Declaration

The composition of this thesis and the experiments described within were the unaided work of the author, except where acknowledgment is made by reference. No part of this work has previously been accepted for any other degree, nor is any part of it being submitted concurrently in candidature for another degree.

Christopher David Smyth
October, 1993
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Publications

ABSTRACTS:


**BOOK CHAPTERS:**


**PEER-REVIEWED RESEARCH PAPERS:**


Presentations


Abstract

Follicular development requires both proliferation and differentiation of the cellular compartments of the maturing follicle. The role of gonadotrophins, FSH and LH, is well accepted, but follicles exposed to the same gonadotrophic stimulation undergo differing fates suggesting that gonadotrophin action is subject to intraovarian modulation. Local regulators may modulate proliferation or differentiation of a developing follicle, either in their own right or by modulating gonadotrophin action. Alternatively they may be concerned with intercompartmental communication, allowing for a tighter linking of cellular populations and thereby facilitating co-ordinated follicular development. This thesis describes a study of the role of local regulators in the modulation of gonadotrophin action.

It was found that in vivo FSH stimulated granulosa cell proliferation and increased ovarian weight, but was incapable of stimulating granulosa cells to proliferate in vitro. This suggests that the action of FSH in vivo is mediated through another factor. However, in the presence of LH, FSH also stimulated the expression of differentiated functions (progesterone and oestradiol production) both in vivo and in vitro demonstrating a direct effect of FSH. In the absence of LH or aromatase substrate, FSH induced the potential for aromatisation but did not increase uterine weight, a marker of oestradiol production. Therefore it is concluded that FSH is the primary stimulus for follicular development but that LH is also required for co-ordinated follicular development.

There is a growing body of indirect evidence to suggest that factors of FSH-stimulated granulosa cell origin may regulate adjacent thecal/interstitial cells. Cytochrome P45017α (17-hydroxylase/C17-20 lyase) in thecal/interstitial cells is a LH-responsive steroidogenic enzyme vital for androgen production. To obtain direct evidence for FSH-stimulated paracrine signalling in the ovary a rat thecal/interstitial cell culture system was validated for the study of the control of androgen production. Using this system and Northern hybridisation the control of androgen production by
gonadotrophins and granulosa cell derived factors was studied. The ~2.0 kb P45017α mRNA signal in ovarian total RNA from intact animals was dose-dependently increased by treatment with recombinant human FSH (rh-FSH). Treatment of hypophysectomised animals with rh-FSH did not consistently alter ovarian P45017α mRNA levels, though in the presence of low levels of LH, FSH increased P45017α mRNA expression. LH treatment alone also increased P45017α mRNA levels. During culture for 48 h in serum-free medium, basal androgen (androstenedione + androsterone) production by thecal/interstitial cells from intact or hypophysectomised animals was unaffected by treatment with rh-FSH in vivo but hLH-stimulated androgen production by cells from intact animals only was enhanced ~2-fold. The ability of rh-FSH treatment in vivo to increase hLH-responsive androgen production in vitro by thecal/interstitial cells from hypophysectomised animals was restored by r-LH treatment in vivo. Similarly, treatment of thecal/interstitial cell cultures with conditioned medium from FSH-treated granulosa cell cultures enhanced LH-responsive but not basal androgen production. It is concluded that treatment of pituitary intact animals with ‘pure’ FSH modulates thecal/interstitial cell androgen production. Granulosa cells but not thecal/interstitial cells possess FSH receptors, and thecal/interstitial cells are principal ovarian sites of P45017α expression. Thus factors produced by FSH-stimulated granulosa cells must function as paracrine signals in vivo. In hypophysectomised animals, absence of any effect of FSH on these parameters of thecal/interstitial cell function emphasises the relevance of paracrine signalling to modulation of LH action rather than any direct regulation of thecal/interstitial function. Factors of granulosa cell origin implicated in the paracrine control of androgen production include IGF-I and inhibin. Both these factors had little or no effect on basal androgen production but dose-dependently increased LH-stimulated androgen production. These data suggest that the effects of FSH in vivo on androgen production could be mediated by one of these factors.

In situ hybridisation of P45017α mRNA expression confirmed that this enzyme was specific to thecal/interstitial cells and was absent from granulosa cells. This observation confirms a basic requirement of the two cell, two gonadotrophin mechanism of follicular oestrogen production. The withdrawal of gonadotrophic support by hypophysectomy for up to 4 days
had no effect on P45017α mRNA expression suggesting that this steroidogenic enzyme is constitutively expressed. Treatment in vivo with DES, a synthetic oestrogen, decreased P45017α mRNA expression consistent with previous observations that oestrogens inhibit androgen production. Treatment with rh-FSH dose-dependently inhibited P45017α mRNA expression in hypophysectomised animals implicating a factor of granulosa cell origin in this down regulation of P45017α. Consistent with previous observations LH stimulated the expression of P45017α mRNA, and FSH, in the presence of a low dose of LH, increased P45017α mRNA expression. These results again underline the modulatory role of FSH-stimulated granulosa cell paracrine signalling on LH-stimulated androgen production.

To establish the paracrine role of inhibin, a system for the culture of individual preantral follicles was used. After 5 days in culture with homologous serum and FSH, follicles had grown to preovulatory size with a commensurate increase in oestradiol secretion but not progesterone. Immunoneutralisation of endogenous inhibin resulted in a significant decrease in oestradiol secretion and an increase in progesterone accumulation. Supplementation of antiserum treated follicles with exogenous inhibin restored oestradiol secretion and reduced progesterone accumulation. Aromatase substrate (androstenedione) levels were too low to measure regardless of antiserum treatment. However, follicles treated with inhibin antiserum in the presence of exogenous androstenedione also exhibited oestradiol levels similar to untreated controls, while progesterone accumulation remained elevated. Based on our previous observations it is proposed that granulosa cell derived inhibin promotes thecal androgen production and hence oestrogen production during preovulatory follicle development. The antiserum-induced increase in progesterone accumulation is most likely explained by reduced metabolism of C21 steroid substrate to androgen in thecal/interstitial cells deprived of inhibin. It is concluded that inhibin is a physiological modulator of follicular steroidogenesis which exerts its effect at the level of theca cell androgen production.

In summary, FSH exerts primary control over follicular development while LH supports and augments the regulatory function of FSH during folliculogenesis. These results provide direct evidence that FSH can influence LH-responsive thecal/interstitial androgen production in vivo and suggest
similar potentials for inhibin and IGF-I of granulosa origin in mediating this action of FSH.
### Abbreviations

<table>
<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>20α-HSD</td>
<td>20α-hydroxyysteroid dehydrogenase</td>
</tr>
<tr>
<td>17β-HSD</td>
<td>17β-hydroxyysteroid dehydrogenase</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>3β-hydroxyysteroid dehydrogenase/Δ5-4 isomerase</td>
</tr>
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<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
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<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>βME</td>
<td>2-mercaptoethanol (β-mercaptoethanol)</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine 3',5'-monophosphate</td>
</tr>
<tr>
<td>CG</td>
<td>chorionic gonadotrophin</td>
</tr>
<tr>
<td>CRE</td>
<td>cyclic AMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic AMP response element-binding protein</td>
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<td>CTP</td>
<td>cytidine 5'-triphosphate</td>
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<td>DAG</td>
<td>diacylglycerol</td>
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<tr>
<td>DCS</td>
<td>donor calf serum</td>
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<tr>
<td>DES</td>
<td>diethylstibestrol</td>
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<td>DHT</td>
<td>5α-dihydrotestosterone (5α-androstane-17β-ol-3-one)</td>
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<td>(c)DNA</td>
<td>(complementary) deoxyribonucleic acid</td>
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<td>DTT</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>(a)FGF</td>
<td>(acidic) fibroblast growth factor</td>
</tr>
<tr>
<td>(b)FGF</td>
<td>(basic) fibroblast growth factor</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
</tr>
<tr>
<td>G-protein</td>
<td>Guanine nucleotide-binding regulatory protein</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine 5'-diphosphate</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
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<td>GnRH</td>
<td>gonadotrophin-releasing hormone</td>
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<td>guanosine 5'-triphosphate</td>
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<tr>
<td>GTPase</td>
<td>guanosine 5'-triphosphatase</td>
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<tr>
<td>h</td>
<td>human</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)</td>
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<td>HMG</td>
<td>human menopausal gonadotrophin</td>
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<td>HRE</td>
<td>hormone response element</td>
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<td>hsp90</td>
<td>90 kilodalton heat-shock protein</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<td>IGF(-I or -II)</td>
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<td>IGFBP</td>
<td>Insulin-like growth factor-binding protein</td>
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<td>IL-1</td>
<td>interleukin-1</td>
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<td>IP3</td>
<td>inositol triphosphate</td>
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<td>IPTG</td>
<td>isopropyl β-α-thiogalactopyranoside</td>
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</tr>
<tr>
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<td>kilodalton(s)</td>
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<td>LB</td>
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<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
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<tr>
<td>LH</td>
<td>luteinizing hormone</td>
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<tr>
<td>LH-R</td>
<td>luteinizing hormone receptor</td>
</tr>
<tr>
<td>α MEM</td>
<td>α-minimum essential medium</td>
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<td>MOPS</td>
<td>3-(N-morpholino)propanesulphonic acid</td>
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<tr>
<td>NADH</td>
<td>β-nicotinamide adenine dinucleotide, reduced</td>
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<td>NADPH</td>
<td>β-nicotinamide adenine dinucleotide phosphate, reduced</td>
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<td>ovine</td>
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<td>P450arom</td>
<td>cytochrome-P450 aromatase</td>
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<td>P450c17α</td>
<td>cytochrome-P45017α-hydroxylase/C17-20 lyase</td>
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<td>P450sccc</td>
<td>cytochrome-P450 side-chain cleavage</td>
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<tr>
<td>(D)PBS</td>
<td>(Dulbecco’s) phosphate-buffered saline</td>
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<td>PIP2</td>
<td>phosphatidylinositol biphosphate</td>
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<td>PKA</td>
<td>protein kinase A</td>
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<td>protein kinase C</td>
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<td>PLC</td>
<td>phospholipase C</td>
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<td>PMNL</td>
<td>polymorpho-nuclear lymphocytes</td>
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<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PMSG</td>
<td>pregnant mare's serum gonadotrophin</td>
</tr>
<tr>
<td>r</td>
<td>recombinant</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
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<tr>
<td>(m or r)RNA</td>
<td>(messenger or ribosomal) ribonucleic acid</td>
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<tr>
<td>RNase</td>
<td>ribonuclease</td>
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<td>SCH 16423</td>
<td>hydroxyflutamide (α,α,α-trifluoro-2-methyl-4'-nitro-m-lactotoluidide)</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>T</td>
<td>testosterone (4-androsten-17β-ol-3-one)</td>
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<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
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<td>TEMED</td>
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<td>TESPA</td>
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<td>tumor necrosis factor-α</td>
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<td>ultraviolet</td>
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<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl β-D-galactopyranoside</td>
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Chapter 1  Literature Review

1. Introduction

The mammalian ovary is a complex organ serving a dual function, being responsible for the provision of fertilisable oocytes, and for the secretion of steroids that regulate female sexual life. The ovary is composed of two main functional units: the developing follicle and the corpus luteum. Follicular development, ovulation and maintenance of corpora lutea are the result of interactions between the hypothalamus, the pituitary and the gonads, as well as the action of peptides and steroids within the ovary.

In the mature female, gonadotrophin releasing hormone, GnRH, is released in a pulsatile manner by cells within the hypothalamus. GnRH stimulates the secretion of luteinizing hormone, LH, and follicle-stimulating hormone, FSH, by the pituitary. These hormones in turn stimulate the two main cell types within the follicle, theca cells and granulosa cells, to develop. Ovarian follicular development is orderly and progresses from primordial through preantral to preovulatory stages. As the follicle grows it differentiates, acquires new functional capabilities and is primarily oestrogen-producing. The oestrogen produced has local regulatory functions within the follicle and also exerts negative feedback on the hypothalamus and pituitary. As oestrogen levels increase the negative feedback becomes positive feedback resulting in a midcycle LH surge which in turn stimulates ovulation. Luteinization is characterised by progesterone secretion and continues until luteolysis is initiated.

As well as endocrine regulation by FSH and LH, the developing follicle is also likely to be subject to paracrine control. This type of regulation involves one cell-type producing and secreting chemical messengers which act specifically on neighbouring cell-types (Franchimont, 1986).

This thesis is primarily concerned with the paracrine regulation of follicular development. The purpose of this review is firstly to provide a background to the ovarian life-cycle and its endocrine control by gonadotrophins, and secondly to outline current knowledge of the local control of the actions of gonadotrophins on the ovary.
2. Ovarian Development

The ovaries are situated in the peritoneal cavity and are surrounded by a bursa that completely encloses the ovary in rats and mice with very little connection with the peritoneum. In pigs and sheep the bursa is open to the peritoneum, and in humans it is virtually absent (Harrison, 1962). The ovary itself is covered with a continuous smooth epithelium, called the surface epithelium. During embryonic development, this epithelium proliferates to give rise to the underlying cortex, in which the germ cells are embedded (Franchi et al. 1962).

Oocytes originate outside the primitive gonad, probably from the hindgut region of the embryo, and migrate as primordial germ cells to the genital ridge resulting in the formation of the primitive ovary (Hardisty, 1978). During foetal life the germ cells in the ovary proliferate by mitosis to form oogonia, and enter the first meiotic division. Once meiosis has begun germ cells are termed primary oocytes and are arrested in diplotene of the first meiotic prophase before spindle formation, and are unable to proceed any further. Therefore the number of germ cells is fixed by birth (or just after birth in the rat) and they are not replaced as they are released. Little is known about the influence, if any, that the oocyte has on follicle development; however it is essential because follicles do not form in their absence (Buccione et al. 1990).

The majority of follicles (~90%) are non-growing throughout life and are termed primordial follicles. Recruitment of primordial follicles initiates dramatic changes in growth, structure and function. The growing follicles are divided into five stages: primary, secondary, tertiary, Graafian and atretic. As described above, the first three stages are gonadotrophin-independent (Hirshfield, 1985) and thus appear to be regulated by intraovarian mechanisms (Eshkol et al. 1970). After a follicle enters the tertiary stage, continued growth and steroidogenesis is gonadotrophin-dependent (Hisaw, 1947).

The primordial follicle is composed of a single layer of granulosa cells and a single immature oocyte arrested in the diplotene stage of the first meiotic division. The primordial follicle is separated from the surrounding
stroma by a layer of spindle-shaped cells with protoplasmic processes that reach the basement membrane, providing a route for nutrient transfer. The oocyte and granulosa cells have no vascular supply and thus exist in a micro-environment enclosed by the basement membrane.

Cuboidal differentiation of the spindle-shaped cells followed by successive mitotic divisions forms a multilayered zona granulosa. The oocyte enlarges and secretes a glycoprotein-containing mucoid substance called the zona pellucida that surrounds the oocyte and separates it from the granulosa cells, forming a primary follicle (Philpott et al. 1987; Lira et al. 1990). The oocyte stops growing, but still produces RNA and protein (Lintern-Moore & Moore, 1979). Re-initiation of meiosis of the oocyte occurs spontaneously in isolated oocytes from primary follicles (Eppig & Downs, 1984), therefore the growth of the oocyte is associated with the development of the ability to resume meiosis. Oocytes do not resume meiosis until just prior to ovulation and thus would appear to be held in meiosis by granulosa-produced factors.

Cyclic AMP can maintain oocytes in meiotic arrest, therefore it is thought that FSH-stimulated granulosa cells pass cAMP through gap junctions (Eppig and Downs, 1984). Purine factors such as hypoxanthine, which is found in high concentrations in follicular fluid, are also implicated by maintaining high cAMP within the oocyte (Downs and Eppig, 1987; Downs et al. 1989).

The secondary follicle is formed by further granulosa cell proliferation and growth. Coincident with granulosa cell proliferation, stromal cells outside the basement membrane differentiate and arrange in concentric cell layers to form the theca interna adjacent to the basement membrane and the theca externa which merge with the surrounding stroma. The secondary follicle acquires an independent vascular supply which terminates in a capillary bed at the basement membrane. The granulosa cells and oocyte thus remain avascular.

Tertiary follicle formation is accompanied by further hypertrophy of theca cells and the appearance of a fluid-filled antrum among the granulosa cells. The antral fluid consists of a plasma transudate and secretory products of granulosa cells. In association with antrum formation the granulosa and theca cells develop intercellular gap junctions (Albertini & Anderson, 1974).
which allow cell-cell communication and synchronised co-ordination of follicular function.

At this stage, the follicle rapidly increases in size under the influence of gonadotrophins to form a Graafian follicle. The antral fluid increases in volume and the oocyte, surrounded by the cumulus oophorus, assumes a polar, eccentric position within the follicle (Gougeon, 1982). The mature Graafian follicle is ready to release ova by ovulation.

Recruited primordial follicles either develop into dominant follicles destined to ovulate or degenerate as a result of atresia (Byskov, 1978). As a result of atresia the oocyte and granulosa cells die and are replaced by fibrous tissue. In contrast the theca cells return to the pool of ovarian interstitial cells (Erickson et al. 1985).

The ovary consists of other cell types other than the ones mentioned above. The cortical stroma contains connective tissue, fibroblasts, blood and lymphatic vessels, and macrophages as well as secretory cells known as interstitial cells. This stroma provides the basic cellular matrix in which the follicles are distributed. Macrophages constitute a major cellular component of the interfollicular ovarian compartment and potentially modulate ovarian function, acting in all likelihood through the secretion of regulatory cytokines (Adashi, 1990). The androgen-producing cells of the interfollicular compartment are heterogenous, and are classified into three classes excluding the thecal/interstitial cells mentioned previously; (1) Primary interstitial cells exist only for a short time during fetal life and have limited steroidogenic capacity. They are not gonadotrophin-responsive and their function is believed to involve the conversion of placental progesterone to androgen (Erickson et al. 1985). (2) The ovary also contains androgen-producing interstitial cells in the hilar region though their function is unknown. (3) Secondary interstitial cells represent hypertrophied theca interna remnants surviving the ongoing process of follicular atresia. They settle in the region of the old follicle but otherwise are functionally indistinguishable from thecal/interstitial cells (Erickson et al. 1985).

