive to FSH and hence develop precociously. This is the case for ewes carrying the Booroola mutation and in ewes treated with low doses of FSH.
(3) In reality both mechanisms may operate simultaneously in some highly fecund breeds, for example, the Finnish Landrace. These hypothetical mechanisms need to be tested against evidence. Two points need to be made: (1) the hypotheses are not mutually exclusive; and (2) the evidence is not strong for either. In reality, the correct experiments have never been done. Having said this, what does the evidence, such as it is, suggest?

(1) Widening the gate through elevated FSH

The fact that exogenous FSH or PMSG can increase the number of ovulatory follicles tells us that this mechanism is feasible, but its feasibility does not conclusively demonstrate

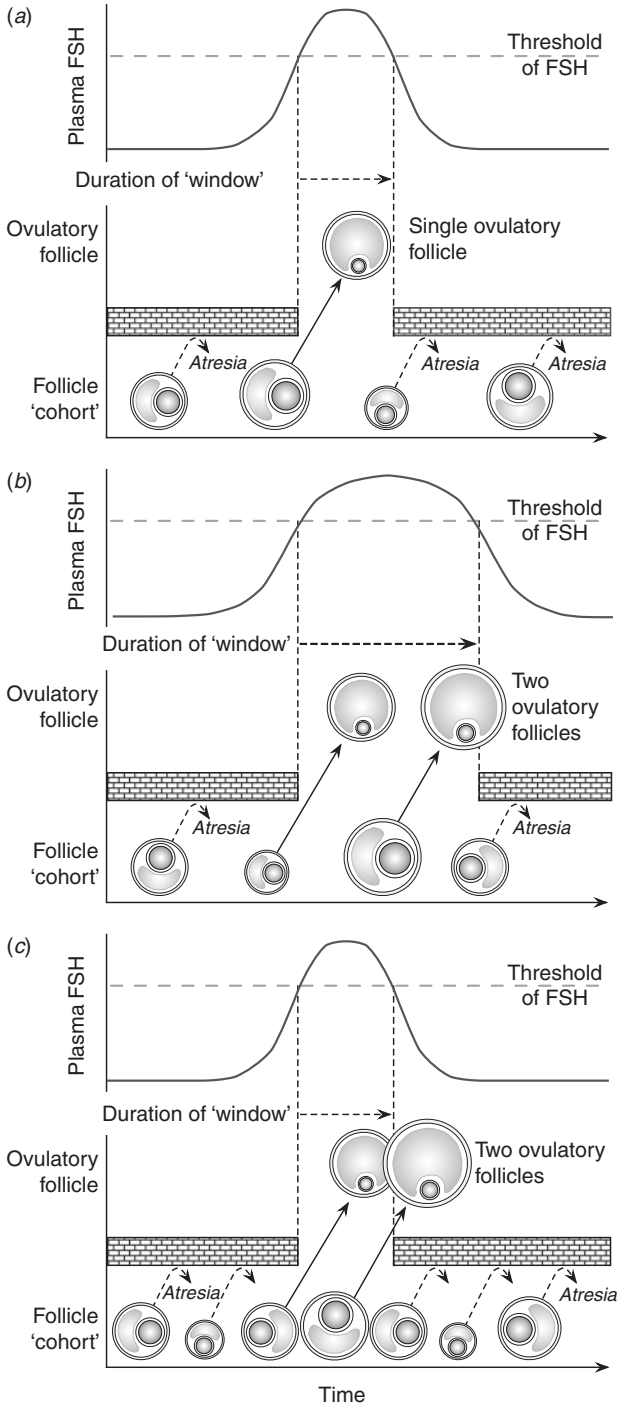

Fig. 5. A model of the interactions that control ovulation rate. To become ovulatory, members of a cohort of gonadotrophin-dependent follicles need their stage of development to coincide with a selection window, the width of which is determined by the period of time that FSH concentrations exceed the critical threshold needed to prevent atresia (*a*). Ovulation rate can be increased by increasing the width of the window (*b*) or by increasing the number of gonadotrophin-dependent follicles that are ready to pass through the window (*c*).

that it is a physiologically normal, regulatory process. Much of the evidence demonstrating that the length of time during which the FSH remains above its threshold before being suppressed by rising oestradiol and inhibin is based on experiments using exogenous FSH. In the ewe, when the concentration of FSH was maintained above its threshold for different lengths of time by the infusion of exogenous FSH there was a time-related stimulation of folliculogenesis (Baird and McNeilly 1987) and in a similar experiment in women (Schipper *et al.* 1998) the continuous infusion of FSH over several days produced a much greater increase in the number of ovulatory follicles than the same dose given as a single injection. These data provide evidence supporting the widening of the window model through elevated FSH.

