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Domestic ruminants as models for the elucidation of the mechanisms controlling ovarian follicle development in humans

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It is necessary to understand the basic physiology underlying the complex process of folliculogenesis to address common causes of infertility and to devise innovative strategies to increase the efficiency of assisted reproduction technologies. Availability of suitable ovarian tissue is a major constraint to research in this area in humans, and monovulatory domestic ruminants represent a physiologically relevant model to elucidate basic mechanisms before more focused clinical investigations. This paper reviews the development of several whole animal and cell culture models in ruminants that have allowed basic investigations into the endocrine and local mechanisms regulating preantral and antral follicle development in monovulatory species. Studies on preantral follicle development using the ovarian autograft model have shown, contrary to accepted dogma, that FSH may mediate the rate at which preantral follicles grow and have provided evidence to support the existence of local regulatory feedback mechanisms that influence the rate of primordial follicle initiation and preantral follicle development. Studies on the endocrine control of antral follicle development using the GnRH-antagonist model have shown that a pulsatile mode of LH delivery is not a requirement for normal patterns of follicle development and ovarian hormone secretion. Studies on the local control of somatic cell differentiation using physiological cell culture models have highlighted the essential relationship between somatic cell communication and expression of differentiative markers. We conclude that the domestic ruminant represents a valuable model system for the elucidation of the endocrine and local mechanisms controlling both early and terminal stages of follicle development in monovulatory

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species. The results of these investigations have direct strategic relevance within clinical medicine.

Introduction

Infertility is a common and major problem in both clinical medicine and animal production. In western countries, one in seven couples will seek medical advice for infertility and infertility affects 8–10% of married or cohabiting couples (Templeton *et al*., 1998). Although infertility does not lead to mortality, the morbidity of infertility includes anxiety, depression, sadness and isolation. The main treatments available to overcome infertility are ovulation induction for women with anovulatory infertility and IVF for couples with male factor (25–32%), unexplained (25%) or female factor infertility (45–50%). At present, most of the hormonal treatments applied to induce ovulation are imprecise and, despite careful monitoring, often lead to under- or over-stimulation of the ovary. IVF still has disappointingly low success rates (mean 17%, range 0–28%) even though its use has increased tenfold in the last decade. Many of the problems associated with assisted reproductive technologies can be attributed to our basic lack of understanding of the physiological process that controls ovarian follicle development and leads to the development of a mature oocyte, within a follicle, that has full developmental potential (Templeton *et al*., 1998).

Ovarian research in humans has been hampered by the shortage of normal tissue from women of reproductive age that has not been subjected to large doses of gonadotrophins *in vivo*. Primates, the closest animal species to humans, can be polyovular, are expensive to keep, their use ethically sensitive, are difficult to handle for experiments involving endocrinological manipulations and monitoring, and yield relatively small amounts of tissue. Laboratory rodents are poor models for studying human ovarian physiology due to their polyovular nature and their small size makes detailed endocrinological and morphological (by ultrasonography) studies difficult. However, large domestic ruminants are predominantly monovular, plentiful, relatively cheap (or have significant sell-on value), require low-grade housing and care, are docile and easily handled and are sufficiently large to allow detailed endocrinological and morphological (via ultrasonography) studies. Although cows, which are almost exclusively monovular, bear the greatest similarity to humans in terms of ovarian physiology (Table 1), the use of sheep as experimental models has the added advantage that the physiological mechanisms that regulate follicle selection involve integration of environmental inputs (nutrition, photoperiod) within a genetically determined range of ovulations.

At present, the mechanisms regulating both early follicle and oocyte development and the recruitment and selection of ovulatory follicles remain obscure and our lack of understanding of these processes prevent the development of more precise ways to induce single or multiple ovulation and the development of new technologies which will allow immature follicles, and the eggs they contain, to be collected, matured and fertilized in the laboratory. Over the last decade, *in vivo* and *in vitro* ruminant experimental models have been developed to allow elucidation of the endocrine and local mechanisms controlling both the preantral and antral stages of follicle development in monovulatory species (Campbell *et al*., 1995; Webb *et al*., 1999) and clinically relevant findings from recent experiments using these model systems are the subject of this review.

Folliculogenesis in monovulatory species

Ovarian follicular growth is a developmental process during which the follicle, and the oocyte it contains, progressively acquire a number of properties at the correct time and

Table 1. Reproductive characteristics of humans and domestic ruminants

Characteristic	Ewes	Cows	Women
Ovulations per cycle	1–3	1	1
Duration of folliculogenesis (months)	6	6	6
Time until antrum formation (months)	4.3	4	4
Size of antrum formation (mm)	0.2	0.2	0.2
Diameter becomes gonadotrophin dependent (mm)	2–3	3–4	3–4
Diameter of granulosa cell LH receptors (mm)	3.5	9–10	8–10
Diameter of ovulatory follicle (mm)	5–7	16–22	19–25
Ovulatory cycle (days)	15–18	19–24	26–29
Follicular phase (days)	2–3	2–3	14
Luteal phase (days)	14	16–19	14
Duration of gestation (days)	142–148	280	280

sequence, each of which is an essential prerequisite for further development. Therefore, follicular development is a continuous process, and at any time the ovaries contain many follicles, many of them morphologically indistinct, at different stages of development and atresia. The mechanisms regulating folliculogenesis are complex and have been the subject of intense experimental investigation for the last 25 years. Most of this time, the focus of this work has revolved around the mechanism controlling antral follicle development in recognition of the strategic importance of anovulatory infertility (for example, polycystic ovary syndrome) and controlled ovarian stimulation. In recent years, early follicle development has come under increasing investigation with the realization that the development of methods that would allow acquisition of developmentally competent oocytes from preantral follicles would represent a major advance in assisted reproductive technologies.