3. Ovarian Function

3.1 Steroidogenesis
A principal function of the ovary is the secretion of sex steroids that regulate female sexual life. The production of sex steroids in a co-ordinated manner is essential for differentiation of the follicle and provision of a fertilisable oocyte. The key steroidogenic enzymes and sex steroids involved in ovarian biosynthesis are illustrated in Fig. 1.1.

Fig. 1.1. Pathways of steroid synthesis in the ovary.
3.1.1 Cholesterol Uptake

All ovarian steroids are derived from cholesterol which tends to be obtained from three sources: (1) pre-formed cholesterol circulating in blood in the form of lipoproteins, (2) cholesterol synthesised de novo within the ovary from acetylcoenzyme A, and (3) cholesterol liberated from cholesterol esters stored within lipid droplets. The preferred source is derived from uptake of plasma lipoprotein cholesterol (Carr et al. 1982) either as low density lipoproteins (LDL) or high density lipoproteins (HDL). In humans LDL is the principal source whereas rats prefer HDL (Miller, 1988; Gwynne & Strauss, 1982).

The mechanism by which cholesterol is taken up from HDL is relatively unknown but is thought to involve an association with recognition sites located on the plasma membrane (Ghosh & Menon, 1986), followed by cholesterol release due to lipase activity. LDL is taken up by a receptor-mediated process, and the lipoprotein degraded in lysosomes. Cholesterol is transported to the inner mitochondrial membrane by cAMP-activated sterol carrier protein (Gwynne & Strauss, 1982).

3.1.2 Ovarian Enzymes of Steroidogenesis

3.1.2.1 Cholesterol Side Chain Cleavage

The rate-limiting step in progesterone biosynthesis is the conversion of C27 cholesterol to C21 pregnenolone by cleavage of the cholesterol side chain. The reaction is catalysed by an enzyme complex located on the inner mitochondrial membrane and consisting of cytochrome P450 side chain cleavage (P450scc) and the electron transport proteins adrenodoxin and adrenodoxin reductase (Waterman & Simpson, 1989). P450scc mRNA and protein are found in both theca and granulosa cells of antral follicles, consistent with the progesterone production capabilities of both cell types (Zlotkin et al. 1986; Goldring et al. 1986; Hedin et al. 1987). Levels of P450scc mRNA are low in small antral follicles, but increase during preovulatory follicular development and reach a maximum during luteinisation (Goldring et al. 1986; Goldschmit et al. 1989; McMasters et al. 1987). Granulosa cells of the cumulus oophorus contain detectable levels of P450scc protein only after ovulation, whereas mural granulosa cells contain detectable levels of
P450SCO protein throughout late preovulatory follicular development (Zlotkin et al. 1986; Goldring et al. 1986; Hedin et al. 1987). The enzyme is subject to regulation by gonadotrophins, via cAMP-dependent kinase, which increase P450SCO, adrenodoxin and adrenodoxin reductase (Tuckey & Stevenson, 1986). These stimulatory effects are enhanced by oestrogen (Richards et al. 1987). Insulin-like growth factors also regulate levels of the enzyme (Veldhuis et al. 1986a, Veldhuis et al. 1986b; Veldhuis & Rogers, 1987).

3.1.2.2 3β HSD

The enzyme complex 3β-hydroxysteroid dehydrogenase/Δ5-4 isomerase (3β-HSD) catalyses the conversion of Δ5-steroids to Δ4-steroids and is found in both theca and granulosa cells of preovulatory follicles as well as corpora lutea (Bogovich & Richards, 1984). The enzyme is microsomal and utilises NAD+ as a co-factor (Miller, 1988). Ovarian reactions catalysed by this enzyme include the conversions of pregnenolone to progesterone, 17-hydroxyprogrenenolone to 17-hydroxyprogesterone, and dehydroepiandrosterone to androstenedione. This reaction is not rate-limiting in any way since ovarian cells are capable of converting large amounts of pregnenolone to progesterone in the absence of any trophic stimulation (Strauss & Miller, 1991). Despite this apparent constitutive expression, gonadotrophins are able to stimulate 3β-HSD enzymic activity (Hsueh et al. 1984).

3.1.2.3 17α-hydroxylase

17α-hydroxylase/C17-20-lyase (P45017α) is the enzyme vital for the conversion of progestogens to androgens (Nakajin & Hall, 1981a; Nakajin & Hall, 1981b; Nakajin et al. 1981). A single peptide is capable of both enzymic activities (Zuber et al. 1986). Electrons for the reaction are supplied by NADPH via P450 reductase for the first reaction, and via P450 reductase or cytochrome b5 for the second reaction (Takemori & Kominami, 1984; Jefcoate et al. 1986). P45017α can utilise either pregnenolone (Δ5-progestogen) or progesterone (Δ4-progestogen) as a substrate to produce either dehydroepiandrosterone or androstenedione, respectively.
Ovarian P45017α expression has been shown to be restricted to theca cells (the major androgen producing cells of the ovary) as shown by northern blot and immunocytochemistry (Human: Voutilainen et al. 1986; Sasano et al. 1989a; Sasano et al. 1989b; Bovine: Rodgers et al. 1986a; Rodgers et al. 1986b; Rodgers et al. 1987). Subtle increases in LH promote theca cell differentiation leading to the induction of P45017α activity in developing follicles (Bogovich et al. 1981). P45017α mRNA, protein content, and bioactivity increase throughout theca cell differentiation while NADPH cytochrome P450 reductase remains constant throughout follicular development (Hedin et al. 1987). In mature Graafian follicles, a surge in LH causes a sharp drop in P45017α mRNA, protein content and bioactivity to levels below those seen in small antral follicles (Hedin et al. 1987). In the rat they remain low throughout the luteal phase and during pregnancy (Hickey et al. 1989). The decline in P45017α activity accounts for the low levels of androgens and consequently oestradiol produced by the corpus luteum despite the abundance of aromatase enzyme during the second half of rodent pregnancy. However, theca cells obtained after the LH surge are capable of regaining their ability to produce androgens (Richards et al. 1986). Cytochrome P45017α is essential for androgen synthesis, and, hence, the provision of aromatase substrates required for oestrogen synthesis. As well as endocrine regulation by LH, thecal/interstitial cell P45017α activity is likely to be subject to paracrine regulation by factors of granulosa cell origin. This thesis will investigate the regulation of P45017α by both LH (directly) and FSH (via granulosa cell-derived paracrine signalling).

3.1.2.4 17β-HSD

17β-hydroxysteroid dehydrogenase is a loosely bound microsomal enzyme responsible for the interconversion of androstenedione to testosterone, and oestrone to oestradiol. The enzyme is constitutively expressed in both theca and granulosa cells of the developing follicle, and therefore does not limit follicular or luteal oestradiol synthesis (Bogovich & Richards, 1984).
3.1.2.5 Aromatase

The aromatase enzyme complex is responsible for the conversion of androgens to oestrogens, and consists of cytochrome P450 aromatase (P450arom) and NADPH cytochrome P450 reductase, located in the smooth endoplasmic reticulum. P450arom catalyses three successive hydroxylations of the steroid A-ring, which are necessary for converting C19 androgens to C18 oestrogens (Cole & Robinson, 1988). In the ovary aromatase activity has been conclusively localised to the granulosa cell layer (Sasano et al. 1989a; Sasano et al. 1989b). P450arom mRNA, protein content, and activity are increased in preovulatory follicles compared to small antral follicles (Hickey et al. 1988) due to the inductive effect of FSH (Steinkampf et al. 1987; Whitelaw et al. 1992). This induction of P450arom mRNA, protein content, and activity is enhanced by oestradiol (Hickey et al. 1988) though oestradiol is not essential for the induction (Whitelaw et al. 1992). Other potential stimulators of P450arom include IGF-I and cAMP analogues (Steinkampf et al. 1988), whilst epidermal growth factor antagonises the stimulatory effects of these agents (Steinkampf et al. 1988).

3.1.2.6 20α-HSD

20α-hydroxylase steroid dehydrogenase activity is found in granulosa cells, and serves a primarily catabolic function: it catalyses the conversion of progesterone to the inert 20α-hydroxyprogesterone (Davoren & Hsueh, 1986). It is found in increasing amounts in regressing rat luteal tissue (Rodway & Kuhn, 1975), and is subject to negative regulation by prolactin (Pupkin et al. 1966), while LH increases 20α-HSD activity (Hsueh et al. 1984).

3.1.2.7 5α-Reductase

This enzyme is involved in steroid catabolism, and also generates the potent androgen, 5α dihydrotestosterone (DHT), from testosterone. Its cellular distribution is not well characterised though it does have a high activity in the stroma (Eckstein & Lerner, 1977; Eckstein & Nimrod, 1977). 5α-reductase activity is inhibited by FSH (Payne et al. 1989), an effect augmented by competitive substrates progesterone and testosterone, and the non-competitive inhibitor, oestradiol. Stromal and granulosa cells are
steroidogenically linked since 5α reduced androgens from stromal cells are competitive inhibitors of granulosa cell aromatase (Hillier et al. 1980) and thus act reciprocally to modulate oestrogen synthesis.

3.1.3 Co-ordination of Steroidogenesis

The co-ordination of steroidogenesis is essential for normal follicular growth and maturation. The regulation of steroidogenesis is achieved by cell-specific enzymic localisation. The key enzymes involved catalyse the conversion of cholesterol to pregnenolone (P450_{SCC}), pregnenolone to progesterone (3βHSD), pregnenolone to androgens (P450_{17α}) and androgens to oestrogens (P450_{arom}). LH regulates the first step in steroidogenesis by controlling the conversion of cholesterol to pregnenolone, whereas FSH controls the conversion of androgens to oestrogens.

Immunoreactive P450_{SCC} is found primarily in the theca interna cells of the follicle, consistent with the requirement of this enzyme for thecal androgen synthesis. In the corpus luteum immunoreactive P450_{SCC} is found in both theca- and granulosa-lutein cells (Sasano et al. 1989b). There is little 3βHSD in follicles but levels are high in the corpus luteum. These parallel increases in P450_{SCC} and 3βHSD are consistent with large levels of progesterone production during the luteal phase of the cycle. Immunoreactive P450_{17α} is exclusively localised to theca interna cells of the follicle and theca-lutein cells of the corpus luteum (Sasano et al. 1989b). Immunoreactive P450_{arom} is localised in granulosa cells consistent with the rise in oestrogen production before ovulation, and is localised in granulosa-lutein cells of the corpus luteum.

The co-ordinated action of these localised enzymatic activities, under the control of FSH and LH, facilitates normal oestrogen synthesis and follicular development (Falck, 1959; Short, 1962; Ryan & Petro, 1966; Bjersing, 1967). Studies of the steroidogenic capacities of isolated granulosa and theca cells led to the proposal of the two-cell, two-gonadotrophin theory of oestrogen synthesis (Armstrong & Dorrington, 1979). This theory proposes that in response to LH the thecal cells produce C_{19} steroids, these steroids traverse the basement membrane and enter granulosa cells, and that FSH stimulates granulosa cells to aromatise C_{19} steroids to oestrogens. The oestrogens produced have a positive feedback effect on LH release (Yen,
1986) and therefore upon their own production, and with each successive LH pulse there is a surge in androgen and oestrogen levels found in the ovarian vein (Baird et al. 1981). It is likely that there is local communication between the thecal and granulosa cells to ensure that the provision of androgens is sufficient for the required production of oestrogen.

As explained in more detail below gonadotrophins increase the activity of steroidogenic enzymes, acting through adenylyl cyclase signalling to regulate the transcription of genes encoding steroidogenic enzymes and related proteins. Intracellular cAMP mediates positive regulation by LH of P450sCC, P45017α and other components of these systems in theca cells, as well as mediating the effects of FSH on P450arom and 17βHSD in granulosa cells. The gonadotrophins differentially regulate enzyme levels using the same second messenger due to the fact that the kinetics of mRNA responses differ. This is due to different sensitivities to cAMP and also to the action of local regulators. Similarly other genes under the transcriptional control of cAMP, and their gene products may then modulate transcriptional control of steroidogenic enzymes. Thus the co-ordinated regulation of steroid hormone synthesis is achieved by enzymic localisation, and differential responses to the concentration of cAMP and the temporal pattern of cAMP accumulation.

3.2 Endocrine Regulation of Ovarian Function

Ovarian follicular development is dependent upon the secretion of FSH and LH from the anterior pituitary. These heterodimeric glycoproteins share a common α-subunit consisting of 89 amino acids and two glycosylation sites. The β-subunit confers specificity to the hormones and they only share 7 amino acids of similarity; the FSH β-subunit also has two glycosylation sites compared to one for the LH β-subunit. FSH has a molecular weight of 32.6 kDa, while LH has a molecular weight of 34 kDa (Johnson & Everitt, 1988).

3.2.1 Control of Gonadotrophin Secretion

Ovarian function is controlled via the hypothalamo-pituitary-ovarian axis involving complex feedback regulation (Knobil, 1980). FSH and LH are secreted by gonadotrope cells in the anterior pituitary in response to
stimulation by the neurosecretory product of neurons in the hypothalamus, gonadotrophin–releasing hormone (GnRH). This factor is a decapeptide produced by a small number of GnRH secreting neurons in the forebrain and defined centres in the hypothalamus, most notably in the medial preoptic area and the arcuate and paraventricular nuclei, where it is then transported to terminals in the median eminence. GnRH is released in a pulsatile manner from the median eminence into the portal system and binds to specific, high affinity G-protein coupled receptors on the cell surface of the pituitary gonadotropes (Clayton & Catt, 1981). Activation of the GnRH receptors stimulates a calcium-dependent release of gonadotrophins from storage granules and during states of enhanced gonadotrophin secretion there is evidence for increased synthesis of α- and β-subunits. LH is released from intracellular stores in pulses in response to GnRH pulses, whereas FSH release is dependent on its rate of synthesis, and thus is released more or less continually (McNeilly, 1988). In addition to stimulating release of LH and FSH, GnRH enhances pituitary responsiveness to subsequent stimulation by GnRH. This 'self-priming' effect is achieved by increasing numbers of its own receptor. All these actions of GnRH are mediated by a calcium-dependent mechanism involving protein kinase C activation (Conn, 1989).

Several feedback control systems operate within the hypothalamo-pituitary-gonadal axis to regulate the secretion of LH and FSH from pituitary gonadotropes. These are superimposed on the pulsatile stimulatory action of GnRH released from the median eminence and include inhibitory and stimulatory inputs from the gonads to the pituitary and hypothalamus. The overall effect of such feedback control is negative as illustrated by the fact that FSH and LH levels increase in gonadectomised animals. Most of the gonadal control of pituitary function is exerted by oestradiol acting at the level of the hypothalamus as well as directly on the pituitary. When circulating levels of oestradiol are low, FSH secretion is inhibited due to the suppression of FSH β-subunit mRNA transcription (Phillips et al. 1988). Transcription of the α-subunit is also inhibited but LH levels remain constant (Baird, 1991) due to the fact that most LH release is from intracellular stores. Oestradiol may also exert stimulatory actions on gonadotrophin secretion by acting in the hypothalamus to promote GnRH synthesis and release at the time of the midcycle LH surge (Fink, 1988). At the same time oestradiol acts
at the level of the pituitary to enhance GnRH sensitivity (Johnson & Everitt, 1988) by increasing the number of GnRH receptors (Laws et al. 1990).

Although the major feedback regulation of gonadotrophin secretion is mediated by the actions of oestradiol at the hypothalamic and pituitary levels, an additional family of non-steroidal regulators have been proposed to be involved. These proteins of ovarian origin are inhibin, activin and follistatin. The evidence for the involvement of inhibin in the mediation of the control of gonadotrophin synthesis is the most compelling; it selectively suppresses FSH synthesis and secretion by pituitary cells in vitro (Robertson et al. 1986; Farnworth et al. 1988) at the level of the anterior pituitary (de Jong, 1987). Immunoneutralisation of inhibin leads to increased plasma FSH levels and increased ovulation rates (Mann et al. 1989a; Rivier & Vale, 1989; Findlay et al. 1989; Wrathall et al. 1990; Brown et al. 1990). These effects are achieved by reducing the GnRH-induced increase in FSH β-subunit (Carroll et al. 1989) by blocking the ‘self-priming’ effect on its own receptor (Braden et al. 1990; Busbridge et al. 1990; Wang et al. 1989). Activin stimulates FSH secretion by cultured pituitary cells (Schwall et al. 1988; Mason et al. 1989), by increasing FSH β-subunit mRNA levels (Attardi & Miklos, 1990; Carroll et al. 1991), and injection of activin into rats increases plasma FSH levels (Rivier & Vale, 1991). Follistatin suppresses pituitary FSH secretion (Ying et al. 1987; DePaolo et al. 1991), and binds activin (Nakamura et al. 1990). Though activin and follistatin exert effects alone in vitro on pituitary cells it is unlikely that there is an in vivo endocrine role for these factors. Follistatin is probably responsible for ensuring that activin intended to have local effects on the gonads does not affect pituitary function.

3.2.2 Role of FSH in the Regulation of Ovarian Function

As indicated by its name, FSH is the main promoter of follicular maturation (Greep et al. 1942; Goldenburg et al. 1972; Goldenburg et al. 1973). It is accepted that FSH receptors are exclusively localised to the granulosa cells, though in the hamster there is evidence for FSH binding in the oocyte (Oxberry & Greenwald, 1982). Therefore it is generally presumed that the biological effects of FSH are exerted upon the maturation and function of granulosa cells. The FSH receptor has been observed in the earliest stages of follicle development and throughout the entire period of follicle
development, though not in primordial follicles nor old corpora lutea (Camp et al. 1991). FSH also stimulates production of its own receptor (Richards et al. 1976; Ireland & Richards, 1978), which sensitises granulosa cells to further stimulation by FSH.