The measurement of FSH concentrations in jugular venous blood throughout the cycle presents a confusing picture that is not always consistent with the widening of the window model. The confusion in the literature is not surprising given what we now know about follicle waves, and studies in which follicle waves are synchronised may help to resolve the confusion. A more relevant approach may be to study the ovulatory wave during the follicular phase of the oestrous cycle. In the ewe, there is a consistent fall in FSH during the follicular phase of the cycle ~24–36 h before ovulation and the number of ovulatory follicles and the ovulation rate can be easily determined. In the luteal phase of the oestrous cycle the major regulator of oestradiol secretion and hence the level of inhibition of FSH is the frequency of LH pulses, which, in turn, is dependent on the level of progesterone.

(2) Widening the gate by lowering the threshold for FSH

The gate will also be widened if the FSH threshold in the follicle is lowered. The evidence for this is almost all indirect and circumstantial and there is no direct evidence that the threshold concentration of FSH necessary to activate medium-size antral follicles is different in ewes with a single or a multiple ovulation. The experimental demonstration of a change in the threshold concentration of FSH *in vivo* is a difficult challenge and we do not know the threshold concentration of FSH. At the Terrigal meeting (Scaramuzzi *et al.* 1993) its value for the mono-ovulatory ewe was estimated as 1.0 ng mL^{-1} of National Institute of Health, Reference preparation 1 (NIH RP-1) and although this estimate has never been confirmed or refuted it seems probable that a single FSH threshold value is unlikely. A suitable model to examine the FSH threshold is the antagonist-suppressed ewe given replacement FSH. Only one limited study has suggested that each ewe has its own FSH threshold (Picton and McNeilly 1991). Thus, it seems likely that the FSH threshold can vary widely among animals depending on age, genotype and other unidentified factors. The indirect evidence, on the other hand, is considerable. Both insulin and IGF-I can, in a dose-dependent manner, alter the sensitivity of granulosa cells to FSH-stimulated oestradiol production as can several other paracrine and autocrine growth factors. Some of these factors (insulin, IGF-I and TGF-β) reduce the dose of FSH required *in vitro* for the maximum stimulation of oestradiol production. Others (EGF, FGF and TFGα) inhibit aromatase activity (Scaramuzzi and Campbell 1990) and thus increase the minimum concentration of FSH required to stimulate oestradiol production.

(3) Increasing the number of gonadotrophin-dependent follicles

This can be achieved by reducing the size at which follicles become gonadotrophin responsive (Fig. 5*c*), as happens, for example, in ewes carrying the Booroola mutation. These ewes are comparable with wild-type controls with respect to the concentrations of FSH and timing of events around ovulation, from luteal regression to the LH surge, from the onset of oestrus to the LH surge and from the LH surge to ovulation. Concentrations of oestradiol are also similar between the wild-type and $FecB^B$ mutant genotypes because the total secretion of oestradiol from several smaller preovulatory follicles of ewes with the $FecB^B$ mutation is approximately the same as from a single large pre-ovulatory follicle in wild-type ewes. Thus, in ewes with the $FecB^B$ mutation follicles become gonadotrophin-responsive and form an antrum at a smaller diameter, thus effectively increasing the size of the pool of gonadotrophin-responsive follicles available to pass through the selection gate without any change in the overall numbers of follicles, and unaltered negative feedback mechanisms.

Alternatively the number of gonadotrophin-dependent follicles may be increased by increasing the population of gonadotrophin-responsive and gonadotrophin-dependent follicles, without changes in follicle size as happens, for example, in the Romanov breed (Fig. 5*c*). Ewes of this breed have ovaries that contain three times more growing follicles (including gonadotrophin-responsive and gonadotrophin-dependent follicles) than those of non-prolific breeds. Thus, more gonadotrophin-responsive follicles pass through the selection gate. In addition, at ovulation, each of their potentially ovulatory follicles has a granulosa cell population of a similar size to that of a single potentially ovulatory follicle in a non-prolific breed. As a consequence of these follicular features, oestradiol concentrations before ovulation are 3-fold higher than in non-prolific breeds. Recently, a reduced sensitivity to oestradiol feedback in this breed has been shown by the observation of a longer latency between the insertion of an oestradiol implant and the LH surge induced by oestradiol in Romanov ewes compared with Île de France ewes (Ben Saïd *et al*. 2007). Roger Land had also shown in 1976 that the ovulation rate in Finish Landrace ewes was less sensitive to the suppressive effect of oestradiol than in Scottish Blackface ewes (Land 1976). The concentrations of FSH in the Romanov and the Île-de-France are similar but the decline of FSH after luteal regression is less profound and delayed in the Romanov. From these data, one concludes that in Finish Landrace and Romanov ewes the hypothalamo–pituitary axis is more resistant to oestradiol. Thus, the available evidence presented above suggests that multiple mechanisms are involved in the determination of ovulation rate in sheep and these mechanisms appear to be additive, at least in some models of extreme prolificacy.