Early folliculogenesis

Over recent years interest in early follicle development has grown due to the realization that (i) primordial follicles represent a vast store of oocytes that could be used for fertility treatment if systems can be developed to overcome the fact that 99.9% of these oocytes are lost to atresia; (ii) the immature oocytes contained within primordial follicles remain viable after cryopreservation whereas more mature oocytes from antral follicles have low developmental competence when frozen, thus presenting the possibility that reproductive function could be restored to individuals with depleted follicle populations due to ageing or cancer treatment; and (iii) the decline in fertility as women age is due to both a quantitative and qualitative decline in oocyte quality and part of the aetiology of this change in oocyte quality may be related to the endocrine changes that accompany depletion of the follicular reserves.

The primary limitation of studying early follicle development in large mammals has been the need to rely on observational rather than experimental data. That is, there have been no *in vivo* and *in vitro* models to allow for the testing of hypotheses and workers have had to rely on observations of the patterns of expression of known local regulators, or their receptors, on follicles at different stages of development, as an indirect means of identifying key regulatory factors (McNatty *et al*., 1999). The picture that has emerged from this work is that early folliculogenesis is controlled by complex interactions between locally produced growth factors that include members of the transforming growth factor β (TGFβ) and insulin-like growth factor (IGF) superfamilies, in a poorly understood intrafollicular cascade.

The role of gonadotrophins during early folliculogenesis is uncertain, as although early follicles acquire FSH receptors quite soon after initiation (McNatty *et al*., 1999), early follicle development is not impaired in hypogonadotrophic individuals (Dufour *et al*., 1979). We have recently used the sheep autograft model to examine the role of gonadotrophins in controlling early folliculogenesis. The cryopreservation and subsequent autografting of patches of ovarian cortical tissue has been developed as a technique to restore fertility in young girls undergoing chemo- or radiotherapy for cancer, treatments that have a high risk of destroying the primordial germ cells in the ovary (Gosden *et al*., 1994). Experimental investigations associated with these studies in sheep have revealed that the ischaemia that occurs during graft revascularization results in the death of all follicles except quiescent primordial and primary follicles and that it takes several months for these follicles to grow to an ovulatory size (Baird *et al*., 1999). This synchronization of early follicle development provides an ideal model to study the initiation of early follicle development and the interactions between the oocyte and somatic cells that control this poorly understood phase of follicle development. As only a small portion of ovary is grafted back into an animal with this technique, it results in a marked depletion in the ovarian follicular population which leads, in turn, to a decrease in the ovarian secretion of inhibin A and a tenfold increase in circulating FSH concentrations (Baird *et al*., 1999; Campbell *et al*., 2000). The experimental paradigm used in these studies involved preventing this increase in FSH using a GnRH-agonist in combination with small oestradiol implants to induce a hypogonadotrophic environment. Ovarian tissue was then recovered at 1, 2, 3 and 4 months after autografting.

The combined agonist and oestradiol treatment was highly effective in preventing the normal increase in FSH observed in autografts with untreated animals having much higher (8.5 ± 0.2 μg l^{-1}; hypergonadotrophic) FSH concentrations compared with treated animals (0.57 ± 0.02 μg l^{-1}; hypogonadotrophic). This difference in FSH resulted in a marked difference in the morphological appearance of the autograft upon retrieval after 3–4 months. Grafts from the hypergonadotrophic group were greatly enlarged and contained numerous small and large antral follicles, whereas grafts from the hypogonadotrophic group remained small and contained no visible antral follicles. Histological examination of the grafts showed that the proportion of ovarian follicles within different size categories had returned to normal as early as 2 months after grafting in hypergonadotrophic animals, but in animals with hypogonadotrophic autografts follicle development beyond the secondary stage of development was absent at 2 months (Fig. 1) and the follicle population did not return to normal in these animals until 4 months after grafting. Therefore, these observations indicate that although FSH may not be essential for the growth of preantral follicles *per se*, it may play a role in regulating the rate of early folliculogenesis. This interpretation is supported by the observations that the proportion of granulosa cells staining for the mitotic marker proliferating cell nuclear antigen (PCNA) is increased in follicles from hypergonadotrophic animals (Campbell *et al*., 2000) and that the graft regeneration in normogonadotrophic autografts is intermediate between hyper- and hypogonadotrophic animals (B. K. Campbell, C. Souza and D. T. Baird, unpublished). This concept has practical implications for the development of culture systems for preantral follicles, as they indicate that the inclusion of FSH at doses of up to ten times the physiological dose *in vitro* might be beneficial in accelerating the rate of preantral follicle development.

Although it is clear from these data that FSH does not affect the rate of primordial follicle initiation or the number of primary and secondary follicles that develop, there was an increase in the number of primary follicles in autografts relative to time 0 controls (Fig. 1), indicating that the rate of primordial follicle initiation is increased in these animals. As the follicular hierarchy has been destroyed in the autografts, this observation supports the hypothesis that the rate of initiation is controlled by factors secreted by more mature growing follicles. This observation