The ability of FSH to orchestrate follicular growth and differentiation depends on its ability to exert multiple actions concurrently. A major action of FSH is to induce the formation of aromatase in the granulosa cell leading to increased conversion of thecal androgen to oestrogen (Dorrington et al. 1975). This effect has been observed both in vivo (Armstrong & Papkoff, 1976) and in vitro (Moon et al. 1975). FSH has also been shown to increase progesterone production (Hsueh et al. 1984).

In vivo or in vitro treatment with FSH has been shown to induce LH receptors in granulosa cells (Erickson et al. 1979) and thus granulosa cells are LH-responsive during the latter stages of follicular development. The ability of FSH to induce LH receptors is augmented by the concomitant presence of oestrogen (Rani et al. 1981). Furthermore this process may be amplified by progesterone and androgen (Rani et al. 1981), LH itself (Ireland & Richards, 1978), and several growth factors (Knecht & Catt, 1983; Erickson et al. 1989).

Although oestrogen is an important mitogen for granulosa cells (Penchartz, 1940), FSH also stimulates follicular growth by promoting granulosa cell division in vivo (Gougeon & Testart, 1990; Roy & Greenwald, 1991). This effect of FSH cannot be reproduced in vitro (Hammond & English, 1987; Skinner et al. 1987; Dorrington et al. 1988), which suggests that this effect of FSH is mediated by an intermediate in vivo.

Other important developments induced by FSH include the production of proteoglycans which contribute to the formation of follicular fluid (Ax & Ryan, 1979; Yanagishita et al. 1981); production of inhibin, activin and follistatin which are probably involved in the feedback control of gonadotrophin secretion and are possible local regulators of follicular development (Bicsak et al. 1986; Hillier et al. 1989; Michel et al. 1990; Michel et al. 1991); production of insulin-like growth factors (Oliver et al. 1989); and also many morphological changes associated with granulosa cell differentiation such as the production of gap junctions (Amsterdam & Rotmensch, 1987).
Studies of the effects of FSH on follicular development have had to take account of the fact that pituitary FSH preparations inevitably contain a significant degree of LH contamination. As such the interpretation of experiments has been difficult. With the availability of recombinant (and therefore 'pure') forms of FSH, it is possible to re-evaluate the roles of FSH and LH in follicular development. This thesis will present the results of a study designed to determine the precise contribution of both FSH and LH to normal follicular development.

3.2.3 Role of LH in the Regulation of Ovarian Function

It is well known that LH enhances FSH-induced follicular development. LH plays a major role in the control of androgen and progestogen synthesis by thecal/interstitial cells during follicular development (Erickson et al. 1985), and stimulates the provision of androgens by thecal/interstitial cells for aromatisation by granulosa cells. LH is therefore essential for normal oestrogen production during follicular development. As the follicle grows, its granulosa cells acquire LH receptors and respond to LH in a similar manner to the way they respond to FSH (Hsueh et al. 1984). LH also maintains the FSH-induced aromatase activity of granulosa cells (Wang et al. 1981).

LH alone stimulates follicular growth (Greep et al. 1942) due to thecal hypertrophy (Erickson et al. 1985). However, in conjunction with FSH, high levels of LH trigger ovulation and the subsequent formation and maintenance of corpora lutea (Greep et al. 1942; Lostroh & Johnson, 1966). The resultant granuloša-lutein cells respond to LH by producing progesterone (Moor, 1974). A major target for LH for the triggering of ovulation is granulosa cells which produce plasminogen activators (Beers et al. 1975), and prostaglandins that activate lysosomal enzymes (Bjersing, 1979) which digest the follicular wall.

3.2.4 Intracellular signalling

Cellular responses to gonadotrophins and peptide factors are controlled by a complex system of intracellular signalling pathways. Membrane-bound
receptors receive and transduce the signal to generate an intracellular signalling cascade.

### 3.2.4.1 cAMP intracellular signalling pathway

The signal transduction pathway of gonadotrophins consists of three membrane-bound components; (1) the hormone receptor, (2) a guanine nucleotide-binding regulatory protein (G-protein) and comprising an $\alpha$-, $\beta$- and $\gamma$-subunit, and (3) the catalytic subunit of the adenylylate cyclase enzyme (Johnson & Dhanasekaran, 1989; Leung & Steele, 1992). When FSH or LH binds to its specific receptor, the hormone-receptor complex binds to the G-protein. This stimulates the $\alpha$-subunit to bind GTP which in turn causes the $\beta$- and $\gamma$-subunits to dissociate from the complex. The remaining complex then binds to the catalytic subunit of the adenylylate cyclase enzyme, resulting in the conversion of ATP to cAMP. The $\alpha$-subunit contains intrinsic GTPase activity, which serves to limit the response. In addition, binding of GTP to the complex can lower the receptor affinity for the hormone and hence limit the response. G proteins may be either stimulatory (G$_{s}$), which couple stimulatory ligand receptors to adenylylate cyclase, or inhibitory (G$_{i}$), which couple inhibitory ligand receptors to adenylylate cyclase (Leung & Steele, 1992). Adenylylate cyclase activity results in the accumulation of cAMP which stimulates cAMP-dependent protein kinase A (PKA). This enzyme exists as a tetramer of two regulatory subunits which bind cAMP, and two catalytic subunits which possess phosphotransferase activity (Beebe et al. 1989). Binding of cAMP to the regulatory subunits suppresses the phosphotransferase activity, and therefore allows phosphorylation of protein substrates which either immediately stimulate steroidogenesis or exert long term effects on cAMP-regulated genes through control of transcription rates (Kurten & Richards, 1989). It is likely that PKA stimulates a cAMP response element binding protein (CREB) which binds as a dimer to an enhancer element known to mediate cAMP responsive gene expression, the cAMP response element (CRE), and thereby stimulates transcription from genes under the regulation of a CRE (Montminy et al. 1990).

The FSH and LH receptors bear high sequence homology with each other and thus they have similar molecular structures. They are large, single chain polypeptides of with seven membrane spanning hydrophobic regions,
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separated by short stretches of hydrophilic amino acid residues (Johnson & Dhanasekaran, 1989). The hormone recognition site of the receptor resides in the long extracellular amino terminus. Glycosylation of the hormone-binding domain of the receptor (Dufau et al. 1989; Minegishi et al. 1989) and the ligand (Petaja Repo et al. 1991) is believed to be important for receptor-ligand interaction. The LH receptor is a 75 KDa (Dufau & Kusuda, 1987), 90 KDa (Keinanen et al. 1987), or a 92 KDa polypeptide (Roche & Ryan, 1989) whose gene is capable of alternative splicing to generate diverse transcripts (Tsai-Morris et al. 1990). It exists as a dimer of two identical hormone-binding subunits associated by covalent interactions (Kusuda & Dufau, 1988). Sequence analysis reveals a 26-residue signal peptide, a 341-residue extracellular domain and a 333-residue region consisting of seven transmembrane segments. The FSH receptor is a 75 KDa polypeptide with a 348-residue extracellular hormone-binding domain (Sprengel et al. 1990).

3.2.4.2 Protein kinase intracellular signalling pathways

Protein kinases are capable of catalysing the phosphorylation of proteins. This covalent modification alters the equilibrium between active and inactive forms of signal proteins, thereby enabling the cell to respond to extracellular stimuli as well as its internal environment. Two major classes of protein kinases are distinguished on the basis of the amino acids that they preferentially modulate;

a) The tyrosine kinases mediate signal transduction for a variety of growth factors such as epidermal growth factor (EGF/TGFα) (Feng et al. 1987) and insulin like growth factor (IGF-I) (Adashi et al. 1985a). The ligand-binding region of the receptor is located on the extracellular portion of the receptor, coupled to the intracellular tyrosine kinase. The receptors exist as single polypeptide chains though in the case of IGF-I the receptor consists of multiple subunits (Sibley et al. 1988). Binding of ligand activates the intracellular tyrosine kinase, promoting phosphorylation of tyrosine residues of proteins involved in cell development (Leung & Steele, 1992).

b) The serine/threonine kinases mediate signal transduction for transforming growth factor β (TGFβ) (Massague et al. 1992) and activin (Mathews & Vale, 1991) and possibly other members of this family such as inhibin. The ligand-binding region of the activin receptor is located on the
very small extracellular domain of the receptor, coupled to the intracellular serine/threonine kinase. This form of intracellular signalling is unusual and has only been seen once before in animal cells, as the product encoded by the developmental gene daf-1 in the nematode C. elegans (Georgi et al. 1990).

3.2.4.3 Phosphatidylinositol intracellular signalling pathways

This intracellular pathway mediates the actions of a number of local regulatory factors that inhibit gonadotrophin-stimulated differentiation in thecal/interstitial cells (Hofeditz et al. 1988) and immature granulosa cells (Kasson et al. 1985; Shinohara et al. 1985) and promote gonadotrophin-stimulated differentiation in luteal granulosa cells (Davis et al. 1989).

The key event in signal transduction is the hydrolysis, mediated by phospholipase-C (PLC), of phosphatidylinositol biphosphate (PIP₂), to yield diacylglycerol (DAG) and inositol triphosphate (IP₃) (Berridge, 1987a). Subsequently, DAG activates PKC and undergoes cleavage to release the prostaglandin precursor, arachidonic acid, while IP₃ mediates the release of sequestered intracellular calcium. PKC is involved in the control of ovarian cell growth and differentiation by inhibiting gonadotrophin-stimulated differentiation in thecal/interstitial cells (Hofeditz et al. 1988) and exerting development-related control over granulosa cell differentiation (Kasson et al. 1985; Shinohara et al. 1985; Davis et al. 1989). Calcium is a ubiquitous second messenger that interacts with specific protein kinases and binds to calmodulin, which regulates many kinase activities as well as functioning as an intracellular calcium receptor. One of the most important target proteins of calcium is the cAMP phosphodiesterase, and activation of this enzyme by calcium release provides a link between the cAMP and phosphatidylinositol intracellular pathways. The forked signalling of IP₃ and DAG and its local regulation is important for the modulation of gonadotrophin-stimulated ovarian follicular development and the activation of gene transcription by local regulators (Berridge, 1987b).

3.3 Local Regulation of Ovarian Function

The observation that ovarian follicles exposed to comparable gonadotrophic stimulation undergo variable fates suggests the existence of
an intraovarian system that modulates the actions of gonadotrophins. The existence of such a system would help to explain differences in rate and extent of follicular development, initiation and arrest of meiosis, and determination of follicular dominance versus atresia. Similarly, early stages of gonadotrophin-independent follicular growth probably involve intraovarian signalling.

Granulosa and theca cells respond to gonadotrophic stimulation by producing a host of steroidal and non-steroidal regulatory factors capable of exerting local control of follicular growth and development. Potential regulatory factors include oestrogens, IGFs, inhibin and activin which will be reviewed below. It is important to remember that the majority of actions that these factors exert require the presence of gonadotrophins, and thus they merely positively or negatively modulate gonadotrophin action at a local level, and do not substitute for gonadotrophins. Most evidence for the role of intraovarian regulators is indirect, and has been obtained from the use of primary cell cultures. This thesis will, however, provide direct evidence for the existence of paracrine signalling in the ovary.

3.3.1 Steroidal Regulation

3.3.1.1 Mechanism of Steroid Action

Steroid action is mediated by specific steroid receptors belonging to a large family of nuclear hormone receptors which includes the thyroid hormone receptor and the vitamin D₃ receptor (Evans, 1988; Parker, 1988). Using mutagenesis (Jenster et al. 1991) and generation of chimaeric receptor proteins (Green & Chambon, 1989) these receptors have been characterised as having three distinct functional domains.

The highly conserved DNA-binding domain is rich in cysteine residues (Green & Chambon, 1986), which coordinate zinc ions to form two zinc fingers (Evans & Hollenberg, 1988; Berg, 1990). The zinc fingers bind specific short genomic DNA palindromic sequences called hormone response elements (HREs) (Ham et al. 1988; Beato et al. 1989; Umesono & Evans, 1989), which act as enhancers (Ptashne, 1988). Binding of the steroid receptor to its HRE leads to enhancement of transcription of the downstream gene, by interacting with other transcription factors at the gene promoter and/or
changing the chromatin structure so these other transcription factors may bind (Beato, 1989). This domain can be considered as the ‘active site’ of the receptor.

The carboxy-terminal steroid binding domain is less well conserved and reflects the difference in structure of the various ligands (Evans, 1988). This domain represses the transcriptional activation potential of the receptor in the absence of any ligand (Hollenberg et al. 1987; Webster et al. 1988) and is involved in dimerisation of occupied receptors (Fawell et al. 1990).

The N-terminal domain of these receptors is variable in size and sequence, and is enigmatic as far as its precise function, though it is necessary for full activity of the receptor (Bocquel et al. 1989; Lees et al. 1989). Recent research suggests that this domain exerts an important influence on on transcriptional modulation, by selectively activating different hormone-regulatable genes (O’Malley & Strott, 1992).

Active hormone-receptor complexes bind as dimers, via their zinc fingers, to their HREs and enhance transcription of hormone-responsive genes (Beato, 1989). Inactive receptors are bound to several proteins including heat shock protein 90 (hsp90) (Ziemiecki et al. 1986) which binds to the DNA-binding domain (Smith et al. 1990) and prevents receptor activation in the absence of steroid (Cadepond et al. 1991) by preventing receptor dimerisation (DeMarzo et al. 1991). Once the steroid ligand binds the receptor is activated due to hsp90 dissociation (DeMarzo et al. 1991) and receptor dimerisation (O’Malley, 1990).

3.3.1.2 Androgens

Aside from serving as substrates for the aromatisation by granulosa cells, androgens exert a variety of receptor-mediated effects at the level of the granulosa cell (Hillier et al. 1977; Hillier & Ross, 1979). Androgens are capable of promoting gonadotrophin-stimulated granulosa cell aromatase activity (Hillier & deZwart, 1981). Thus androgens augment FSH-stimulated aromatase activity not only by acting as a substrate, but also by exerting a direct paracrine effect, resulting in the up-regulation of aromatase activity. Androgens also promote progesterone synthesis (Armstrong & Dorrington, 1976; Lucky et al. 1977). Androgens enhance many other FSH-stimulated effects such as the induction of LH-responsiveness (Shaw et al. 1989), inhibin
production (Hillier et al. 1989; Michel et al. 1991) and plasminogen activator secretion (Jia et al. 1990). However, high follicular concentrations of reduced androgens such as dihydrotestosterone (DHT) may act as competitive inhibitors of granulosa cell aromatase activity (Hillier et al. 1980).

In the absence of gonadotrophins, androgens promote follicular atresia and antagonise oestrogen-supported ovarian weight gain in hypophysectomised immature rats (Hillier & Ross, 1979; Leung & Armstrong, 1979). Similarly in vitro or in vivo treatment with DHT abolishes the ability of FSH to induce granulosa cell LH receptors (Farookhi, 1980). It has been postulated that these and other effects may be due to an androgen-receptor mediated decrease in the ovarian oestrogen receptor content. These findings are in keeping with the observation that an increased androgen : oestrogen ratio is associated with the morphologic features of atresia. Although androgens undoubtedly play regulatory roles at the level of the thecal/interstitial cell, there is no published experimental information to-date of any effects of androgens on thecal/interstitial cells. The function of androgens in vivo is not certain, since administration of antiandrogens does not disrupt rat oestrous cycles (Neumann et al. 1970), nor does it affect FSH-responsiveness in rats (Zeleznik et al. 1979), and androgen resistant mice undergo apparently normal cycles (Lyon & Glenister, 1974). However, intraovarian androgen implants reduce progesterone secretion by granulosa cells in vitro (Schomberg et al. 1978).

3.3.1.3 Oestrogens

Oestrogens, in addition to their multiple systemic effects, exert a variety of critical actions at the level of the ovary (Goldenberg et al. 1972; Goldenberg et al. 1973). Both granulosa and theca cells appear to be sites of oestrogen action (Payne & Hellbaum, 1955; Bradbury, 1961). At the level of the granulosa cell, oestrogens promote cellular division (Rao et al. 1978), exert a direct antiatretic effect (Harman et al. 1975), and stimulate expression of inhibin α- and β-subunit mRNAs (Turner et al. 1989). In addition oestrogens play important roles in the promotion of intercellular gap junction formation (Merk et al. 1972), in the enhancement of antrum formation (Richards et al. 1980), and in the augmentation of the oestrogen receptor content (Richards, 1980). Importantly, oestrogens also synergise with gonadotrophins at several
levels, including the promotion of ovarian growth (Reiter et al. 1972), LH and FSH receptor formation (Richards et al. 1979; Louvet & Vaitukaitis, 1976), augmentation of FSH-induced inhibin production by cultured rat granulosa cells (Bicsak et al. 1988), the enhancement of aromatase activity (Adashi & Hsueh, 1982), and augmentation of FSH-stimulated progesterone production (Ratoosh & Richards, 1985). The ability of oestrogens to augment aromatase contributes to the exponential preovulatory rise in the circulating levels of oestradiol. This form of self amplification may also play a role in establishing follicular dominance. The augmenting actions of oestrogens on FSH-stimulated parameters of granulosa cell function are believed to be mediated at the level of cAMP generation or breakdown. Both in vivo and in vitro, oestrogens amplify cAMP levels found in granulosa cells in response to FSH (Richards et al. 1979).

However, oestrogens can also inhibit ovarian androgen production by blocking the conversion of C21 progestogens to C19 androgens (Leung & Armstrong, 1979; Magoffin & Erickson, 1981; Magoffin & Erickson, 1982a) and thus oestrogen may be involved in suppressing thecal androgen synthesis in response to the LH surge (Erickson et al. 1985). The exact mechanism of oestrogen action in thecal/interstitial cells remains unclear. This inhibitory action does not involve altered cAMP generation by theca cells (Hunter & Armstrong, 1987; Morley et al. 1989), and is probably due to inhibition of P45017α (Magoffin & Erickson, 1982a). This thesis will address the question of how oestrogens inhibit ovarian androgen production during follicular development.