(4) The link with nutrition

What is the relationship between nutrition and the mechanisms that determine ovulation rate? It is undisputed that nutrition stimulates folliculogenesis in ruminants. Furthermore, it would be reasonable to assume that the mechanism of this effect will be

one of those proposed for the control of ovulation rate in general. Available evidence suggests that nutrition (1) increases the number of small and medium-sized gonadotrophin-responsive follicles; and (2) reduces atresia among large gonadotrophin-dependent follicles without a change in FSH, suggesting that nutrition increases the number of ovulatory follicles by increasing the cohort size of gonadotrophin-responsive follicles (Fig. 5*c*). Reduced atresia amongst gonadotrophin-dependent follicles can be regarded as a direct consequence of a widened gate (Fig. 5*b*) associated with a low FSH threshold rather than increased concentrations of circulating FSH.

A model for nutritional and metabolic influences on folliculogenesis

A mechanistic model for the influence of nutrition on folliculogenesis is shown in Fig. 6. This model attempts to integrate the known influences of nutrition and body condition with the metabolic effects on folliculogenesis that we consider to be the more significant. The model is a conceptual framework that attempts to make physiological sense of known facts and highlight critical areas where knowledge is deficient or contradictory, thus providing direction and focus for future research. It is clear that body condition and nutrition can act throughout the