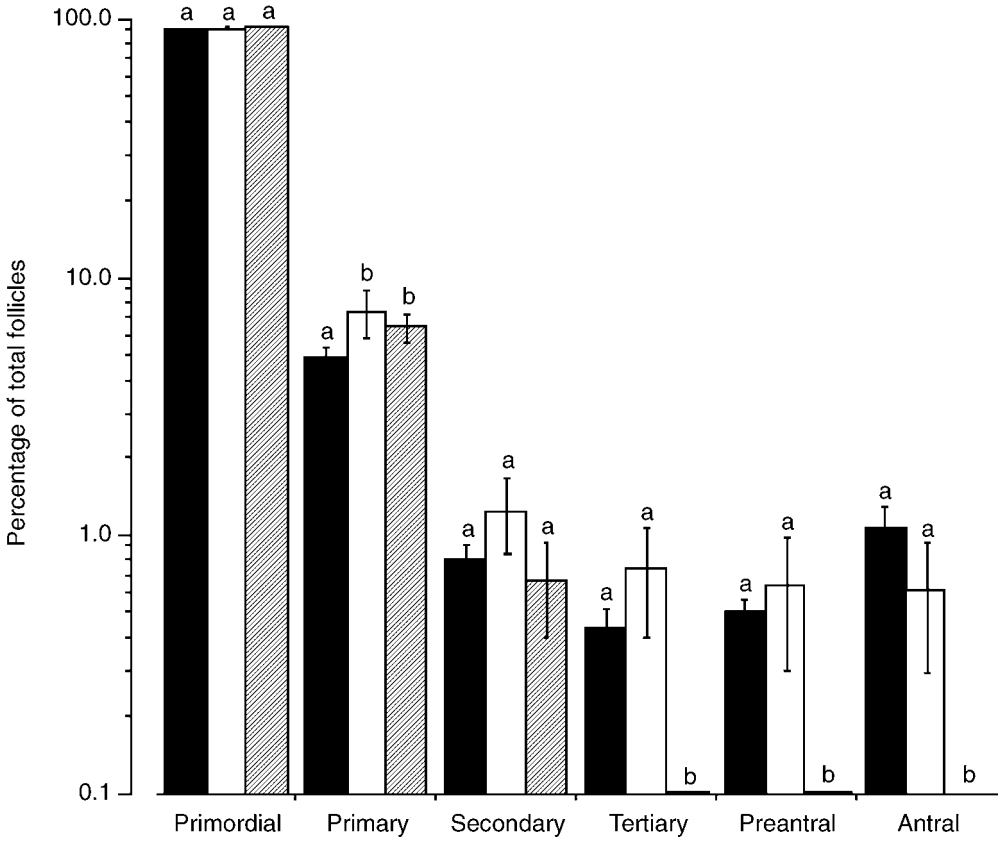

Fig. 1. Percentage of total follicles per size class in ewes with ovarian cortical autografts that either did not receive treatment (hypergonadotrophic, □) or received treatment with GnRH-agonist and oestradiol to render the animals hypogonadotrophic (▨). The percentage of follicles in both groups in ovarian tissue recovered before grafting (t = 0, ■). Data for tissue recovered after 1 and 2 months have been combined. Within follicle class, columns with different letters are significantly different ($P < 0.05$).

has been supported by more recent experiments that focused on the period immediately after grafting to show a marked but transient increase in the rate of initiation of primordial follicle development in ovarian cortical strips recovered 2 weeks after transplantation (D. T. Baird, E. E. Telfer, C. Souza and B. K. Campbell, unpublished). A similar increase in the rate of initiation is also observed in cultured cortical strips (Picton *et al.*, this supplement). These observations all support the existence of a gonadotrophin-independent intraovarian feedback loop regulating both the rate of primordial follicle initiation and the rate of early follicle development. This hypothesis explains the common observation in many species that the rate of initiation is inversely correlated with the size of the growing pool of follicles (Peters, 1978; Driancourt, 1987).

The identity of this putative factor is unknown, but one strong candidate is activin. In sheep, expression of inhibin βA subunit protein is observed in all follicle size classes from the primary stages onwards, whereas mRNA encoding inhibin βA subunit is first observed in early antral follicles (McNatty *et al.*, 1999). Observation of the pattern of expression for inhibin βA subunit protein in small antral follicles has shown that diffuse expression is

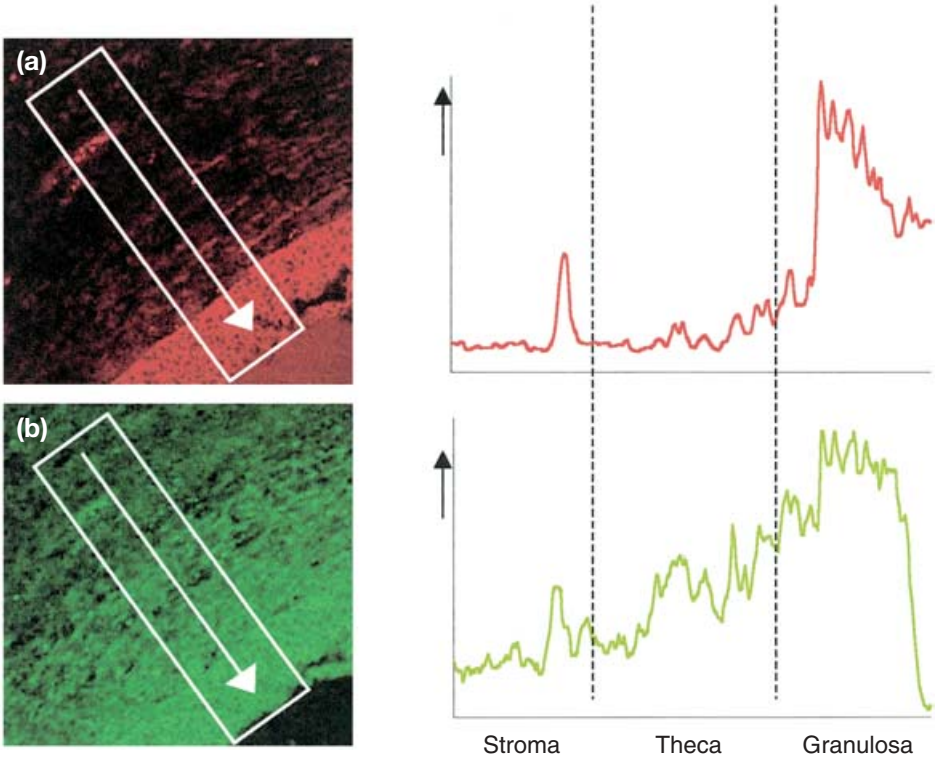

Fig. 2. Expression of inhibin subunit protein across the wall of a sheep antral follicle. Expression of (a) inhibin α subunit is confined to the granulosa cells, whereas (b) inhibin β A subunit exhibits intense staining in the granulosa cell layer with declining expression in the theca and stroma cell layer indicating diffusion from the granulosa cell layer. (A. J. Mitchell, C. J. H. Souza, B. K. Campbell, N. P. Groome and D. T. Baird, unpublished)

observed in the thecal cell layer in the absence of mRNA expression, indicating diffusion of inhibin–activin from these larger follicles (Fig. 2). Importantly, no such diffusion was observed in the related protein, Müllerian stimulating hormone (A. J. Mitchell, C. J. H. Souza, B. K. Campbell, N. P. Groome and D. T. Baird, unpublished). However, further studies using physiological culture systems for preantral follicles and cortical strips are required to prove a role for activin in mediating the rate of early follicle development in monovulatory species. Elucidation of these mechanisms has immense clinical significance in terms of development of systems for *in vitro* growth of preantral follicles (Picton *et al*., this supplement) and the development of therapies for delaying the menopause in women.