3.3.1.4 Catecholoestrogens

The enzyme, oestrogen-2-hydroxylase converts oestrogens to 2-hydroxyoestradiol, a catecholoestrogen, in the ovary. Catecholoestrogens have been detected in human and horse follicular fluid (Dehennin et al. 1984; Spicer & Hammond, 1989b), and in porcine follicles the enzyme, oestrogen-2-hydroxylase, is abundant in both theca and granulosa cells, although not in corpora lutea (Spicer & Hammond, 1989b). 2-hydroxyoestradiol stimulates basal production as well as augments FSH-induced synthesis of progesterone by rat and pig granulosa cells, and augments androgen aromatisation (Hudson & Hillier, 1985; Spicer & Hammond, 1987a; Spicer &
Hammond, 1987b). 2-hydroxyoestradiol can also inhibit proliferation of granulosa cells (Spicer & Hammond, 1989a). In pig thecal cells, 2-hydroxyoestradiol can inhibit androgen production (Morley et al. 1989) by reducing P45017α activity, in much the same way as oestrogens act. The action of 2-hydroxyoestradiol is distinct from that of oestradiol though since its potency is greater than that of oestradiol (Spicer & Hammond, 1987a). These observations suggest that catecholestrogens have a role in the local control of follicular development.

3.3.2 Non-steroidal Regulation

Over recent years it has become clear that the ovary produces several non-steroidal regulatory agents such as growth factors (e.g. IGF-I), cytokines (e.g. TNFα), and developmental factors (e.g inhibin and activin). Although originally identified for their effects on cellular proliferation, within the immune system, and during embryogenesis and organogenesis (e.g. Roberts et al. 1988), they have been shown to be capable of regulating the activity of steroidogenic cells in the ovary. To be considered putative intraovarian regulators such factors must meet the following criteria: (1) local production, (2) local reception, and (3) local action. Ideally, a demonstration of the indispensability of these factors in vivo for normal follicular development is also necessary.

3.3.2.1 IGFs

Insulin-like growth factor-I (IGF-I) and its structural homologue, IGF-II, are low molecular weight single-chain polypeptides that promote cellular proliferation and differentiation in a variety of systems (Slack, 1989) and are believed to play a role in cyclic ovarian follicular development (Giudice, 1992). The IGFs have a close homology with insulin (Froesch et al. 1985) and these three factors cross react with each others' receptors (Rechler & Nissley, 1985). While the IGFs are produced by the liver under the control of growth hormone (GH) (Slack, 1989) they are also produced by the ovary and exert local actions (Adashi et al. 1985a; Giudice, 1992). In the rat (Hernandez et al. 1989; Oliver et al. 1989) and pig (Hammond et al. 1985; Hsu & Hammond, 1987a) the granulosa cells are the principal site of ovarian IGF-I gene expression and protein synthesis. Porcine granulosa cell IGF-I production is
stimulated by FSH, LH, GH and oestradiol via the cAMP second messenger pathway (Hsu & Hammond, 1987a; Hsu & Hammond, 1987b). IGF-I production by porcine granulosa cells is also modulated by other growth factors (Mondschein et al. 1988) and may also be inhibited by FSH (Mondschein & Hammond, 1988). Receptors for IGF-I have been demonstrated in rat (Adashi et al. 1986a), sheep (Monget et al. 1989), pig (Veldhuis & Furlanetto, 1985) and human (Gates et al. 1987) granulosa cells, and in rat theca cells (Hernandez et al. 1988b). Expression of granulosa cell IGF-I receptors is also stimulated by FSH, LH and GH in vivo and in vitro (Adashi et al. 1988; Adashi et al. 1986a).

Having demonstrated local production and reception, the next criterion to satisfy is that of local action. Although multiple ovarian actions have been assigned to IGF-I, its main role is the amplification of gonadotrophin action (Adashi et al. 1985a). IGF-I augments FSH-stimulated progesterone production in cultured granulosa cells of several species (rat: Adashi et al. 1985b; pig: Veldhuis & Furlanetto, 1985) by increasing P450scc mRNA levels (Veldhuis et al. 1986a) and thus protein levels (Veldhuis et al. 1986b). FSH-stimulated oestrogen synthesis is augmented by IGF-I (Davoren et al. 1985) accompanied by an increase in P450arom gene expression (Steinkampf et al. 1988). Other FSH-stimulated differentiated functions that are augmented by IGF-I include inhibin production (Bicsak et al. 1986), LH receptor induction (Maruo et al. 1988) and proteoglycan synthesis (Adashi et al. 1986b).

In purified rat thecal cell studies it has been demonstrated that IGF-I alone does not stimulate androgen or progesterone synthesis (Magoffin et al. 1990). However, in the presence of LH, there is a synergistic increase in thecal cell production of both androgen and progesterone without a change in thecal cell sensitivity to LH stimulation (Magoffin et al. 1990). The mechanism of this synergism probably involves effects of IGF-I on several components of the LH-responsive signalling pathway. IGF-I also increases LH receptor content of thecal cells both in the presence and absence of LH (Cara et al. 1990) by increasing transcription and translation of the LH receptor gene. In cultured human thecal cells the effect of IGF-I on thecal androgen synthesis is greater than that of LH, though IGF-I synergises with LH to increase androgen production also (Hillier et al. 1991b; Hillier et al.
Therefore IGF-I has the potential to exert paracrine control over androgen synthesis as well as autocrine control in the granulosa cell, and thereby coordinate follicular oestrogen synthesis.

The IGF-II gene is expressed by thecal cells (Hernandez et al. 1990b), and in humans IGF-II is found in significant amounts in the follicular fluid (Ramasharma et al. 1986). Similarly, granulosa cells produce IGF-II in vitro in response to FSH, LH, GH and dibutyryl cAMP (Voutilainen & Miller, 1987). These observations suggest a potential local role for IGF-II during follicular development.

The hormonal actions of IGFs are subject to further modulation by the local production of low-molecular weight binding proteins (IGFBPs) which bind to IGFs during transport in the blood (Giudice, 1992). There are at least six different IGFBPs, all of which are produced by different cell types at various stages of development and have a variety of modulatory effects on IGF action (Rosenfeld et al. 1990). Both stimulatory and inhibitory effects of IGFBPs have been shown (Adashi et al. 1991), but most available evidence suggests that IGFBPs negatively regulate IGF action and thus are antigonadotrophic in nature. In a study of the expression of IGFBP-1, 2, 3 and 4 in the rat ovary (Nakatani et al. 1991b) it was shown that IGFBP-1 is not found anywhere in the ovary in agreement with porcine studies (Mondschein et al. 1990) but in contrast to the human granulosa cells (Jalkanen et al. 1989). IGFBP-2 was expressed only in thecal/interstitial cells in larger follicles, IGFBP-3 was found only in corpora lutea, and IGFBP-4 was found only in granulosa cells of atretic follicles (Nakatani et al. 1991b; Erickson et al. 1992a). In another study IGFBP-5 (Erickson et al. 1992b) was localised to granulosa cells of atretic preantral follicles. Similarly in sheep increased IGFBP expression is associated with atresia (Monget et al. 1989). The thecal/interstitial cells are a prominent site of rat ovarian IGFBP-6 gene expression (Rohan et al. 1993). The level of IGFBP transcript is inhibited by FSH and DES, and IGFBP-6 has limited antigonadotrophic actions on rat granulosa cell function (Rohan et al. 1993). The actions of IGFBPs observed so far are similar to the effects of a specific antiserum to IGF-I and suggest a complex level of control of ovarian function that requires more attention in order to develop a fuller understanding.
3.3.2.2 EGF/TGFα

Transforming growth factor α (TGFα), and a structural analogue, epidermal growth factor (EGF), are single chain polypeptides of c. 50 amino acids, that are capable of binding to an apparently common granulosa cell EGF/TGFα receptor (Massague, 1983; Derynck, 1986). Not only do they share the same receptor, but they are of equal potency in systems studied to-date. The ovarian content of EGF/TGFα receptors is increased by FSH or LH (St Arnaud et al. 1983). EGF does not appear to be expressed in the ovary (Skinner & Coffey, 1988), but TGFα is expressed in thecal cells (Skinner & Coffey, 1988).

At the ovarian level, EGF and TGFα exert regulatory effects on granulosa cell proliferation and differentiation in a variety of species (May & Schomberg, 1989). EGF is a mitogen in vitro (Gospodarowicz & Bialecki, 1979; Skinner et al. 1987; May et al. 1987; Mondschein et al. 1988), though rat granulosa cells are insensitive to this action of EGF (Gospodarowicz & Bialecki, 1979). In rats, EGF inhibits FSH-stimulated oestradiol production (Dorrington et al. 1987; Adashi et al. 1987), inhibin production (Bicsak et al. 1986), progesterone production (Dodson & Schomberg, 1987) and LH receptor induction (Dodson & Schomberg, 1987). These effects are mediated via a suppression of cAMP production (Adashi et al. 1987), though EGF is also able to block the action of cAMP analogues (Adashi et al. 1987). In pigs and humans the effects of EGF are similar (Steinkampf et al. 1988; Michel et al. 1991) except that EGF enhances progesterone production (Tapanainen et al. 1987; Urban et al. 1990), IGF-I and IGFBP production (Mondschein et al. 1990), and increases FSH receptor number (May et al. 1987). TGFα has also been shown to be an inhibitor of gonadotrophin-supported granulosa cell differentiation (Skinner & Coffey, 1988).

EGF is an inhibitor of LH-stimulated thecal cell androgen biosynthesis (Erickson & Case, 1983), which in turn leads to decreased follicular oestrogen production. These observations suggest that EGF/TGFα may exert paracrine effects at the level of the adjacent granulosa cell, as well as exerting autocrine effects on thecal/interstitial cells. Thus TGFα of thecal/interstitial cell origin may orchestrate follicular activities at both granulosa and thecal/interstitial cell levels.
3.3.2.3 TGFB

Transforming growth factor β is a 25 KDa homodimeric protein, originally identified for its ability to elicit a reversible phenotypic transformation of mammalian cells (Massague, 1985). TGFB exists in three forms (TGFB1, TGFB2, TGFB3) and is a member of a family of related proteins that include inhibin, activin, and Mullerian duct inhibiting substance (MIS) (Knecht & Catt, 1989), all of which are involved in embryonic development. TGFB is produced by both granulosacells (Kim & Schomberg, 1989; Bendell & Dorrington, 1991) and theca cells (Skinner et al. 1987; Bendell & Dorrington, 1988) of the ovary in most species, but pig granulosa cells do not produce it (Kim & Schomberg, 1989). The regulation of TGFB production has not been extensively studied to-date but it is known that oestradiol does stimulate TGFB production by rat granulosa cells (Bendell & Dorrington, 1991).

The effects of TGFB on granulosa cells have been studied extensively in both the rat and pig, but it has generally opposite effects in these two species. In the rat, TGFB increases FSH-stimulated oestradiol (Ying et al. 1986; Bendell & Dorrington, 1988), progesterone (Dodson & Schomberg, 1987) and inhibin production (LaPolt et al. 1989), but has little effect in the absence of gonadotrophin. It also increases EGF and LH receptor induction (Feng et al. 1986; Blair et al. 1988). These effects are partially due to enhanced cAMP accumulation (Knecht et al. 1986; Blair et al. 1988) but also lead to increased amplification of the actions of cAMP (Knecht et al. 1987). In rats TGFB also stimulates granulosa cell proliferation, both alone and in synergy with FSH (Dorrington et al. 1988). Indeed the action of FSH on proliferation can be blocked by immunoneutralising TGFB (Bendell & Dorrington, 1991).

In pigs the actions of TGFB are generally inhibitory. It inhibits FSH-stimulated inhibin production (Michel et al. 1991), progesterone synthesis (Mondschein et al. 1988), and proliferation (Mondschein et al. 1988). It also inhibits EGF-stimulated IGF-I production (Mondschein & Hammond, 1988) and basal IGFBP secretion (Mondschein et al. 1990).

In rat thecal cells, TGFB potently inhibits LH-stimulated androgen synthesis (Magoffin et al. 1989; Hernandez et al. 1990a), but augments LH-stimulated progesterone production (Magoffin et al. 1989). In the absence of gonadotrophins there is little effect of TGFB unless IGF-I or insulin are
present at the same time (Hernandez & Adashi, 1988). These observations suggest potential autocrine and paracrine roles for TGFβ during follicular development in regulating both the effects of gonadotrophins and other growth factors on granulosa and theca cells.

3.3.2.4 Inhibin and Activin

Mature inhibin is a 32 KDa heterodimeric protein that has been isolated from follicular fluid as one of two forms consisting of an α-subunit and either a β₂A- or β₂B-subunit (Ling et al. 1985; Miyamoto et al. 1985; Rivier et al. 1985; Robertson et al. 1985; Vale et al. 1988; Ying, 1988). Treatment of pituitary cell cultures with inhibin suppresses secretion of FSH, whereas treatment with the β-subunit homodimer (β₂A-β₂A or β₂A-β₂B, called activin) stimulates release of FSH (Tsonis & Sharpe, 1986).

The principal site of ovarian inhibin expression is the granulosa cells, where all three inhibin subunits are produced (Meunier et al. 1988). In rats, cows and sheep, the α-subunit is expressed in healthy follicles of all sizes, but the β-subunits are only expressed in healthy antral follicles (Meunier et al. 1988; Woodruff et al. 1988; Rodgers et al. 1989). The corpus luteum of these species do not express inhibin (Woodruff et al. 1988; Rodgers et al. 1989; Campbell et al. 1991), though in the case of the rat some inhibin α-subunit expression is detected in newly formed corpus luteum (Meunier et al. 1988). In the primate, β₂B-subunit is expressed earlier in follicular development than the other two subunits (Schwall et al. 1990). Primate corpora lutea do however express inhibin subunits (Smith & Fraser, 1991). The theca cells of rats and cows express only very low levels of inhibin α-subunit (Meunier et al. 1988), but express neither of the other two subunits.

The inhibin subunits are encoded by separate genes (Mason et al. 1985; Mason et al. 1986; Esch et al. 1987) whose expression is developmentally regulated and induced by FSH (Woodruff et al. 1987; Woodruff et al. 1988; Turner et al. 1989). The α- and β₂B- subunit mRNAs are also inducible by in vitro treatment with oestradiol (Turner et al. 1989). In vitro studies have established that granulosa cell production of inhibin is stimulated by FSH in rat (Bicsak et al. 1986), pig (Michel et al. 1991) and primate (Hillier et al. 1989), while cow and sheep granulosa cells appear not to be responsive to FSH or
LH (Henderson & Franchimont, 1981; Campbell et al. 1991). LH in low doses stimulates inhibin production by mature granulosa cells (Bicsak et al. 1986) and is inhibitory at high concentrations (Zhang et al. 1988). Inhibin production is also subject to regulation by a variety of other steroidal (e.g. androgen) and non-steroidal factors (e.g. IGF-I and TGFβ) as outlined throughout this section. The bioavailability of inhibin and activin is regulated by binding proteins for both factors (Krummen et al. 1993), most notably follistatin (Nakamura et al. 1990) and α2-macroglobulin (Vaughan & Vale, 1993).

As well as their endocrine functions, both inhibin and activin are putative intraovarian regulators of ovarian function (Burger & Findlay, 1989; Mather et al. 1992). The effects of these factors are mediated by cell surface receptors (Mathews & Vale, 1991; Shinozaki et al. 1992). Intrabursal injection of inhibin or activin in immature rats (Woodruff et al. 1990) showed that inhibin stimulates follicular development and tritiated thymidine uptake into granulosa cells, whereas activin caused atresia, and decreased granulosa cell thymidine uptake. Progesterone production by Graafian follicles in vitro is not affected by addition of inhibin (Tsafiriri et al. 1989b), though the production of progesterone may be limited at a point other than inhibin availability.

In rat granulosa cell cultures, inhibin has no effect at all (Hutchinson et al. 1987), though a slight inhibition of FSH-stimulated cAMP generation has been reported (Ying et al. 1986), which leads to a reduction in oestradiol and progesterone production. Activin, on the other hand, stimulates granulosa cell function. It enhances progesterone and inhibin production as well as FSH-induced aromatase activity (Hutchinson et al. 1987; Xiao et al. 1990), and cAMP formation (Xiao & Findlay, 1991), LH receptor expression (Sugino et al. 1988), and FSH receptor expression (Hasegawa et al. 1988; Nakamura et al. 1993). Activin alone stimulates basal inhibin secretion while not affecting progesterone or oestradiol production. Activin also stimulates proliferation of luteinized human granulosa cells (Rabinovici et al. 1990b) and inhibits luteinized function in luteinizing cow granulosa cells (Shukovski & Findlay, 1990). Thus in granulosa cells, activin promotes early follicular development but inhibits luteinization.
In vitro regulation of theca cell androgen production by activin and inhibin has been examined in the rat (Hsueh et al. 1987) and human (Hillier et al. 1991b; Hillier et al. 1991c). In both species inhibin augments LH-stimulated androgen production in vitro by thecal cells, whereas activin inhibits LH-stimulated androgen production, while these two factors antagonise each other when incubated together with LH. Therefore both activin and inhibin exert potentially significant autocrine and paracrine effects in the regulation of follicular function, though the indispensability of either of these factors to normal follicular development has yet to be demonstrated. This thesis presents evidence that locally produced inhibin is crucial to normal follicular oestrogen synthesis.

3.3.2.5 Follistatin

Follistatin is a single chain peptide that suppresses FSH release from pituitary cells, and exists in various forms of either 32 or 35 KDa in porcine follicular fluid (Ueno et al. 1987), and 31, 35, or 39 KDa in bovine follicular fluid (Robertson et al. 1987). These size variants are the products of alternative splicing (Michel et al. 1990). Follistatin binds activin (Nakamura et al. 1990) and thus antagonises many actions of activin. Follistatin has an endocrine function as a suppressor of FSH secretion stimulated by GnRH or activin (Robertson et al. 1987; Ueno et al. 1987; DePaolo et al. 1991), both by inhibiting FSH release (Ying, 1989), and by reducing levels of FSH-β mRNA (Carroll et al. 1989).