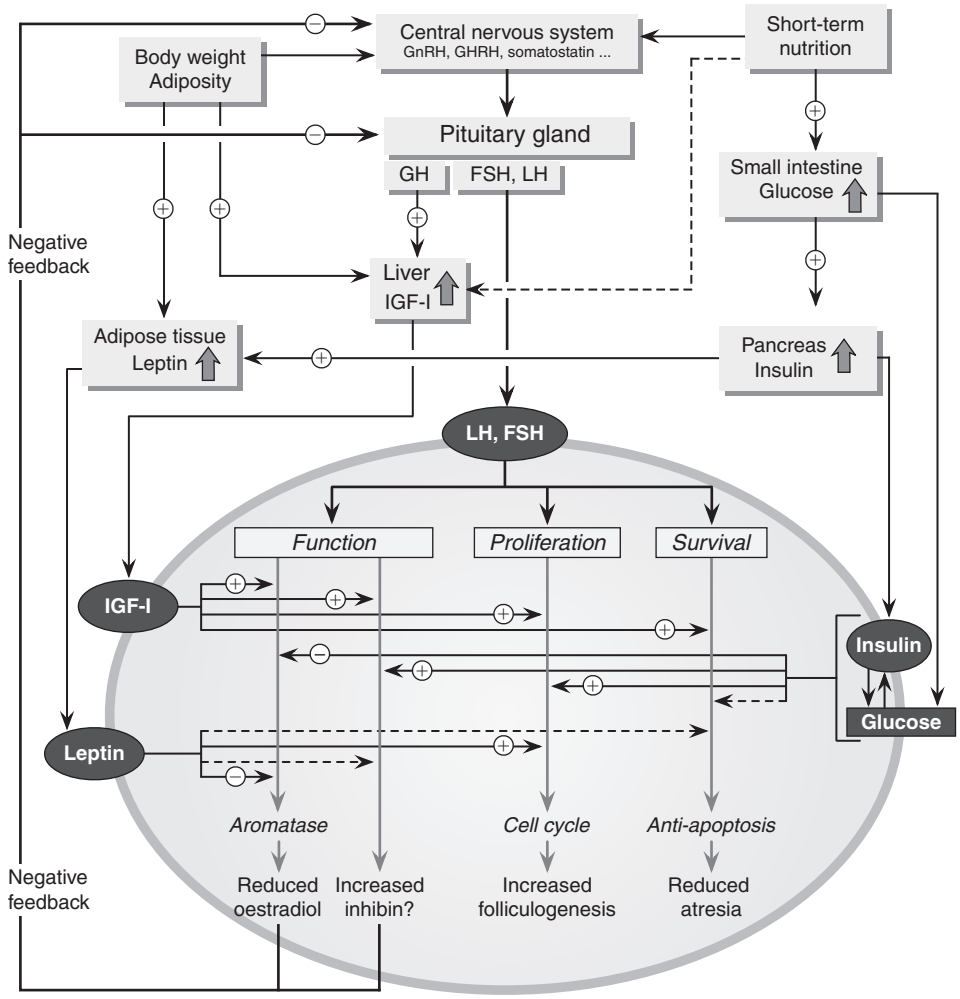

Fig. 6. A model for nutritional and metabolic inputs into folliculogenesis. Short-term nutritional influences are mediated primarily by glucose–insulin while the effect of bodyweight or adiposity is mediated by leptin and IGF. Short-term nutritional treatment that leads to the increased post-ruminal absorption of glucose stimulates folliculogenesis by direct intrafollicular effects of insulin or insulin-mediated glucose uptake; these inhibit oestradiol and probably stimulate inhibin. The feedback consequences of altered secretion of follicular hormones are unclear but they appear to have little effect on FSH secretion. Thus, increased folliculogenesis resulting from short-term nutrition may be the result of subtle changes in negative feedback or the result of direct follicular action of insulin or glucose. The effect of insulin may also be mediated via leptin. The effect of short-term nutrition on the IGF system is uncertain (dashed line). High bodyweight and adiposity lead to increased circulating IGF-I and leptin. The leptin inhibits oestradiol secretion and stimulates folliculogenesis but its effect on the follicular inhibin system is not known. The effect of GH folliculogenesis is probably mediated by IGF-I. Known intra-ovarian interactions are shown as solid lines while dashed lines indicate speculative interactions that have not been confirmed and may not exist. Some of the intracellular mediators for these interactions have been elucidated and are discussed in the text.

hypothalamus–pituitary–ovarian axis to affect the follicle and that the later stages of folliculogenesis have a primary requirement for LH and FSH (Webb and Campbell 2007). It is probable that nutritional influences on folliculogenesis involve both direct intra-follicular and negative feedback mechanisms (Scaramuzzi *et al.* 2006), but with a primary effect at the level of the follicle that evokes alterations in negative feedback.

Three effects of nutrition on folliculogenesis have been described. Increased bodyweight and higher body condition scores are both associated with increased folliculogenesis. The rate of increase of bodyweight is also associated with increased ovulation rate but this is a difficult experimental model to work with. At this point we assume without supporting data that its effect is also mediated by increased folliculogenesis; it is not included in the model. Short-term nutritional stimulation that does not affect bodyweight or adiposity also stimulates folliculogenesis in sheep and cattle without significant changes in peripheral gonadotrophin concentrations. This plethora of experimental models has led to many superficially comparable nutritional studies that are, in fact, difficult to compare because the experimental manipulation can involve short-term supplements, prolonged changes in body mass, static differences in body mass and fasting.

There are some important omissions in the model; these are the effect of nutrition on the oocyte and the effects of dietary polyunsaturated fatty acids (PUFAs) on folliculogenesis. These have been deliberately excluded either because of limited data or because of a need to impose limits on the extent of the review. Importantly, the follicle is the vehicle for the oocyte. There is increasing but limited evidence that nutrition affects the developmental potential of the oocyte and the subsequent survival of the embryo and may be a significant factor affecting the success of assisted reproduction. Thus, interesting questions arise: is the oocyte a direct target for nutrition and is the nutritional influence on folliculogenesis mediated by the oocyte?

Recently it has been suggested that, in ruminants, the composition of the diet is also important (Chagas *et al.* 2007) and two aspects of dietary composition appear to be noteworthy. The first is the variable ability of dietary carbohydrates to resist digestion in the rumen thus increasing post-ruminal absorption of glucose and of dietary PUFAs to avoid saturation in the anaerobic environment of the rumen. The second is the nature of the dietary PUFAs themselves (Abayasekara and Wathes 1999).

Conclusions, future directions and problems

Technical limitations

Several technical bottlenecks are slowing the advance of our understanding of folliculogenesis in ruminant species. First, there is a need for reliable biologically active reagents relevant to ruminant species, particularly inhibin, FSH and growth factors from the TGF-β superfamily (GDF9, BMP15). Second, we need improvements to some existing technologies; for example, imaging technology (e.g. to track follicle development and monitor follicular blood flow), micro-array technology and methods of genomic analysis (e.g. gene libraries for defining stages of follicular development and testing the value of the cattle genome for other ruminants) and proteomics specific to ruminants. Third, there is a need for improved *in vitro* systems for single follicles and ovarian cells. Technical breakthroughs in these areas will help us to overcome several conceptual barriers. Most attractive is the ultimate goal of being able to track the dynamic, functional history of a follicle in real time, either *in vitro* or *in vivo*. Implicit in this is the ability to study the processes that determine the rate of recruitment of primordial follicles.

Conclusions

We have only recently begun to appreciate how folliculogenesis is affected by the functional interactions between the oocyte and granulosa and cumulus cells. We need to further develop this field of study so we can address questions such as how the developing endocrine functions of the follicle are coordinated among the oocyte, cumulus and granulosa cells as well as between the granulosa and theca cells, why granulosa cells undergo apoptosis preferentially and why oocytes are protected during early atresia? Additionally we need to understand the internal workings of the follicle to explain, for example, the high degree of redundancy, the local diffusion of regulatory factors, the role of the extracellular matrix and the basal lamina in regulating diffusion (Irving-Rodgers and Rodgers 2006), the origin and role of the theca and its androgens and the role of TGF signalling (BMP, AMH).

Acknowledgements

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