Overall, these data provide strong evidence that FSH, contrary to accepted dogma, can affect the development of preantral follicles in a large monovulatory species with a prolonged period of preantral follicle development. The data generated so far from these studies would appear to indicate that FSH may be affecting the rate at which preantral follicle development proceeds rather than acting as a permissive factor. Such a hypothesis is consistent with the known action of FSH in stimulating proliferation of undifferentiated granulosa cells (Richards *et al*., 1995; Campbell *et al*., 1996) and the observation that antral follicles can be observed in hypogonadotrophic animals (Wang and Greenwald, 1993). However, further studies are needed to determine the point at which the rate of follicle development deviates in these hypo- and hypergonadotrophic individuals.

Terminal follicle development

In humans and domestic ruminants, such as sheep and cows, each reproductive cycle is characterized by an extraordinary process in which one or two follicles are selected from a morphologically identical cohort of 10–20 small antral follicles, exposed to the same endocrine environment, to become dominant (that is actively suppress the development of the cohort) and ovulate a fertilizable oocyte. The mechanism of this selection process is still not fully understood, but the clinical importance of this area is that: (i) stimulation regimens designed to induce multiple follicle development in ART programmes involve the circumvention of these selection mechanisms; (ii) to avoid the induction of multiple births the stimulation of ovulation in anovular women requires the artificial recreation of these selection mechanisms, and (iii) the major cause of anovulatory infertility in women is the polycystic ovary syndrome which is characterized by a failure in the normal mechanisms of ovulatory follicle selection and dominance (Templeton *et al*., 1998).

The mechanism of follicle selection has been the focus of our research in Edinburgh for many years (Baird and McNeilly, 1981; Baird *et al*., 1983, 1991; Campbell *et al*., 1995; Baird and Campbell, 1998; Webb *et al*., 1999). Accordingly, this section of the manuscript will summarize the major mechanisms regulating follicle selection and identify gaps in our knowledge that require further experimentation.

Gonadotrophic control of follicle selection

The development of antral follicles to diameters beyond 2–4 mm in humans and large domestic ruminants is entirely dependent on the pituitary gonadotrophins LH and FSH, and these hormones provide the primary mechanisms that control follicle recruitment, selection and dominance via negative inhibitory feedback loops with the hypothalamo–pituitary unit. FSH is the main hormone controlling follicle growth and its secretion is in turn controlled via the main secretory products of a large dominant follicle, oestradiol and inhibin A. In contrast to the human corpus luteum, the corpus luteum in sheep and cattle does not secrete either oestradiol or inhibin and the feedback loop between the ovary and the brain is open during the luteal phase and during periods of seasonal anoestrus (sheep). The result is that follicle development is characterized by a series of waves of follicle development that are preceded by peaks of FSH secretion (Souza *et al*., 1996, 1997a–c; Webb *et al*., 1999). These fluctuations are controlled by the reciprocal secretion of oestradiol and inhibin A by the dominant follicle (Souza *et al*., 1997a–c).

When examining domestic ruminants as possible models for humans, cows have many similarities in ovarian and reproductive physiology (Table 1), but one of the fundamental differences is that the follicular phase is much shorter in cows (3 days) than in humans (14 days). The reason for this has long been thought to be due to the fact that concentrations of FSH in the luteal phase are suppressed in humans by secretions of the corpus luteum (oestradiol and inhibin A), whereas in cows, as discussed above, the corpus luteum secretes only progesterone with the result that FSH concentrations remain sufficiently high to allow the development of large antral follicles throughout the ovarian cycle. This hypothesis was tested by prolonged suppression of FSH in cattle by combined GnRH-agonist, oestradiol and inhibin treatment followed by FSH infusion to mimic the intercycle day 3 FSH peak in humans. The treatment resulted in a significant increase in the time of follicle recruitment and the development of a single dominant follicle in a manner that was highly analogous to the human follicular phase (Fig. 3). Thus, this modified cow model provides an ideal non-primate model to study the control of terminal follicle development in humans.