Expression of follistatin in the ovary is similar to the pattern of inhibin-α; it is confined to granulosa cells of developing follicles from early antral stages (Shimasaki et al. 1989; Nakatani et al. 1991a), with a maximum being reached in the preovulatory follicle. Expression declines after the LH surge, and is completely absent by the time of luteal regression (Nakatani et al. 1991a). Follistatin production by granulosa cells is stimulated by FSH or PMSG (Shimasaki et al. 1989; Michel et al. 1990; Saito et al. 1991).

Follistatin inhibits aromatase activity and inhibin production, and augments FSH-stimulated progesterone production (Xiao et al. 1990), but has little effect on progesterone secretion by cultured preovulatory follicles (Tsafriri et al. 1989b). These effects of follistatin seem to be mediated through
a suppression of cAMP accumulation (Xiao & Findlay, 1991). There are no published data of the effects of follistatin on thecal cell function to-date.

3.3.2.6 GnRH

Gonadotrophin-releasing hormone is a potential local regulator of follicular development, as well as its primary function of stimulating pituitary gonadotrophin secretion. GnRH has a large number of potential roles in the modulation of ovarian function (Jones, 1989). GnRH has been detected in sheep and cow ovaries (Aten et al. 1987), as well as the rat ovary (Oikawa et al. 1990). Receptors for GnRH have been demonstrated on rat granulosa and theca cells (Hsueh & Schaeffer, 1985; Maruo et al. 1985) and in the granulosa cells of dominant follicles (Latouche et al. 1989). The recently cloned GnRH receptor cDNA (Eidne et al. 1992) has enabled the expression of GnRH receptor mRNA to be examined (Whitelaw et al. 1993). The GnRH receptor mRNA was detected in the corpus luteum, and granulosa cells at varying stages of follicular development, including some but not all primary follicles. It was found to be expressed at an earlier stage than the LH receptor. Treatment with FSH enhanced expression of the receptor. GnRH causes a rapid increase in granulosa cell inositol triphosphate, diacylglycerol and calcium (Davis et al. 1986) and therefore the action of GnRH is probably mediated through the protein kinase C pathway. GnRH also affects cAMP metabolism by reducing gonadotrophin-stimulated cAMP generation, suggesting that the protein kinase A and C pathways are linked. Indeed these two pathways appear to be antagonistic; granulosa cell differentiation is stimulated through the protein kinase A system and inhibited through the protein kinase C system (Shinohara et al. 1985; Shinohara et al. 1986).

The modulation of gonadotrophin action is not limited to the granulosa cell compartment. GnRH treatment inhibits LH action (Hsueh & Jones, 1981) on androgen biosynthesis. In contrast, GnRH treatment alone stimulates progesterone and androgen production by cultured preovulatory follicles (Popkin et al. 1983).

3.3.2.7 FGFs

Fibroblast growth factors are single chain heparin binding growth factors (Burgess & Macaig, 1989) which are involved in embryonic development. There are two classes of FGFs; acidic (aFGF) and basic (bFGF). In the ovary they are recognised for their angiogenic properties and recent studies have detected bFGF mRNA in cultured granulosa cells at all stages of development (Grothe & Unsicker, 1989) and corpora lutea of cows (Stirling et al. 1991), though expression of FGF mRNA is believed to occur predominantly in endothelial cells (Koos & Olsen, 1989; Koos & Seidel, 1989). FGF stimulates bovine granulosa cell proliferation (Neufeld et al. 1987), but rat granulosa and theca cells do not proliferate in response to FGF (Gospodarowicz & Bialecki, 1979).

Basic FGF exerts inhibitory effects on FSH-stimulated oestradiol (Baird & Hsueh, 1986), inhibin (LaPolt et al. 1990b), and LH receptor induction (Baird & Hsueh, 1986) by granulosa cells. It also inhibits LH-stimulated androgen production by rat theca cells (Hurwitz et al. 1990). In contrast bFGF stimulates plasminogen activator expression, progesterone production (Baird & Hsueh, 1986), oocyte maturation and prostaglandin production (LaPolt et al. 1990c). All these events are associated with ovulation and thus suggest that FGF is involved in this process.

The angiogenic properties of FGFs implicate them in the process of corpus luteum establishment after ovulation. Similarly, the vascularisation of the thecal cell layer in the preovulatory follicle is likely to be subject to paracrine regulation by FGFs (Gospodarowicz & Ferrara, 1989).

3.3.2.8 Cytokines

Direct actions of a number of monocyte- and macrophage-derived cytokines such as the interferons, interleukins and tumor necrosis factor on
ovarian function have been demonstrated recently (Adashi, 1990). Macrophages, in particular, are known to constitute a significant cellular component of the ovarian interfollicular compartment (Hume et al. 1984).

Interleukin-1 (IL-1), a polypeptide cytokine predominantly produced and secreted by activated macrophages, has been shown to have a wide range of biological activities as well as playing a role as an immunological mediator. At the level of the ovary IL-1 suppresses the functional and morphological luteinization of cultured porcine and murine granulosa cells (Fukuoka et al. 1987; Fukuoka et al. 1989) and also inhibits LH-stimulated androgen production by theca cells (Hurwitz et al. 1991); its antigonadotrophic effect involving sites both distal and proximal to cAMP generation. The ability of IL-1 to inhibit granulosa cell differentiation is inversely related to its ability to promote granulosa cell growth (Fukuoka et al. 1989). The gene expression of IL-1 is progesterone dependent (Cannon & Dinarello, 1985); low levels of progesterone upregulate IL-1 gene expression (Polan et al. 1988) whilst higher concentrations significantly inhibit IL-1 activity.

Tumor necrosis factor (TNFα), a 157 amino acid polypeptide, is capable of attenuating the differentiation of cultured granulosa cells from immature rats by virtual neutralisation of FSH action at sites proximal but not distal to cAMP generation (Roby & Terranova, 1990). TNFα alters progesterone and androstenedione (Roby & Terranova, 1990) but not oestradiol production. This ability of TNFα to diminish the gonadotrophin supported accumulation of progesterone by granulosa cells is largely due to attenuation of key biosynthetic steps leading to progesterone production. Atretic granulosa cells have been implicated as possible sites for TNFα gene expression (Roby & Terranova, 1988), though granulosa cells enhance the release of IL-1 and TNFα from co-cultured macrophages.

Although the relevance of cytokine factors has yet to be established, the study of interactions between the immune system and the ovary is an area of intense recent attention. Given the actions of just two immunoregulatory peptides mentioned above, the immune cells of the ovary seem likely to be involved in the regulation of its function.
3.3.2.9 Other Factors

Apart from the local regulators mentioned previously in this section there are many other factors that potentially qualify for a role in the regulation of ovarian function. Many of the potential effects have only been observed \textit{in vitro} and more information is required before any speculation of physiological significance is possible.

The theca and secondary interstitial cells are directly innervated by adrenergic nerves though the significance of this innervation remains unclear. Catecholamines stimulate androgen production by theca cells (Dyer & Erickson, 1985), while noradrenaline causes a modest increase in basal androgen production (Hernandez et al. 1988a).

Free inhibin \(\alpha\) (Knight et al. 1989) and \(\beta\)-subunits (Robertson et al. 1992) have been shown to have potential regulatory roles in ovarian follicular development. Similarly, combinations of these inhibin subunits, as well as splice variations, have been identified in follicular fluid (Miyamoto et al. 1986; Sugino et al. 1989; Sugino et al. 1992) and thus potentially have a regulatory function in ovarian development.

Other factors implicated in the local regulation of ovarian function include platelet derived growth factor (May et al. 1990), nerve growth factor (Lara et al. 1990), components of the renin-angiotensin system (Lightman et al. 1989), substance P (Dees et al. 1985), vasoactive intestinal peptide (Ojeda et al. 1989), relaxin (Too et al. 1984), arginine vasopressin (Khan-Dawood & Dawood, 1989), plasminogen activators (Knecht, 1988), and inhibitors (Ny et al. 1985), extracellular matrix (Amsterdam et al. 1989) and many other factors for which effects have been observed on ovarian function.

4. Coordination of ovarian function: The ovarian cycle

The development of cyclic, regular ovulatory ovarian cycles results from regulated interactions of the hypothalamus, pituitary, and gonads as described above. By a process of feedback to the hypothalamus and pituitary, the ovary itself determines the length of the ovarian cycle. The mammalian ovarian cycle consists of a repeated process of follicular recruitment, selection, ovulation and corpus luteum formation then
The human menstrual cycle is usually divided into two phases: a follicular, or proliferative, phase and a luteal, or secretory, phase.

The follicular phase is the part of the cycle when small follicles are stimulated to grow in response to a rise in FSH levels and tonic LH levels. The required number of follicles which begin to secrete oestrogen are ‘selected’, while the remainder become atretic. During this phase, tonic LH levels are maintained while FSH levels fall in response to rising oestrogen secretion by the dominant follicle(s). The transition to the luteal phase is marked by a gonadotrophin surge, followed by ovulation some hours later. The ovulated follicle becomes the corpus luteum, which in humans secretes progesterone, oestradiol and inhibin. The life-span of the corpus luteum during a non-fertilised cycle is the length of the ensuing luteal phase. After the degeneration of the corpus luteum, gonadotrophin levels rise again and another cycle begins.

The duration of the ovarian cycle and its constituent follicular and luteal phases are different between species in both the absolute length of the ovarian cycle and in the relative duration of its follicular and luteal components. These differences, however, mask a fundamentally similar organisation and result only from minor but significant modifications to a basic pattern.

In humans and primates the follicular and luteal phases of the cycle are approximately the same duration and gonadotrophin-dependent development of small follicles does not begin until the degeneration of the corpus luteum. This is probably due to oestrogen, inhibin and progesterone production by the corpus luteum (Bassett et al. 1990; Baird, 1991), which feeds back to suppress gonadotrophin secretion from the pituitary.

In other species such as pigs, cows and sheep, the luteal phase is equivalent to the human, but their follicular phase is much shorter. The corpus luteum of these species does not secrete oestrogen or inhibin (Greenwald, 1979; Henderson & Franchimont, 1983; Mann et al. 1989b), and thus FSH levels are much higher during the luteal phase, which allows follicular development to occur despite the presence of a corpus luteum.

In species such as rats, mice and hamsters the corpora lutea do not secrete progesterone and degenerate in 2-3 days unless coitus has occurred. Follicular development in rats is triggered by a second FSH surge on the
morning of oestrus, at about the same time as ovulation (Barraclough & Wise, 1979). Therefore rats have a cycle of only 4-5 days. A functional luteal phase can be induced by mating the female with an infertile male to induce pseudopregnancy.

Rabbits and cats have permanent follicular phases, with repeated waves of antral follicular development and atresia occurring until mating, which triggers a LH surge, leading to ovulation and a luteal phase.

The differences in lengths of the two phases between species reflect different ovarian cycles: Primates have menstrual cycles where the endometrium is shed at the end of every luteal phase, and this endometrium must be regenerated before the next ovulation in preparation for embryo implantation. Thus the follicular phase is extended by suppressing gonadotrophin-dependent follicular development until the corpus luteum has degenerated.

4.1 Recruitment

Throughout life there is a continuous number of follicles that begin gonadotrophin-independent growth in response to unknown cues. Follicles do not respond to gonadotrophins until they have developed a theca cell layer (Goldenburg et al. 1973). By antral stages of development these follicles have granulosa cell FSH receptors and theca cell LH receptors (Oxberry & Greenwald, 1982; Uilenbroek & Richards, 1979). After the LH surge in rats and sheep the preovulatory follicle produces less oestradiol and inhibin (Barraclough & Wise, 1979; Watanabe et al. 1990; Campbell et al. 1990) and therefore FSH levels are allowed to peak for a second time (Butcher et al. 1974; Pant et al. 1977; McNeilly, 1988). It is this FSH surge that is responsible for the recruitment of the small antral follicles present in the ovary at that time, and thus follicular development begins during the luteal phase of the previous cycle. In primates the corpus luteum secretes oestradiol and inhibin and therefore gonadotrophin levels are suppressed due to negative feedback by these factors. It is only when the corpus luteum has degenerated that FSH levels are allowed to rise and the next wave of small follicles is recruited.

Theca and granulosa cells proliferate in response to the gonadotrophins and the theca cells produce androgens in response to the tonic LH levels. In
the mid to late follicular phase most follicles will become atretic and only a small number (species-specific) are destined to ovulate.

The recruited follicle contains a heterogenous population of granulosa cells; mural granulosa cells are the most differentiated and express steroidogenic enzymes (Goldschmit et al. 1989) and LH receptor (Oxberry & Greenwald, 1982; Richards et al. 1987; Whitelaw et al. 1992); granulosa cells surrounding the oocyte and lining the antrum are mainly proliferative (Hirshfield, 1986) and they express greatest amounts of mRNA for IGF-I (Oliver et al. 1989) which is implicated in the control of granulosa cell proliferation. This gradient of differentiated function is probably due to the greater access of mural granulosa cells to blood-borne FSH and other regulatory factors from both the theca and blood.

4.2 Selection

In response to FSH, the steroidogenic capacity and inhibin production of the developing granulosa cells increases. Throughout the follicular phase the plasma concentrations of oestradiol and inhibin increase, and feedback to the pituitary to suppress FSH levels. At this stage trophic support is decreasing and the growing follicle responds to this decline in different ways. Many of the follicles become atretic because they need higher levels of FSH to maintain their development. The most advanced, dominant, follicles with high gonadotrophin responsiveness continue to grow despite the declining trophic support. An important factor in the selection of a dominant follicle is a high rate of oestradiol synthesis for negative feedback to decrease pituitary FSH production. Equally important is the role of local intraovarian factors in mediating the effects of gonadotrophins at the level of the ovary, and thus follicles exposed to the same gonadotrophin concentrations respond differently. It should also be noted that the FSH-induced acquisition of LH receptors on mature granulosa cells enables these cells to respond to tonic levels of LH in the same way that they would respond to FSH. In this way LH can stimulate intracellular cAMP accumulation and act as a substitute for the lowered FSH levels.
4.3 Ovulation

As the dominant follicle develops, it produces increasing amounts of oestradiol, which eventually switch from negative to positive feedback control of the pituitary. This leads to a massive surge of gonadotrophins, in particular LH. The LH surge stimulates resumption of meiosis as well as production of progesterone (Moor, 1974) and prostaglandins (Morioka et al. 1989; Wong et al. 1989) by the granulosa cells of the preovulatory follicle. Progesterone promotes ovulation (Baranczuk & Fainstat, 1976; Greenwald, 1977; Tsafiriri et al. 1987) by interfering with collagen synthesis in the follicle wall (Tjugum et al. 1984). Prostaglandins are also capable of inducing ovulation (Strickland & Beers, 1976); an effect that is blocked by an inhibitor of prostaglandin synthesis, indomethacin (Canipari & Strickland, 1986). Prostaglandins increase blood flow to the follicle and increase vascular permeability (Brannstrom & Janson, 1991) which increases the volume of the follicular fluid. The last phase of preovulatory follicular growth is due to this increase in follicular fluid volume (Brannstrom & Janson, 1991).

The major class of proteolytic enzymes produced by granulosa and theca cells are plasminogen activators (Curry et al. 1989; Cajander, 1989) which are produced in response to gonadotrophins (Galway et al. 1990) and are maximal at the time of ovulation (Canipari & Strickland, 1985). Expression of collagenases is also stimulated by human chorionic gonadotrophin (hCG) (Reich et al. 1991). These enzymes degrade connective tissue in the apical region of the follicle wall; ovulation may be blocked partially by the administration of antibodies to plasminogen activator (Tsafiriri et al. 1989a). The gonadotrophin stimulation of plasminogen production is mediated through prostaglandin synthesis (Canipari & Strickland, 1986). Progesterone also augments this effect of gonadotrophins (Ny et al. 1985).

Prior to ovulation, gap junctions between the oocyte and the cumulus cells break down (Lindner et al. 1977), and the cumulus mass expands due to the intercellular hyaluronic acid accumulation (Eppig, 1991). The oocyte then re-initiates meiosis, possibly due to the removal of inhibitory factors such as cAMP or purines produced by the granulosa cells. The prostaglandin-induced increase in vascular permeability sustains a positive intrafollicular
pressure and provides the driving force for extrusion of the oocyte. A gradual reduction in the tensile strength of the follicular wall eventually leads to its complete rupture; the flow of follicular fluid and vascular transudate then carries the cumulus-enclosed oocyte out of the follicle on to the ovarian surface.

4.4 Corpus Luteum

After extrusion of the oocyte, the follicle collapses, the basement membrane fragments (Yoshinaga Hirabayashi et al. 1990) and the cells of the follicle wall fill the antrum, to form the corpus luteum. The new structure is invaded by blood vessels due to the release of angiogenic factors such as bFGF (Findlay, 1986). The corpus luteum secretes progesterone, and in primates it secretes oestradiol and inhibin as well (Savard et al. 1965; McLachlan et al. 1987; Fraser et al. 1989). The function of this is to provide a secretory endometrium capable of supporting pregnancy until the placenta is developed enough to take over this role. Accordingly, the granulosa cells of the former follicle cease proliferating, undergo hypertrophy and accumulate cytoplasmic lipid droplets and large numbers of mitochondria; all characteristics of steroid-secretory cells. This luteinization is the final stage of differentiation for granulosa cells. The granulosa-lutein cells lose their FSH receptors (Richards et al. 1976; Lee & Takahashi, 1977; Oxberry & Greenwald, 1982; Camp et al. 1991) but remain LH-responsive. The theca-lutein cells remain LH-responsive and secrete progesterone. In rats and mice, another pituitary hormone, prolactin, also stimulates luteal steroidogenesis (Hilliard, 1973), but its luteotrophic action is less clear in other species. The steroidogenic capacity of the luteal cells is at its greatest at the beginning of the luteal phase and declines thereafter, although their LH responsiveness may increase (Fisch et al. 1989). However, progesterone concentrations continue to rise during the luteal phase, probably due to the increasing vascularisation of the corpus luteum during the first half of the luteal phase (Niswender et al. 1976). During the second half of the luteal phase of a non-fertile cycle, progesterone secretion by the corpus luteum falls until the corpus luteum degenerates completely. The life-span of the corpus luteum is fixed in different species; in sheep the luteal phase occupies most of the
cycle, whereas in the rat there is no real luteal phase unless coitus takes place (Barraclough & Wise, 1979). The degeneration of the corpus luteum is independent of LH levels and is thought to be pre-programmed (Zeleznik, 1991). In the event of pregnancy chorionic gonadotrophin (CG) is secreted by the placenta of some species such as the human. This hormone acts via the LH receptor to rescue the corpus luteum and prolong its lifespan. The greater half-life of CG compared to LH (Rizkallah et al. 1969) and slower internalisation of CG-receptor complexes by luteal cells (Mock & Niswender, 1983) are believed to explain why LH cannot rescue the corpus luteum while CG can via the LH receptor.

5. Summary

The start of the ovarian cycle is characterised by the growth of primary follicles under gonadotrophic control. A rise in FSH stimulates the granulosa cell layer to proliferate and express differentiated functions, including LH receptor induction and production of inhibin and oestradiol. This oestradiol and inhibin exerts negative feed back on the pituitary to decrease FSH secretion. The decrease in trophic support leads to follicle degeneration in the majority of cases. However, some follicles are "selected" and progress to ovulation.

The fact that all follicles are exposed to comparable gonadotrophic stimulation, and yet undergo variable fates, suggests the existence of additional intraovarian modulatory systems that determine the fate of the follicle. Both steroidal (e.g. oestradiol) and non-steroidal (e.g. inhibin and IGF-I) regulatory factors of both granulosa and theca cell origin are implicated in this system. These factors engage in subtle intraovarian modulation of gonadotrophin-stimulated growth and differentiation, acting either in their own right, or more often as positive or negative modulators of gonadotrophin action. In this way the different cellular compartments of the follicle may be linked in order to facilitate coordinated follicular growth. This thesis concentrates on the paracrine actions of granulosa cell-derived inhibin on thecal/interstitial cell androgen synthesis. By means of such a paracrine mechanism the granulosa cells may regulate the adjacent thecal/interstitial
cell function, and thus the supply of androgen substrate for granulosa cell aromatisation during normal follicular oestrogen synthesis.

6. Structure of the Thesis

Gonadotrophins exert a primary endocrine regulation on follicular development in the ovary. This endocrine control is believed to be subject to a secondary local level of control by factors of ovarian origin. The two-cell, two-gonadotrophin model of oestrogen synthesis requires FSH to stimulate granulosa cell oestradiol production from androgens derived from LH-stimulated theca cells. The major steroidogenic enzyme under FSH control during oestrogen synthesis is granulosa cell P450arom, while LH controls thecal cell P45017a activity. The latter activity is vital for follicular androgen production, and thus the provision of substrates for granulosa cell aromatisation. This thesis describes the effects of gonadotrophins on aspects of follicular growth and differentiation, then provides evidence for the existence of a paracrine level of control of gonadotrophin action, and then finally tests the hypothesis that inhibin is involved in such paracrine signalling.

Firstly, the endocrine control of follicular development by FSH and LH was investigated. Previous studies of the effects of FSH on follicular development have used pituitary or urinary gonadotrophin preparations containing finite amounts of LH contamination and thus interpretation of data has been difficult. The availability of recombinant forms of gonadotrophins has enabled experiments to be designed to dissect their precise role in follicular development. Chapter 1 presents the results of a study designed to determine the contributions of FSH and LH to normal follicular growth and differentiation, using both in vivo and in vitro techniques.

Secondly, the local control of gonadotrophin action on the ovary was investigated. During the ovarian cycle the levels of LH remain constant throughout except for a midcycle surge, whereas the levels of FSH are variable throughout the cycle. In order for the thecal/interstitial cells to respond appropriately to LH during the ovarian cycle, they presumably rely
on paracrine signals from FSH-stimulated granulosa cells to modulate the actions of constant levels of LH. Therefore, the actions of LH on thecal/interstitial function (androgen synthesis) would appear to be regulated by FSH via granulosa cell-derived paracrine signalling. Chapter 2 describes the validation of a thecal/interstitial cell culture system for the study of the control of thecal androgen synthesis. Chapter 3 provides direct \textit{in vivo} evidence for FSH-activated granulosa\textrightarrow{theca} signalling in the ovary, and also describes a study of the modulation of LH-stimulated thecal cell androgen synthesis by gonadotrophins and putative regulatory factors of granulosa cell origin. Chapter 4 presents the results from an \textit{in situ} hybridisation study to determine the control, by gonadotrophins, of thecal cell P45017α mRNA expression, the enzyme that is essential for thecal/interstitial androgen synthesis. Inhibin, because of its development-related pattern of expression by FSH-stimulated granulosa cells and its positive modulatory effects on LH-stimulated thecal androgen production, is a potential paracrine signal in the growing follicle. The final chapter describes the results from a study to test the effects of the removal of inhibin, by immunoneutralisation, on rat ovarian follicles cultured \textit{in vitro}. 
Chapter 2. Relative Roles of Gonadotrophins

1. Introduction

Development of a follicle from the preantral to the preovulatory stage requires both cell proliferation (Peters et al. 1975) and differentiation (Hsueh et al. 1984). Preantral follicles contain relatively few, non-differentiated, granulosa cells, whereas preovulatory follicles contain many more granulosa cells which are morphologically and biochemically differentiated (Hirshfield, 1985). For normal follicular differentiation and oestrogen synthesis both FSH and LH are clearly necessary (Fevold, 1941; Lostroh & Johnson, 1966), however the precise role of each gonadotrophin in the control of cellular proliferation and follicular development is not entirely clear.

The pituitary gonadotrophins, FSH and LH, have been available in a ‘purified’ form for over fifty years. Using purified ovine pituitary gonadotrophins it was demonstrated that both FSH and LH were necessary for normal follicular development and steroidogenesis (Fevold, 1941; Greep et al. 1942). However until the availability of recombinant (and therefore ‘pure’) forms of gonadotrophins it was not possible to design experiments to dissect out the precise requirements for each gonadotrophin to stimulate preovulatory follicular development and steroid synthesis in the ovary.

FSH stimulates growth of ovarian follicles in vivo (Greep et al. 1942) by inducing proliferation of granulosa cells (Roy & Greenwald, 1991) and directly causes granulosa cells to differentiate (Hsueh et al. 1984). This differentiated state is expressed as development of steroidogenic activity, mainly progesterone (Hillier et al. 1978; Richards et al. 1979) and oestradiol (Dorrington et al. 1975; Moon et al. 1978), and the expression of LH receptors (Zeleznik et al. 1974; Richards et al. 1976). LH alone has been reported to stimulate limited follicular growth (Greep et al. 1942) due in the most part to thecal hypertrophy (Erickson et al. 1985), probably caused by the atretic actions of androgens (Hillier & Ross, 1979). In combination with FSH it triggers ovulation and the subsequent formation and maintenance of corpora lutea (Greep et al. 1942; Lostroh & Johnson, 1966). LH acts in the developing follicle to stimulate thecal cell production of androgens (Erickson et al. 1985),
which are substrates for oestrogen synthesis by granulosa cells (Hillier & deZwart, 1982). As the follicle grows FSH stimulates granulosa cell LH-receptor induction (Zeleznik et al. 1974; Richards et al. 1976) and these cells respond by producing more steroids (Hsueh et al. 1984). High levels of LH induce ovulation and granulosa cell luteinization.

It has been reported that recombinant human FSH (rh-FSH) alone can induce ovulation (Galway et al. 1990), though since the experimental animals received exogenous oestrogen it is possible that oestrogen (probably not normally produced in the absence of LH) may have affected the action of FSH. It has also been demonstrated that follicular development and aromatase activity are increased by rh-FSH (Mannaerts et al. 1991). The same report showed that plasma levels of oestradiol are only increased by FSH in the presence of a surrogate LH, hCG. This chapter describes a study designed to determine the precise contribution of each gonadotrophin and the paracrine factors under their control to normal follicular development and oestrogen synthesis, using rh-FSH and LH in vitro and in vivo.

2. Materials and Methods

2.1 Animals

Intact or hypophysectomised (Charles River, Margate, Kent, UK.) immature female Wistar rats were housed in a temperature and light-controlled room. Animals were fed on proprietary rat food ad libitum and hypophysectomised animals also received a 1% (w/v) dextrose solution (The Boots Company Plc., Nottingham, UK.). Experimental groups (n≥6) received treatment with ovine FSH (NIADDK-o-FSH-16; FSH bioactivity 20 IU/mg, LH bioactivity 0.04 x NIH-LH-S1), (National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, USA) given as four 12-hourly subcutaneous (SC) injections (total dose 80 μg/animal); rh-FSH (GONAL-F™, 13,096 IU FSH/mg), (Serono Laboratories UK Ltd, Welwyn Garden City, Herts. UK) given as four 12-hourly SC injections (total dose 3-100 IU/animal) and r-LH (13,108 IU LH/mg), (Serono Laboratories UK Ltd) given as four 12-hourly SC injections (total dose 0.1-30 IU/animal). For comparison, other groups of animals received human menopausal gonadotrophin (HMG)
(Pergonal™, Serono Laboratories UK Ltd.) at a total dose of 40 IU of both LH and FSH or a LH-containing gonadotrophin preparation as a single injection (15 IU) of PMSG (Sigma, Poole, Dorset, UK). Where hCG was administered in vivo, to mimic a LH surge, 20 IU hCG (Sigma) were injected subcutaneously 12 h before killing the animals. Controls received injection vehicle alone, which was phosphate buffered saline (PBS) containing 0.1% (w/v) bovine serum albumin (BSA) (ICN Biomedicals, High Wycombe, Bucks, UK). Some animals were also treated with diethylstilboestrol (DES) (Sigma) using 1 x 0.3 cm silastic tubing capsules containing solid DES, implanted SC under general anaesthesia in the throat region of the neck. All animals were killed by carbon dioxide asphyxiation at 25 (intact) or 27 (hypophysectomised) days of age, 48 h after initiating treatment. Ovaries were immediately removed and dissected free from fat and other extraneous tissue.

2.2 Granulosa cell culture

Granulosa cells from preantral and early antral follicles were harvested in culture medium by gentle puncture with a hypodermic needle. Cells were then centrifuged at 1500 rpm for 10 min and resuspended in 1.0 ml of medium. To disperse the cells and to eliminate clumps, the suspension was drawn through a pipette tip several times, made up to 10 ml with medium, and allowed to settle for 1 min. The cells remaining in suspension were then centrifuged, resuspended and triturated again as before. An aliquot of the cell suspension was removed and mixed with an equal volume of trypan blue solution, and cells in this mixture were counted using a haemocytometer. Viability was assessed based on the exclusion of the dye, and was >40%. Cells were cultured in 500 μl Medium 199 containing 25 mM HEPES buffer, extra (2 mM) L-glutamine, penicillin (50 IU/ml) and streptomycin (50 mg/ml) (all from Gibco Ltd, Paisley, Renfrewshire, UK) with 0.1% (w/v) bovine serum albumin (BSA). Multiwell plastic dishes (Linbro Space Savers, Flow Laboratories, Rickmansworth, Herts, UK) were precoated with donor calf serum (Gibco) and washed twice with 1 ml Dulbecco’s phosphate buffered saline (DPBS; Oxoid) before use (Hillier &
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deZwart, 1981). The culture wells were inoculated with replicate 250 µl portions of cell suspension containing 40,000 viable granulosa cells. The granulosa cells were incubated for 48 h. In vitro treatments were with human pituitary FSH (LER 8/116; FSH bioactivity 900 IU/mg, LH bioactivity 0.5 IU/mg), (generously donated by Prof L.E. Reichert, Jr.) at a final concentration of 0.1-100 ng/ml; ovine LH (oLH) (NIADDK-oLH-26; NIADDK-NIH, Bethesda, MD) at a final concentration of 0.1-10 ng/ml; a 10⁻⁴ M solution of testosterone (17β-hydroxyandrost-4-en-3-one; Sigma) in ethanol at a final concentration of 10⁻⁶ M; rh-FSH at a final concentration of 0.1-1000 ng/ml; rh-IGF-I (CGP-35M26, donated by Drs. H.H. Peter and K. Scheibli, Ciba-Geigy) at a final concentration of 30 ng/ml; rh-activin-A (Genentech Inc, San Francisco, CA) at a final concentration of 30 ng/ml; rh-inhibin-A (Genentech Inc) at a final concentration of 30 ng/ml. The spent culture medium was collected and stored frozen at -20°C.

2.3 Measurement of DNA synthesis

Tritiated thymidine uptake was determined using the method of May et al. (1988). After removing spent medium, the cell monolayer was washed once with Dulbecco’s PBS and incubated for a further 18 h at 37°C in 500 µl fresh medium containing 1.0 µCi [methyl-³H]thymidine (1.5 Ci/mmol; Amersham). The monolayers were then washed twice with ice-cold Dulbecco’s PBS and fixed by incubation for 20 min in 1.0 ml 5% (w/v) trichloroacetic acid. After washing once with trichloroacetic acid and twice with ice-cold methanol, the cells were dissolved in 500 µl 0.5 M NaOH. The digest was then neutralised by addition of 500 µl 0.5 M HCl. The solution was then aspirated and 3 ml scintillation cocktail (RIALUMA, Lumac LSC b.v., Olen, Belgium) was added. The tritium content of the cultures were measured by liquid scintillation spectrophotometry (RackBeta, LKB Wallac, Turku, Finland).

2.4 Bromodeoxyuridine (BrdU) measurement

To study the effects of gonadotrophins on cell proliferation in vivo we localised incorporated thymidine analogue, BrdU, using monoclonal
antibodies. Three hours before they were killed, animals received a SC injection of 0.1 ml BrdU at a concentration of 1mg/10g animal in PBS containing 0.1% BSA. After sacrifice ovaries were removed and dissected free from fat and other extraneous tissue. Ovaries were fixed for one hour in "3+1" (3 parts absolute ethanol and 1 part glacial acetic acid) followed by overnight at 4°C in 4% (w/v) paraformaldehyde (Sigma) dissolved in PBS. The tissue was then automatically processed overnight (Shandon Processor 2LE; Shandon Scientific Ltd, UK) through graded ethanol solutions, followed by xylene, 1:1 xylene-paraffin, then paraffin wax. Prepared tissues were then placed in moulds containing melted paraffin, which was then allowed to solidify.

Slides were cleaned and coated to help tissue section retention. Firstly slides were left overnight in chromic acid (9 parts 2% potassium dichromate, 1 part sulphuric acid) (both Sigma). This was followed by a 5 h wash in running water, then a rinse in distilled water and the slides were allowed to air dry. Once the slides had dried they were twice dipped in a 2% (v/v) solution of 3-aminopropyltriethoxysilane (TESPA) in acetone for 10 sec, followed by two 10 sec washes in acetone. The slides were then rinsed in distilled water then baked dry at 42°C.

Using a standard rotary microtome (American Optical Corporation), tissue was sectioned at 5μm thickness to produce a ribbon of sections. This ribbon was floated on the surface of water in a water bath. Sections were mounted by inserting a coated slide into the water bath near the sections at a 45° angle to the water surface, touching an edge of the ribbon to the slide and slowly withdrawing the slide from the bath. Slides were placed in an oven overnight at 40°C to ensure sections had adhered to the slide.

Tissue sections were deparaffinised and rehydrated by immersing slides in two changes of xylene for 10 min each time. The slides were then passed sequentially, for 2 min each, through 100%, 100%, 90% (v/v), 75% (v/v), 50% (v/v), and 30% (v/v) ethanol then 1 min in water, then slides were washed three times in PBS. To each section was added 70 μl of anti-BrdU antibody (IgG2a antibody and nuclease in tris buffered saline pH9 containing 1.0 % BSA, magnesium chloride and preservative) (Amersham International plc, Buckinghamshire, UK) and slides were incubated for 2 h. Slides were washed three times in PBS, and to each section was added 70 μl
of peroxidase anti-mouse IgG (peroxidase conjugated antibody in PBS containing 1.0 % BSA and preservative) (Amersham) and slides were incubated for 30 min. Slides were washed three times in PBS and immersed in diamino benzidine (DAB) staining solution for 10 min. After three washes in water, sections were dipped in a cytoplasmic counterstain, light green. Slides were then dehydrated through successively increasing alcohol solutions; 1 min in each of 30% (v/v), 50% (v/v), 75% (v/v), 90% (v/v) and 100% ethanol followed by 5 min in xylene. Coverslips were then mounted using Eukitt (O. Kindler GmbH & Co, Freiburg, Germany) which was then allowed to dry for 4 h.

2.5 Measurement of ovarian and uterine weight

Ovaries and uteri were dissected of all fat and extraneous tissue and then weighed on an electronic balance (Cahn TA 4100; Cahn, Cerritos, CA). Results are expressed as weight of ovarian pair (mg) or intact uterine horn (mg).

2.5 Steroid assays

2.5.1 Progesterone Radioimmunoassay

Progesterone (4-pregnene-3,20-dione) was measured in culture media by specific radioimmunoassay. The assay buffer was PBS/gelatin (0.05M sodium phosphate, 0.15M NaCl, 0.1% gelatin). The antibody, anti-progesterone-11α-hemisuccinate-BSA (R31/12), was provided by Medical Research Council, Mill Hill. Antibody was used at a working dilution of 1:4,500. Cross-reaction of the antibody with oestriadiol-17β, ICI 164,384, ICI 164,275 or SCH 16423 was <0.01 % (Turner, 1992). Cross-reaction with testosterone, 5α-dihydrotestosterone and 2-hydroxyoestradiol was 0.04 %, 0.05 % and 0.02 %, respectively. Tracer, [1,2,6,7-3H]-Progesterone (80-110 Ci/mmol, Amersham), was stored at -20°C in ethanol, at a concentration of 10 μCi/ml. Before use this stock was diluted 1:400 in assay buffer to give ~10,000 cpm/500 μl. A standard curve covering the range of 0.04 to 5.1
pmoles/tube progesterone (Sigma), made up in culture medium, was used. Samples (5-100 µl) or standards (in 200 µl) were placed in tubes, and the volume of samples was adjusted to 200 µl. Quality controls (0.26 and 2.7 pmoles/tube) were also included at the beginning and end of each assay. Antibody (300 µl) and tracer (500 µl) were added, and tubes were incubated for 3 h at room temperature. Tubes were placed on ice for 30 min before addition of 200 µl dextran-coated charcoal (1.25 % (w/v) dextran, 25 % (w/v) charcoal in assay buffer) to separate bound and free progesterone. Tubes were mixed and incubated for 10 min on ice, then centrifuged at 3000 rpm for 10 min at 4°C. Supernatants were decanted, 3 ml scintillation cocktail (RIALUMA) was added, and bound tracer was measured by liquid scintillation counting (RackBeta). The β-counter had a 36 % efficiency as assessed by external standardisation. Standard curves were calculated using a commercial computer program (AssayZap, Biosoft, Cambridge, UK). Samples which were outside the detection limits were re-assayed at the appropriate volume. The detection limit of the assay was within the range 0.05-0.06 pg/tube. Inter- and intra-assay coefficients of variation were <15%.