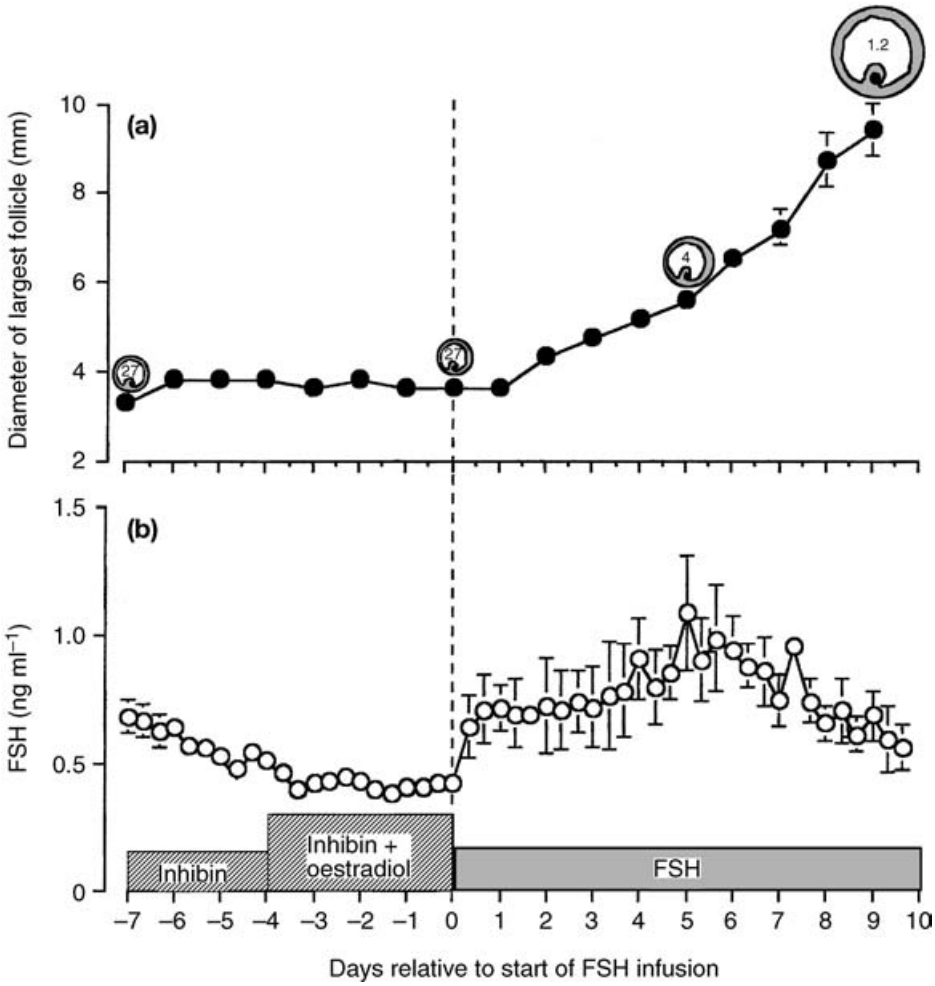

Fig. 3. Response of profoundly suppressed cows to FSH treatment. (b) Mean (± SEM) FSH concentrations in six heifers pre-treated with GnRH agonist (buserelin) for 6 weeks followed by inhibin (10 ml bovine follicular fluid in 8 h) for 3 days (days −7 to −4) and inhibin plus oestradiol (1 mg oestradiol benzoate in 8 h) for a further 4 days (days −4 to 0) before stimulation with low dose FSH (2.5 μg oFSH-17 h^{-1}) for 10 days. (a) Ovarian response to treatment in terms of the mean (± SEM) diameter of the largest follicle and the mean number of antral follicles within 2 mm of the diameter of the lead follicle (number within follicle representation). Further suppression with inhibin and oestradiol had no effect on the apparent antral follicle population, but depressed FSH to below 0.5 ng ml^{-1}. FSH infusion resulted in a gradual increase in FSH concentrations that was accompanied by recruitment of only four follicles of 4–6 mm in diameter per cow after 5 days of treatment and just 1.2 follicles of 8–10 mm in diameter per cow after 10 days of treatment. This compared with 14 follicles of > 15 mm in diameter after receiving the same FSH treatment in controls that were suppressed by GnRH-agonist alone.

Role of LH during preovulatory follicle development

Although LH is thought of as primarily a steroidogenic hormone it can also influence folliculogenesis via indirect and direct mechanisms. Acting indirectly through LH receptors on thecal cells, LH mediates androgen substrate supply for oestradiol secretion and, hence,

FSH secretion from the pituitary gland (Campbell *et al*., 1999). Conversely, as oestradiol induces the formation of LH receptors on the granulosa cells of a dominant follicle, LH may mediate oestradiol secretion and growth of the preovulatory follicle directly via these receptors. Furthermore, surge concentrations of LH provide the physiological signal for germinal vesicle breakdown and progression of meiosis to metaphase II in the oocyte before ovulation although the intrafollicular mechanisms underlying this action are obscure (Mattioli and Barboni, 2000).

Conceptually, all ovarian stimulation protocols work on the principle of artificially increasing circulating concentrations of FSH to above threshold concentration for a protracted period of time by administering gonadotrophin preparations that contain various concentrations of FSH and LH. This treatment, depending on the dose and duration, recruits a variable proportion of the gonadotrophin-responsive follicles present in the ovaries to an ovulatory stage and the oocytes within these follicles can either be retrieved by ultrasound guided oocyte pick-up before ovulation or flushed from the oviducts after ovulation. Commonly, an exogenous ovulatory stimulus (GnRH, hCG) is applied to synchronize final maturation to allow for a convenient timing of oocyte collection. Despite our knowledge of the gonadotrophic control of ovulatory follicle development across all species, induction of multiple follicle development has two main problems: (i) large variation in the ovarian response and (ii) poor oocyte and embryo quality leading to poor conception rates after transfer (Fauser and Van Heusden, 1997; Driancourt, 2001; Thatcher *et al*., 2001). To some extent, the ovarian response is a function of the number of gonadotrophin-responsive follicles available for stimulation, but it is likely that poor oocyte quality results from deficiences in the gonadotrophic stimulation regimen leading to an inappropriate and unphysiological endocrine environment. As detailed above, LH plays a crucial role during normal folliculogenesis and oocyte maturation, but there has been an increased tendency in both animal science and clinical medicine to design stimulation regimens with highly purified FSH as these systems apparently yield oocytes that result in higher conception rates. However, in both of these situations, endogenous LH is either unsuppressed (animal) or incompletely suppressed (human with GnRH-agonists) and it is possible that these apparently negative effects of exogenous LH are indirect, mediated through ovarian steroids acting within the follicle or on the uterus to effect gamete transport or uterine receptivity. We have used ewes with ovarian autotransplants, often in combination with GnRH-analogue suppression models to control the pattern of gonadotrophic stimulation applied to the ovary, to examine the role of LH during normal and stimulated preovulatory follicle development.