2.5.2 Oestradiol Radioimmunoassay

Oestradiol-17β was measured in culture media by specific radioimmunoassay. The assay buffer was PBS/gelatin (0.05M sodium phosphate, 0.15M NaCl, 0.1% gelatin). The donkey K3 anti-oestradiol-17-hemisuccinyl-BSA antiserum was provided by Dr. G. Read, Tenovus Institute, Cardiff. Antibody was used at a working dilution of 1:75,000. Major cross-reactions were: oestradiol-17β, 100 %; oestrone, <10% and <0.5 % for all other steroids tested. Tracer, [125I]-Oestradiol-3-carboxymethylether, was diluted in assay buffer to give ~12,000 cpm/200µl. A standard curve covering the range of 0.015 to 1.8 pmoles/tube oestradiol-17β (Sigma), made up in culture medium, was used. Samples (5-100 µl) or standards (in 100 µl) were placed in tubes, and the volume of samples was adjusted to 100 µl. Quality controls (0.17 and 0.37 pg/tube) were also included at the beginning and end of each assay. Antibody (200 µl) and tracer (200 µl) were added, and tubes were incubated at room temperature for 3 h. Tubes were placed on ice for
15 min before addition of 500 µl dextran-coated charcoal to remove unbound steroid. Tubes were mixed and incubated for 10 min on ice, then centrifuged at 3000 rpm for 10 min at 4°C. Supernatants were decanted and bound tracer was measured by γ-counting (NE1600; Nuclear Enterprises, Edinburgh, Scotland). Standard curves were calculated using a commercial computer program (AssayZap). Samples which were outside the detection limits were re-assayed at the appropriate volume. The detection limit of the assay was in the range 0.015-0.018 pg/tube. Inter- and intra-assay coefficients of variation were less than 15%.

2.5.3 Androstenedione Radioimmunoassay

Androstenedione in spent culture medium was determined by specific radioimmunoassay. The assay buffer was PBS/gelatin (0.05M sodium phosphate, 0.15M NaCl, 0.1% gelatin). The androstenedione antiserum was rabbit anti-androst-4-ene-3,17-dione-7α-carboxyethylthioether-BSA (Hillier et al. 1991b). Major cross-reactions were: androsterone 46.3 %; 5α-androstane-3,17-dione 50 %; testosterone 37 %; and less than 0.5 % for all other steroids tested. Tracer, [1,2,6,7 3H]-Androst-4-ene-3,17-dione, was diluted in assay buffer to give ~10,000 cpm/500 µl. A standard curve covering the range of 0.025 to 6.4 pmoles/tube androstenedione (Sigma), made up in culture medium, was used. Samples (5-100 µl) or standards (in 200 µl) were placed in tubes, and the volume of samples was adjusted to 200 µl. Quality controls (0.08 and 2.4 pmoles/tube) were also included at the beginning and end of each assay. Antibody (300 µl) and tracer (500 µl) were added, and tubes were incubated at 37°C for 1 h then at 4°C for 1 h. Tubes were placed on ice before addition of 200 µl dextran-coated charcoal (1.25 % dextran 25 % charcoal in assay buffer) to separate bound and free steroid. Tubes were mixed and incubated for 10 min on ice, then centrifuged at 3000 rpm for 10 min at 4°C. Supernatants were decanted, 3 ml scintillation cocktail (RIALUMA) was added, and bound tracer was measured by liquid scintillation counting (RackBeta). Standard curves were calculated using a commercial computer program (AssayZap). Samples which were outside the detection limits were re-assayed at the appropriate volume. The detection limit of the assay was in
the range 0.03-0.04 pmoles/tube. Inter- and intra-assay coefficients of variation were less than 15%.

2.6 Gonadotrophin levels in serum

2.6.1 Follicle stimulating hormone and Luteinizing hormone

Human FSH and LH in serum were measured by specific immunoradiometric assay (Serono FSH MAIAclone™ and Serono LH MAIAclone™). The 125I anti-FSH and -LH reagent consisted of fluorescein and 125I labelled mouse monoclonal antibodies to the gonadotrophin, <10 μCi per vial in the FSH assay and <8 μCi per vial in the LH assay, in PBS containing normal sheep serum, BSA, inert dye and 0.2 % (w/v) sodium azide. The zero standard was bovine serum with 0.2 % (w/v) sodium azide. A standard curve covering the range of 0.5 to 150 mIU/ml of gonadotrophin per ml (FSH; 2nd IRP 78/549. LH; 1st IRP 68/40) in bovine serum with 0.2 % (w/v) sodium azide was used. Samples (100 μl) or standards (in 100 μl) were placed in tubes. Serotest™ quality control (FSH; 7.13 U/l. LH; 6.86 U/l) was also included at the beginning and end of each assay. The 125I anti-gonadotrophin reagent was added, and tubes were incubated at room temperature for 1 h. 200 μl of MAIAclone separation reagent (sheep antiserum to fluorescein covalently bound to magnetic particles in Tris buffer with BSA and 0.1 % (w/v) sodium azide) was added to each tube and tubes were incubated for 5 min at room temperature. Particles were magnetically separated for 2 min on a MAIA magnetic separator, the supernatant was decanted and 500 μl of wash buffer (Tris buffer and 0.1 % (w/v) sodium azide) was added, the tubes were vortexed and then allowed to separate magnetically for a further 2 min. Supernatants were decanted and tubes were measured by γ-counting (NE1600). Standard curves were calculated using a commercial computer program (AssayZap). Samples which were outside the detection limits were re-assayed at the appropriate volume. The sensitivity of the FSH assay was < 0.25 mIU/ml, while that of the LH assay was < 0.15 mIU/ml.
2.7 Statistics

Statistical analysis was carried out using commercial software (CLR ANOVA; Clear Lake Research Inc., Houston, TX, USA). Analysis of variance with the Newman-Keuls test was used to analyse differences between experimental and control observations. Differences assigned a $P$ value of $<0.05$ were regarded as statistically significant.

3. Results

3.1 Effect of gonadotrophins and DES on BrdU localisation in vivo

Bromodeoxyuridine uptake by proliferating cells was used to determine the effects of gonadotrophins and DES on the proliferating population of cells in the ovary. Basal proliferation in intact animals as estimated by BrdU localisation was found to be low (Fig. 2.1A). The administration of oFSH caused an increase in the number of antral follicles and a large increase in proliferation in granulosa cells throughout these follicles (Fig. 2.1C). Many thecal/interstitial cells appeared to be labelled also. The administration of oFSH and hCG caused an increase in the number of preovulatory follicles and a centripetal pattern of proliferation whereby antral granulosa cells were mainly proliferating and only a few cells at the granulosa-theca cell boundary were proliferating (Fig. 2.1E).

The administration of DES (Fig. 2.1B) increased basal levels of proliferation though the effect of FSH (Fig. 2.1D) was no greater than in the absence of DES. Again treatment with FSH/hCG caused preovulatory follicles to exhibit a centripetal pattern of proliferation (Fig. 2.1F). The corpus luteum showed no signs of proliferation (Fig. 2.1G) and the only BrdU labelling was by blood vessels.
Fig. 2.1. Bromodeoxyuridine labelling of rat ovaries in vivo showing dividing granulosa cells. Three hours before they were killed animals received a subcutaneous injection of 0.1 ml bromodeoxyuridine (BrdU) at a concentration of 1mg/10g body weight in PBS containing 0.1% (w/v) BSA. Ovaries were removed, fixed and incorporated BrdU was localised using monoclonal antibodies. Bar = 100μm. A Section from an untreated immature rat ovary showing low levels of BrdU incorporation. C Section from an oFSH treated (total dose 80 μg/animal) rat ovary showing increased BrdU incorporation due to FSH treatment. E Section from an oFSH (total dose 80 μg/animal) then hCG treated (20 IU) rat ovary showing BrdU incorporation predominately in antral granulosa cells in a centripetal pattern of labelling. B Section from a DES treated immature rat ovary showing high levels of BrdU incorporation due to DES treatment. D Section from a DES and oFSH treated (total dose 80 μg/animal) rat ovary showing further increased BrdU incorporation due to FSH treatment. F Section from a DES and oFSH (total dose 80 μg/animal) then hCG treated (20 IU) rat ovary showing BrdU incorporation predominately in antral granulosa cells in a centripetal pattern of labelling. G Corpus luteum showing background incorporation of BrdU only at the site of blood vessels.
3.2 Effect of gonadotrophins on granulosa cell proliferation and differentiation in vitro

3.2.1 Thymidine uptake

Thymidine uptake (Fig. 2.2) was used as a measure of granulosa cell proliferation in vitro. Animals were treated with FSH (to produce differentiated granulosa cells), FSH+hCG (to produce preovulatory granulosa cells) or injection vehicle alone (to produce non-differentiated granulosa cells) in vivo and then these cells were treated in vitro with FSH or LH with or without testosterone. Differentiated granulosa cells exhibited basal levels of thymidine uptake approximately one half of levels observed in non-differentiated granulosa cells, whilst preovulatory granulosa cells have an even lower level of basal thymidine uptake. The addition of FSH or LH in vitro approximately halved the thymidine uptake by non-differentiated, differentiated or preovulatory granulosa cells. Likewise, testosterone treatment in vitro also halved thymidine uptake by granulosa cells at all stages of differentiation. Interestingly, FSH in the presence of testosterone further decreased thymidine uptake by granulosa cells at all stages of differentiation. However, LH only further decreased thymidine uptake in the presence of testosterone by differentiated and preovulatory granulosa cells whilst it had no measurable effect on non-differentiated granulosa cells treated with testosterone.

Overall, a pattern was established whereby gonadotrophin treatment in vitro decreased thymidine uptake by granulosa cells. Similarly granulosa cells of increasingly differentiated state in vivo showed decreasing levels of thymidine uptake in vitro.
Fig. 2.2. Effects of gonadotrophins and/or testosterone on tritiated thymidine uptake by non-differentiated (control), differentiated (FSH-treated) and preovulatory (FSH/hCG-treated) granulosa cells. Granulosa cell cultures (40,000 viable cells per well) were incubated for 48 h in serum-free medium containing hFSH (30 ng/ml), oLH (30 ng/ml) and/or testosterone (1μM). Tritiated thymidine uptake was determined after 48 h of culture as described in section 2.3. Values are mean ± SE, data are representative of 3 experiments. Asterisks denote significant inhibition by in vitro treatment vs. corresponding control (C or T): *, P < 0.01.
3.2.2 Progesterone production

Progesterone production in vitro (Fig. 2.3) was used as an indication of granulosa cell differentiation. Non-differentiated, differentiated and preovulatory granulosa cells were treated in vitro with FSH or LH with or without testosterone. Differentiated granulosa cells exhibited higher basal levels of progesterone production than levels observed in non-differentiated granulosa cells, whilst preovulatory granulosa cells had a much greater level of basal progesterone production. The addition of FSH or LH increased progesterone production by non-differentiated, differentiated or preovulatory granulosa cells. Testosterone increased progesterone production by differentiated and preovulatory granulosa cells. FSH or LH in the presence of testosterone further increased progesterone production by granulosa cells at all stages of differentiation. Granulosa cells from preovulatory follicles were more responsive to LH+T than FSH+T in vitro. There was no significant difference in the way that differentiated and non-differentiated granulosa cells responded to gonadotrophin treatment in vitro in the presence of testosterone.

The general pattern that was established showed that gonadotrophin treatment in vitro increased progesterone production by granulosa cells. Similarly granulosa cells of increasingly differentiated state in vivo showed increasing levels of progesterone production in vitro.

3.2.3 Oestradiol production

Oestradiol production in vitro (Fig. 2.4) was used as a measure of granulosa cell differentiation. Non-differentiated, differentiated and preovulatory granulosa cells were treated in vitro with FSH or LH with or without testosterone. In the absence of aromatase substrate, testosterone, there was no measurable oestradiol production. Testosterone increased basal levels of oestradiol production; differentiated and preovulatory granulosa cells produced much higher levels of oestradiol than non-differentiated cells. FSH in the presence of testosterone further increased oestradiol production by granulosa cells at all stages of differentiation, especially by non-differentiated granulosa
Fig. 2.3. Effects of gonadotrophins and/or testosterone on basal progesterone synthesis by non-differentiated (control), differentiated (FSH-treated) and preovulatory (FSH/hCG-treated) granulosa cells. Granulosa cell cultures (40,000 viable cells per well) were incubated for 48 h in serum-free medium containing hFSH (30 ng/ml), oLH (30 ng/ml) and/or testosterone (1μM). Progesterone in the spent culture medium was determined by RIA; values are mean ± SE, data are representative of 3 experiments. Asterisks denote significant stimulation by in vitro treatment vs. corresponding control (C or T): *, $P < 0.01$; **, $P < 0.05$. 
Fig. 2.4. Effects of gonadotrophins and/or testosterone on basal oestradiol synthesis by non-differentiated (control), differentiated (FSH-treated) and preovulatory (FSH/hCG-treated) granulosa cells. Granulosa cell cultures (40,000 viable cells per well) were incubated for 48 h in serum-free medium containing hFSH (30 ng/ml), oLH (30 ng/ml) and/or testosterone (1 μM). Oestradiol in the spent culture medium was determined by RIA; values are mean ± SE, data are representative of 3 experiments. Asterisks denote significant stimulation by in vitro treatment vs. corresponding control (C or T): *, \( P < 0.01 \).
cells. LH in the presence of testosterone only increased oestradiol production by non-differentiated granulosa cells.

Generally, gonadotrophin treatment in vitro increased oestradiol production by granulosa cells. Similarly granulosa cells of increasingly differentiated state in vivo showed increasing levels of oestradiol production in vitro.

3.3 Effect of FSH and LH on granulosa cell proliferation and differentiation in vitro

The dose-dependence of FSH (pituitary and rh-FSH) on thymidine uptake (Fig. 2.5), progesterone (Fig. 2.6) and oestradiol production (Fig. 2.7) was established in experiments on intact animals not treated with DES. Both pituitary and recombinant FSH dose-dependently decreased thymidine uptake (Fig. 2.5). Maximum responses occurred in the presence of 30 ng/ml pituitary FSH and 1000 ng/ml rh-FSH with the ED50 being ~3 ng/ml and ~20 ng/ml respectively. Both pituitary and recombinant FSH dose-dependently increased progesterone and oestradiol production by granulosa cells in vitro (Fig. 2.6 and 2.7). Maximum responses occurred in the presence of 30 ng/ml pituitary FSH and 300 ng/ml rh-FSH with the ED50 being ~3 ng/ml and ~30 ng/ml respectively. LH at the doses tested had no significant effect on any of the parameters measured. In all cases an approximately 10-fold higher dose of rh-FSH than pituitary FSH was necessary to elicit a similar level of response.

3.4 Effect of DES on granulosa cell proliferation and differentiation in vitro

Results achieved in the presence or absence of DES were not qualitatively different from each other except for the results observed with non-differentiated cells. Untreated granulosa cells did not respond to LH, whereas DES treatment in vivo rendered granulosa cells LH-responsive in vitro (Table 2.1).
Fig. 2.5. Effects of pituitary hFSH, rh-FSH and oLH on tritiated thymidine uptake by non-differentiated granulosa cells. Granulosa cell cultures (40,000 viable cells per well) were incubated for 48 h in serum-free medium containing pituitary hFSH (0.1-100 ng/ml), rh-FSH (0.1-1000 ng/ml) or oLH (0.1-10 ng/ml). Tritiated thymidine uptake was determined after 48 h of culture as described in section 2.3. Composite data from 5 experiments are expressed as mean ± SE. Asterisks denote significant inhibition by in vitro treatment vs. untreated control (C): *, P < 0.01.
Fig. 2.6. Effects of pituitary hFSH, rh-FSH and oLH on progesterone production by non-differentiated granulosa cells. Granulosa cell cultures (40,000 viable cells per well) were incubated for 48 h in serum-free medium containing pituitary hFSH (0.1-100 ng/ml), rh-FSH (0.1-1000 ng/ml) or oLH (0.1-10 ng/ml). Progesterone in the spent culture medium was determined by RIA; Composite data from 5 experiments are expressed as mean ± SE. Asterisks denote significant stimulation by in vitro treatment vs. untreated control (C): *, P < 0.01.
Fig. 2.7. Effects of pituitary hFSH, rh-FSH and oLH on oestradiol production by non-differentiated granulosa cells. Granulosa cell cultures (40,000 viable cells per well) were incubated for 48 h in serum-free medium containing 1.0 μM testosterone (aromatase substrate) and pituitary hFSH (0.1-100 ng/ml), rh-FSH (0.1-1000 ng/ml) or oLH (0.1-10 ng/ml). Oestradiol in the spent culture medium was determined by RIA; Composite data from 5 experiments are expressed as mean ± SE. Asterisks denote significant stimulation by in vitro treatment vs. untreated control (C): *, P < 0.01; **, P < 0.05.
Table 2.1. Effect of DES treatment in vivo on LH-induced thymidine uptake and oestradiol synthesis in vitro. Granulosa cell cultures (40,000 viable cells per well) were incubated for 48 h in serum-free medium containing 1.0 μM testosterone (aromatase substrate) and oLH (30 ng/ml). Oestradiol in the spent culture medium was determined by RIA. Tritiated thymidine uptake was determined after 48 h of culture as described in section 2.3. Values are expressed as a percentage ± SE of control values in vitro. Asterisks denote significant difference due to DES treatment: *, P < 0.01.