Unlike FSH, LH alone will not stimulate follicular development from the gonadotrophin-responsive phases of follicle development (1–2 mm in sheep: McNeilly *et al*., 1991) but, depending on the intensity of FSH stimulation, LH pulses can partially inhibit the stimulatory action of FSH on follicular growth (McNeilly *et al*., 1991). Furthermore, gonadotrophin-dependent follicles can transfer gonadotrophin dependence from FSH to LH and this is thought to be one of the key mechanisms of follicle selection (Campbell *et al*., 1999; Webb *et al*., this supplement). Once ovulatory follicles have become LH dependent, withdrawal of LH support will result in rapid atresia (Dobson *et al*., 1997).

If FSH concentrations are maintained at values above threshold in GnRH-antagonist suppressed ewes, physiological regimens of pulsatile LH stimulation have little effect on either the number of ovulatory follicles that grow in response to FSH (Campbell *et al*., 1998a) or the number of follicles that ovulate in response to an exogenous ovulatory stimulus (Campbell *et al*., 1999). However, consistent with its steroidogenic role, LH has a major effect on ovarian oestradiol secretion and animals that receive pure FSH only are markedly hyposteroidal (Campbell *et al*., 1998a, 1999), whereas LH in combination with FSH resulted in a

hypersteroidal endocrine environment. Therefore, these results indicate that many of the adverse effects of ovarian stimulation regimens may be mediated through ovarian steroids acting within the follicle or on the uterus to effect gamete transport or uterine receptivity. This idea is supported by recent data from humans as the use of human recombinant FSH is contraindicated in patients that are hypogonadotrophic or also receiving GnRH-antagonists, which can profoundly suppress LH (Balen, 1998). Furthermore, data from sheep has shown that suppression of endogenous LH with GnRH-antagonists depresses the developmental potential of embryos derived from these animals, an effect that was reversed by exogenous LH provided in a physiological manner (Oussaid *et al*., 1999).

LH is secreted physiologically as a series of pulses and both pulse amplitude and frequency vary according to the stage of the oestrous cycle in response to changes in ovarian steroid secretion. A variation in the antagonist model has been used to examine the role of the pattern of LH stimulation on ovarian steroid secretion and ovulatory follicle development. Ewes with ovarian autotransplants were treated with GnRH antagonist at the time of induction of luteal regression and received either no LH, pulsatile LH designed to mimic the normal follicular phase, which consisted of injection (i.v.) of 2.5 μg oLH-26 at 2 h intervals for 12 h followed by 1.25 μg oLH-26 at 1 h intervals for the next 48 h ($n=8$) or a constant infusion of the same dose of LH (1.25 μg h^{-1} i.v.) for 60 h ($n=8$). After 60 h, an ovulatory stimulus was applied in the form of 100 μg LH and 500 iu hCG. The ovarian response to these treatments was assessed each day by ultrasonography and determination of the ovarian secretion of oestradiol and inhibin A. In sheep that did not receive LH, FSH concentrations remained constant throughout the 'follicular phase' and although the large antral follicles present at the time of luteal regression persisted throughout this period they did not ovulate. The ovarian secretion of oestradiol was undetectable in these animals (Fig. 4) but there was a constant baseline of inhibin A secretion. Animals stimulated with either pulsatile or constant LH had normal patterns of oestradiol, inhibin A and FSH secretion in the follicular phase with a marked increase in oestradiol (seven- to tenfold; Fig. 4; $P<0.001$) and inhibin A (two- to fourfold, $P<0.01$) and a concomitant decline (40%, $P<0.01$) in FSH. Furthermore, these animals all ovulated the normal number (one to two) of follicles in response to LH–hCG. Overall, ovarian secretion of oestradiol was higher ($P<0.05$) in animals that received constant LH when compared with pulsatile LH, but there were no differences in inhibin A and FSH concentrations between these two groups. These data show that although LH is required for normal ovulatory follicle development and induction of the follicular phase depression in FSH, a pulsatile mode of LH delivery is not a requirement for normal patterns of follicle development and ovarian hormone secretion. Therefore, more research is needed to define the role of LH within stimulation regimens to develop protocols that will lead to more controlled responses yielding higher quality oocytes and embryos.

In vitro models to study gonadotrophin-induced somatic cell differentiation

The differential expression of local factors that modulate the actions of gonadotrophins provides the mechanism whereby individual follicles respond in different ways to the same endocrine stimulus (Campbell *et al*., 1995; Webb *et al*., 1999). The orderly expression of these factors at the correct time and in the correct sequence has been termed the intrafollicular cascade and the elucidation of this cascade is a key research priority. Physiological serum-free cell culture systems have been developed in which the problem of spontaneous luteinization has been largely overcome (Campbell *et al*., 1996, 1998b; Gutierrez *et al*., 1997; Picton *et al*., 1999) and these systems allow differentiation *in vitro* in response to physiological doses of gonadotrophins in a manner which closely mimics responses to similar stimuli

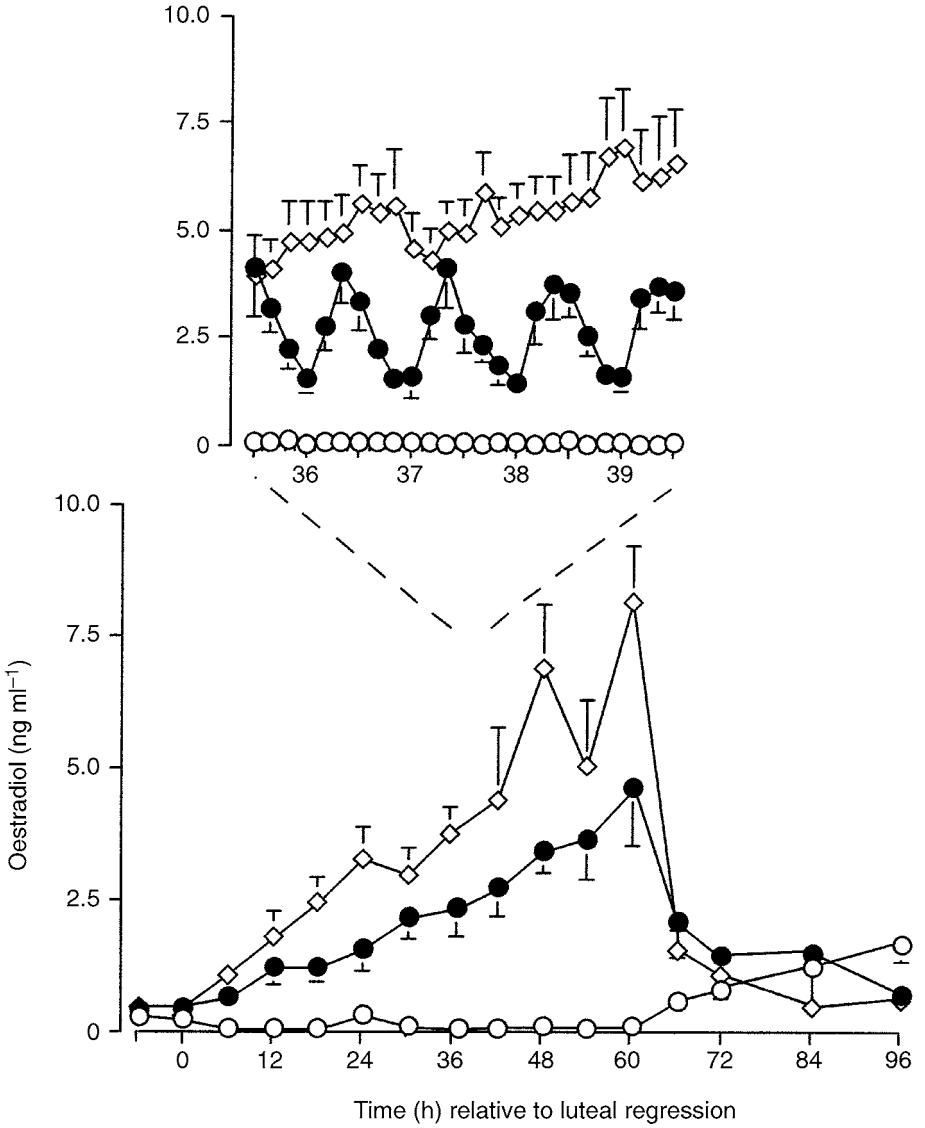

Fig. 4. Ovarian secretion of oestradiol (mean ± SEM) by ewes treated with GnRH antagonist at the time of luteal regression and subsequently stimulated with either no LH (○), pulsatile LH in a physiological regimen (1.25 μg oLH-s26 h^{-1}; ●) or the same dose of LH infused constantly (◇). Insert shows pattern of oestradiol secretion generated between 35.8 and 39.3 h after luteal regression estimated by blood samples collected at 10 min intervals. All ewes received hCG as an ovulatory stimulus 60 h after luteal regression.

in vivo. The use of these culture systems allows investigation into the intrafollicular response to specific gonadotrophic stimulation and the intrafollicular paracrine and autocrine dialogue between follicular cells and the oocyte. Recent experiments have related temporal changes in the expression of mRNA encoding receptors for FSH receptor (FSHr) and the IGF type 1 receptor (IGF1r) to the enzymic activity and expression of cytochrome P450 aromatase, the enzyme responsible for oestradiol production, during FSH-induced differentiation of granulosa

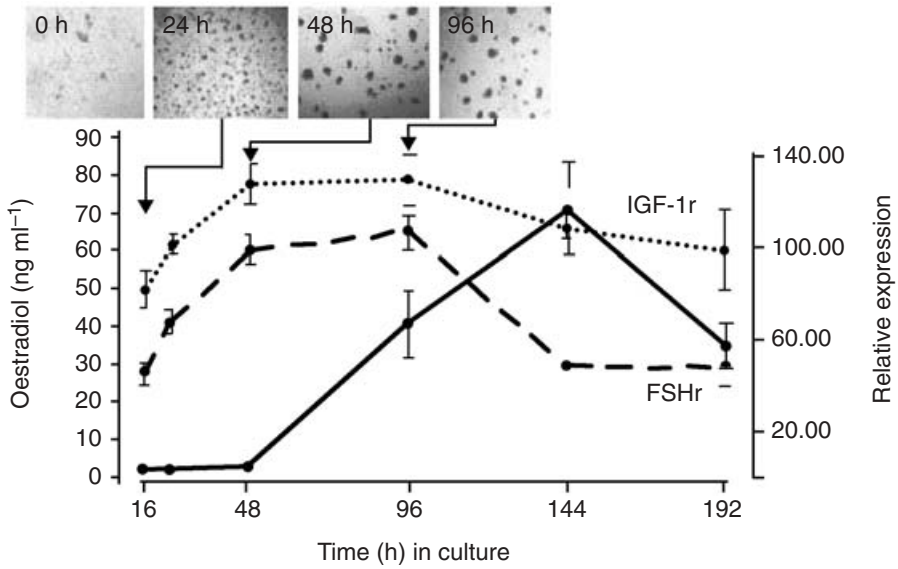

Fig. 5. Temporal relationships between FSH receptor (FSHr) (- - - - -) and insulin-like growth factor 1 receptor (IGF-1r) (· · · · · ·) receptor expression (expressed relative to freshly isolated granulosa cells = 100) and oestradiol production (——) by bovine granulosa cells from small antral follicles cultured under optimum conditions to induce cellular differentiation. The top panels show changes in cellular morphology during initial stages of culture. Note the increase in receptor expression before induction of aromatase activity and the relationship between re-initiation of intercellular communication and functional status; that is receptor expression is re-initiated when cells have regrouped from a dispersed monolayer ($t = 0$) to form characteristic interconnected clumps (24–48 h). (Adapted from Marsters *et al*., 2003.)

cells from small (< 4 mm) bovine follicles (Marsters *et al*., 2003). The results (Fig. 5) show a marked decline in gene expression for all three differentiative markers during the early stages of culture, when the cells had been dissociated into a dispersed monolayer, followed by the sequential re-initiation of gene expression for these markers that was temporally related to both the re-initiation of intercellular communication as the cells form clumps and the induction of steroidogenic activity. Overall, the results indicate a relationship between granulosa cell morphology and gene expression with the cells reverting to an immature phenotype during the early stages of culture followed by rapid *in vitro* differentiation that appears to parallel FSH-induced follicular differentiation *in vivo*.

The observed relationship between re-initiation of intercellular communication and differentiation explains the observation that cellular plating density is a critical determinant of the ability of gonadotrophins to induce somatic cell differentiation *in vitro* (Campbell *et al*., 1996, 1998b). Further evidence for the importance of cellular morphology comes from experiments in which exposure of granulosa cells to the copper chelator, thiomolybdate, results in a dose responsive decrease in oestradiol production concomitant with a dispersed cellular phenotype in culture (Kendall *et al*., 2003). The possible explanation for these observations is that copper is an essential component of enzymes, such as lysyl oxidase, that control formation of extracellular matrix proteins. Examination of the patterns of expression of the extracellular matrix proteins collagen type IV, laminin and fibronectin showed abundant expression of all three proteins in granulosa and theca cells, both *in vivo* within antral follicles and *in vitro*

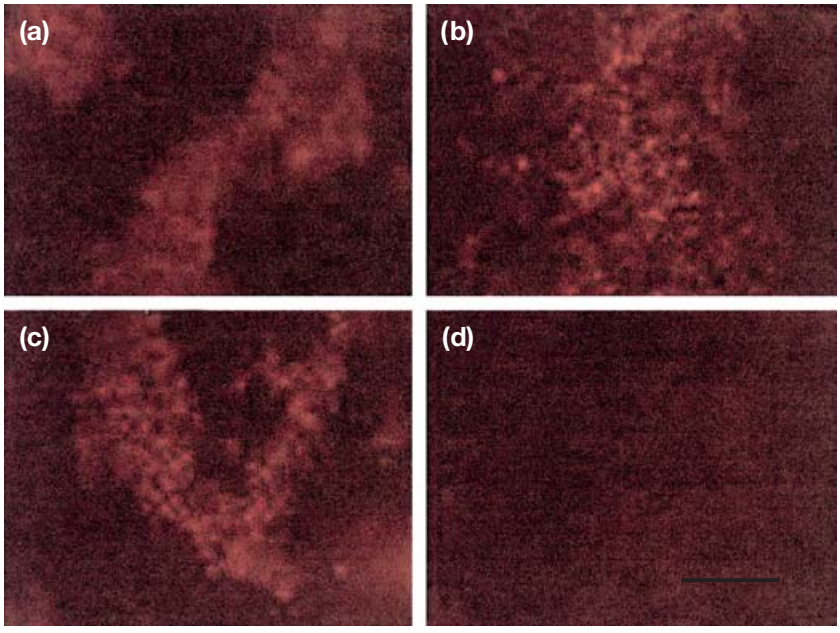

Fig. 6. Expression of connexin 43 protein in bovine granulosa cells cultured in serum-free media in the presence of increasing doses of oFSH ((a) no FSH; (b) 0.1 ng FSH ml^{-1}; (c) 1.0 ng FSH ml^{-1}) as described by Campbell *et al*. (1996) and Guitierrez *et al*. (1997) or (d) in the presence of 1% fetal calf serum for 144 h. Granulosa cells were plated at a density of 350 000 cells per well in Labtek culture slides and fixed with methanol after 144 h of culture. The primary antibody used was mouse monoclonal anti-connexin 43 (Chemicon) and protein was visualized by Texas red labelled second antibody (Jackson Immunoresearch Ins). Granulosa cells have assumed their characteristic clumped phenotype when cultured in this serum-free system and FSH has stimulated connexin 43 in a dose responsive manner. No expression was evident when cells were cultured in the presence of serum, in which the cells appear as a confluent fibroblastic monolayer. Scale bar represents 50 μm.

around granulosa and theca cells maintained in serum-free culture (B. K. Campbell and G. Robinson, unpublished). However, a causal effect of thiomolybdate on extracellular matrix protein expression has not yet been established.

Extensive intercellular bridges or gap junctions have been observed between granulosa cells in Graafian follicles (rat: Amsterdam *et al*., 1976; sheep: Hay and Moor, 1975). These junctions represent a means of intercellular communication for metabolic exchange and transport of molecules, such as cAMP (Schultz, 1985), and it has been suggested that the membrana granulosa can be considered as a 'functional syncytium' (Merk *et al*., 1972). Therefore, it can be hypothesized that the observed relationship between intercellular communication and somatic cell differentiation is most likely mediated through the loss and formation of gap junctions between somatic cells during the early stages of culture. Recent studies examining the expression of the gap junction protein connexin 43 in cultured bovine granulosa cells support this hypothesis, with abundant expression being detected within clumps of cells exposed to doses of FSH known to induce cellular differentiation in serum-free culture (Fig. 6). Importantly, expression of connexin 43 was not observed in luteinized granulosa cells cultured in the presence of serum that had assumed a characteristic confluent fibroblastic monolayer *in vitro*. Together, these results confirm that cellular morphology in terms of expression of extracellular

matix proteins and gap junctions represent an important component of the intrafollicular cascade of events that occur during gonadotrophin-induced development and growth of ovarian follicles which ultimately regulate follicular recruitment, selection and dominance in monovulatory species.

Conclusions

It is necessary to understand the basic physiology underlying the complex process of folliculogenesis in order to address common causes of infertility and to devise innovative new strategies to increase the efficiency of assisted reproduction technologies. The domestic ruminant represents a valuable model system for the elucidation of the endocrine and local mechanisms controlling both early and terminal stages of follicle development in monovulatory species. The results of these investigations have direct strategic relevance within clinical medicine but much more research is needed to allow elucidation of the complex endocrine, paracrine and autocrine mechanisms that regulate ovarian follicle and oocyte development.

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