3.5 Effect of peptide growth factors on granulosa cell proliferation and differentiation in vitro

3.5.1 Thymidine uptake

The effect of peptide growth factors on granulosa cell proliferation in vitro was tested. Non-differentiated, differentiated and preovulatory granulosa cells were treated in vitro with IGF-I, inhibin or activin. Differentiated granulosa cells exhibited basal levels of thymidine uptake approximately one quarter of levels observed in non-differentiated granulosa cells, whilst preovulatory granulosa cells have an even lower level of basal thymidine uptake (Fig. 2.8). Treatment with IGF-I or inhibin reduced levels of thymidine uptake by granulosa cells at all stages of maturity; especially non-differentiated cells. Activin also reduced non-differentiated granulosa cell thymidine uptake dramatically, however, it reduced differentiated and preovulatory granulosa cell thymidine uptake to even lower levels.

3.5.2 Progesterone production

The effect of peptide growth factors on granulosa cell progesterone production in vitro was tested. Non-differentiated, differentiated and preovulatory granulosa cells were treated in vitro with IGF-I, inhibin or
Fig. 2.8. Effects of IGF-I, inhibin and activin on tritiated thymidine uptake by non-differentiated (control), differentiated (FSH-treated) and preovulatory (FSH/hCG-treated) granulosa cells. Granulosacell cultures (40,000 viable cells per well) were incubated for 48 h in serum-free medium containing IGF-I, inhibin and activin (all 30 ng/ml). Tritiated thymidine uptake was determined after 48 h of culture as described in section 2.3. Values are mean ± SE, data are representative of at least 2 experiments. Asterisks denote significant inhibition by in vitro treatment vs. untreated control (C): *, P < 0.01; **, P < 0.05.
activin. Differentiated granulosa cells exhibit higher basal levels of progesterone production than non-differentiated granulosa cells, whilst preovulatory granulosa cells had an even higher level of basal progesterone production (Fig. 2.9). Non-differentiated granulosa cell progesterone production was increased by IGF-I or inhibin and was unaffected by activin. Differentiated granulosa cell progesterone production was unaffected by IGF-I, inhibin and only marginally increased by activin. Preovulatory granulosa cell progesterone production was increased by IGF-I or inhibin, but it was decreased by activin.

3.5.3 Oestradiol production

The effect of peptide growth factors on granulosa cell oestradiol production in vitro was tested. Non-differentiated, differentiated and preovulatory granulosa cells were treated in vitro with IGF-I, inhibin or activin in the presence of 1.0 μM testosterone. Differentiated and preovulatory granulosa cells exhibit higher basal levels of oestradiol production in the presence of testosterone as an aromatase substrate than non-differentiated granulosa cells (Fig. 2.10). Oestradiol production at all stages of granulosa cell differentiation was unaffected by treatment with IGF-I or inhibin. Non-differentiated and differentiated granulosa cell oestradiol production was increased (P < 0.05) by activin treatment, but preovulatory granulosa cell oestradiol production was unaffected by the presence of activin.

3.6 Effect of gonadotrophins on levels of oestradiol and androstenedione in blood serum

The effect of recombinant gonadotrophins on steroid levels in rat serum was tested. Table 2.2 shows the effect of rh-FSH, r-LH and gonadotrophin preparations containing both FSH and LH activities (PMSG and Pergonal) on the levels of oestradiol and androstenedione in the blood of hypophysectomised immature rats. Recombinant human FSH alone did not increase the levels of either steroid in the blood. LH however, in the presence of a fixed dose of FSH, dose-dependently increased the levels of both steroids found in the blood. Similarly, FSH in
Fig. 2.9. Effects of IGF-I, inhibin and activin on basal progesterone synthesis by non-differentiated (control), differentiated (FSH-treated) and preovulatory (FSH/hCG-treated) granulosa cells. Granulosa cell cultures (40,000 viable cells per well) were incubated for 48 h in serum-free medium containing containing IGF-I, inhibin and activin (all 30 ng/ml). Progesterone in the spent culture medium was determined by RIA; values are mean ± SE, data are representative of at least 2 experiments. Asterisks denote significant difference due to in vitro treatment vs. untreated control (C): *, $P < 0.01$; **, $P < 0.05$. 
Fig. 2.10. Effects of IGF-I, inhibin and activin on basal oestradiol synthesis by non-differentiated (control), differentiated (FSH-treated) and preovulatory (FSH/hCG-treated) granulosa cells. Granulosa cell cultures (40,000 viable cells per well) were incubated for 48 h in serum-free medium containing IGF-I, inhibin and activin (all 30 ng/ml) and 1.0 μM testosterone (aromatase substrate). Oestradiol in the spent culture medium was determined by RIA; values are mean ± SE, data are representative of at least 2 experiments. Asterisks denote significant stimulation by in vitro treatment vs. untreated control (C): **, $P < 0.05$. 
### Table 2.2. Effects of administration of recombinant gonadotrophins in vivo on oestradiol, androstenedione, rh-FSH and r-LH levels in plasma.

Hypophysectomised immature female rats were treated in vivo with injection vehicle alone, rh-FSH (total dose 3-100 IU) and/or r-LH (total dose 0.1-30 IU), Pergonal (40 IU each of LH and FSH) with four 12-hourly injections, or a single injection of PMSG (15 IU). After 48 h the animals were killed and blood was sampled from the posterior vena cava and analysed by specific RIA (oestradiol and androstenedione) or specific IRMA (rh-FSH and r-LH). Results are expressed as mean ± SE, n ≥ 6. Asterisks denote significant stimulation by in vivo treatment vs. corresponding control: *, P < 0.01; **, P < 0.05. ND: Not Determined.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oestradiol (pM)</th>
<th>Androstenedione (pM)</th>
<th>FSH (mIU/ml)</th>
<th>LH (mIU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt 1 - C</td>
<td>50</td>
<td>0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>FSH 72</td>
<td>1533</td>
<td>6.95</td>
<td>226</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>PERGONAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt 2 - C</td>
<td>66</td>
<td>ND</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>FSH 3</td>
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<td>ND</td>
<td>46</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>FSH 10</td>
<td>72</td>
<td>ND</td>
<td>141</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>FSH 30</td>
<td>68</td>
<td>ND</td>
<td>123</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>FSH 100</td>
<td>67</td>
<td>ND</td>
<td>394</td>
<td>&lt;0.5</td>
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<tr>
<td>PERGONAL</td>
<td>997</td>
<td>ND</td>
<td>111</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Expt 3 - C</td>
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<td>0.46 ± 0.03</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>FSH 0 LH 1</td>
<td>&lt;50</td>
<td>0.77 ± 0.02 **</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>FSH 0 LH 10</td>
<td>&lt;50</td>
<td>3.50 ± 0.18 *</td>
<td>&lt;0.5</td>
<td>17 ± 1.7</td>
</tr>
<tr>
<td>FSH 30 LH 0</td>
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<td>0.35 ± 0.05</td>
<td>244 ± 43</td>
<td>&lt;0.5</td>
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<tr>
<td>FSH 30 LH 1</td>
<td>346 ± 65 *</td>
<td>0.69 ± 0.06 **</td>
<td>211 ± 16</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>FSH 30 LH 10</td>
<td>1168 ± 42 *</td>
<td>6.26 ± 0.19 *</td>
<td>258 ± 30</td>
<td>18 ± 1.3</td>
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<tr>
<td>FSH 72 LH 0</td>
<td>&lt;50</td>
<td>1.15 ± 0.04 *</td>
<td>392 ± 77</td>
<td>&lt;0.5</td>
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<tr>
<td>FSH 72 LH 1</td>
<td>1236 ± 134 *</td>
<td>1.68 ± 0.16 *</td>
<td>350 ± 50</td>
<td>&lt;0.5</td>
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<tr>
<td>FSH 72 LH 10</td>
<td>1850 ± 454 *</td>
<td>7.55 ± 0.27 *</td>
<td>347 ± 115</td>
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<td>PMSG</td>
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<td>&lt;0.5</td>
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<td>ND</td>
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<td>FSH 30 LH 0</td>
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<td>ND</td>
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<td>2.30 ± 0.86</td>
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<td>ND</td>
</tr>
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<td>229 ± 1 *</td>
<td>1.66 ± 0.21</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FSH 30 LH 1</td>
<td>250 ± 30 *</td>
<td>5.59 ± 3.00 *</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FSH 30 LH 3</td>
<td>446 ± 41 *</td>
<td>6.31 ± 1.96 *</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FSH 30 LH 10</td>
<td>750 ± 104 *</td>
<td>5.03 ± 1.10 *</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FSH 30 LH 30</td>
<td>1059 ± 151 *</td>
<td>10.20 ± 5.17 *</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PMSG</td>
<td>1111 ± 124 *</td>
<td>7.20 ± 1.60 *</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
the presence of a fixed dose of LH dose dependently increased oestradiol and androstenedione levels found in the blood. The table also shows the levels of rh-FSH and r-LH found in the blood after 48 h of gonadotrophin treatment.

3.7 Effect of FSH on hypophysectomised rat ovarian and uterine weight and granulosa cell aromatase activity.

The effect of rh-FSH, in the absence of any other gonadotrophic stimulus, on ovarian weight, uterine weight and aromatase activity in vitro was tested. Treatment with a fixed dose of rh-FSH approximately doubled the ovarian weight of hypophysectomised immature rats while having no measurable effect on uterine weight (Fig. 2.11). Treatment with Pergonal (40 IU of both FSH and LH) caused a further doubling of ovarian weight and a ~3-fold increase in uterine weight.

Basal oestradiol production in the absence of a substrate, testosterone, was increased by FSH treatment in vivo and was further increased by Pergonal treatment (Fig. 2.12). FSH-stimulated oestradiol production was unaffected by in vivo gonadotrophin treatment. Basal and FSH-stimulated aromatase activity (oestradiol production in the presence of testosterone) was increased by rh-FSH treatment in vivo to levels similar to those found in cells treated in vivo with Pergonal (Fig. 2.12).

The dose-dependence of rh-FSH action on ovarian and uterine weight and aromatase activity was established in experiments on hypophysectomised animals. Recombinant human FSH dose-dependently increased ovarian weight while having no effect on uterine weight (Fig. 2.13). Basal oestradiol production was increased by increasing doses of rh-FSH but FSH-stimulated oestradiol production was unaffected. Likewise basal and FSH-stimulated aromatase activity was dose-dependently increased by rh-FSH treatment in vivo (Fig. 2.14).
Fig 2.11 Effect of rh-FSH administration in vivo on ovarian and uterine weight. Hypophysectomised immature female rats were treated in vivo with rh-FSH (total dose 72 IU), Pergonal (40 IU each of LH and FSH) or injection vehicle alone with four 12-hourly injections. After 48 h the animals were killed and the ovaries and uteri were immediately removed and cleaned of all extraneous material. Results are expressed as weight of a single ovary ± SE, or weight of uterine horn ± SE, n ≥ 12. Asterisks denote significant stimulation by in vivo treatment: *, P < 0.01.
Fig 2.12 Effect of rh-FSH administration \textit{in vivo} on aromatase activity. Hypophysectomised immature female rats were treated \textit{in vivo} with rh-FSH (total dose 72 IU), Pergonal (40 IU each of LH and FSH) or injection vehicle alone with four 12-hourly injections. Granulosa cell cultures (40,000 viable cells per well) were incubated for 48 h in serum-free medium with or without 1.0 \( \mu \text{M} \) testosterone (aromatase substrate) and/or hFSH (30 ng/ml). Oestradiol in the spent culture medium was determined by RIA; Results are expressed as pmol oestradiol produced per 1000 cells per h \pm SE, \( n = 6 \). Asterisks denote significant stimulation by \textit{in vivo} treatment vs. corresponding control: *, \( P < 0.01 \).
Fig 2.13 Effect of rh-FSH administration *in vivo* on ovarian and uterine weight. Hypophysectomised immature female rats were treated *in vivo* with rh-FSH (total dose 3-100 IU), Pergonal (40 IU each of LH and FSH) or injection vehicle alone with four 12-hourly injections. After 48 h the animals were killed and the ovaries and uteri were immediately removed and cleaned of all extraneous material. Results are expressed as weight of a single ovary ± SE, or weight of uterine horn ± SE, n ≥ 6. Asterisks denote significant stimulation by *in vivo* treatment vs. corresponding control: *, *P* < 0.01.
Fig 2.14 Effect of rh-FSH administration in vivo on aromatase activity. Hypophysectomised immature female rats were treated in vivo with rh-FSH (total dose 3-100 IU), Pergonal (40 IU each of LH and FSH) or injection vehicle alone with four 12-hourly injections. Granulosa cell cultures (40,000 viable cells per well) were incubated for 48 h in serum-free medium with or without 1.0 μM testosterone (aromatase substrate) and/or hFSH (30 ng/ml). Oestradiol in the spent culture medium was determined by RIA; Results are expressed as pmol oestradiol produced per 1000 cells per h ± SE, n = 3. Asterisks denote significant stimulation by in vivo treatment vs. corresponding control: *, P < 0.01.
3.8 Effect of LH on hypophysectomised rat ovarian and uterine weight and granulosa cell aromatase activity.

The effect of r-LH, in the presence of a fixed dose of rh-FSH, on ovarian weight, uterine weight and aromatase activity in vitro was tested. Recombinant LH in the presence of rh-FSH (total dose 30 IU) increased ovarian weight at total doses above 3 IU, and increased uterine weight at total doses above 0.1 IU r-LH (Fig. 2.15). Basal and FSH-stimulated oestradiol production was increased by increasing doses of r-LH (Fig. 2.16). However, basal and FSH-stimulated aromatase activity (oestradiol production in the presence of testosterone) was unaffected by increasing doses of r-LH in vivo (Fig. 2.17).

4. Discussion

These data provide evidence that FSH is required for normal follicular development, though it is unlikely that this is entirely a direct effect of the hormone. For normal follicular differentiation and oestrogen synthesis both FSH and LH are necessary, as previously suggested by the two-cell, two-gonadotrophin theory of follicular oestrogen synthesis (Armstrong & Dorrington, 1979). These actions of gonadotrophins are subject to local regulation by factors of both granulosa and thecal cell origin.

In the absence of gonadotrophins, follicular growth can continue to the preantral stage (Hirshfield, 1986). The present study demonstrated that FSH is capable of stimulating the rate of granulosa cell proliferation in vivo, especially in antral follicles. It was observed in animals treated with FSH and hCG, to induce preovulatory follicle development, that granulosa cells abutting the basement membrane no longer incorporated detectable levels of BrdU, whereas antral granulosa cells were labelled. This centripetal pattern was more striking as follicular size increased. It is possible that the mural granulosa cells have left the cell cycle and have become differentiated. Indeed mural granulosa cells express a higher level of LH receptor mRNA, a marker of differentiation, than antral
Fig 2.15 Effect of rh-LH administration *in vivo* on ovarian and uterine weight. Hypophysectomised immature female rats were treated *in vivo* with rh-FSH (total dose 30 IU) plus r-LH (total dose 0-30 IU), Pergonal (40 IU each of LH and FSH) or injection vehicle alone with four 12-hourly injections. After 48 h the animals were killed and the ovaries and uteri were immediately removed and cleaned of all extraneous material. Results are expressed as weight of a single ovary ± SE, or weight of uterine horn ± SE, n ≥ 6. Asterisks denote significant stimulation by *in vivo* treatment vs. corresponding control: *, P < 0.01.
Fig 2.16 Effect of rh-LH administration in vivo on aromatase activity. Hypophysectomised immature female rats were treated in vivo with rh-FSH (total dose 30 IU) and r-LH (total dose 0-30 IU), Pergonal (40 IU each of LH and FSH) or injection vehicle alone with four 12-hourly injections. Granulosa cell cultures (40,000 viable cells per well) were incubated for 48 h in serum-free medium with or without hFSH (30 ng/ml). Oestradiol in the spent culture medium was determined by RIA; Results are expressed as pmol oestradiol produced per 1000 cells per h ± SE, n = 3. Asterisks denote significant stimulation by in vivo treatment vs. corresponding control: *, P < 0.01.
Fig 2.17 Effect of rh-LH administration *in vivo* on aromatase activity. Hypophysectomised immature female rats were treated *in vivo* with rh-FSH (total dose 30 IU) and r-LH (total dose 0-30 IU), Pergonal (40 IU each of LH and FSH) or injection vehicle alone with four 12-hourly injections. Granulosa cell cultures (40,000 viable cells per well) were incubated for 48 h in serum-free medium containing 1.0 μM testosterone (aromatase substrate) and/or hFSH (30 ng/ml). Oestradiol in the spent culture medium was determined by RIA; Results are expressed as pmol oestradiol produced per 1000 cells per h ± SE, n = 3. Oestradiol production appears to be substrate limited since incubating cells with 1.0 μM testosterone (500 pmoles) can only produce a maximum of 500 pmoles oestradiol. In this experiment 8 pmoles/h over a period of 48h is equivalent to about 400 pmoles oestradiol from 500 pmoles of substrate.
granulosa cells (Whitelaw et al. 1992). This is consistent with a general principle of biology that proliferation and differentiation are inversely related. These results suggest that while early follicular growth is gonadotrophin-independent, later stages are subject to gonadotrophic regulation. Gonadotrophins affect follicular growth by altering the proportion of proliferating granulosa cells in large follicles. Cyclic hormonal fluctuations may thus regulate the number of follicles that will ovulate and ensure that the number of granulosa cells that will luteinize is adequate to form a corpus luteum capable of sustaining pregnancy.

FSH does not stimulate granulosa cells to proliferate in vitro which suggests that in vivo the growth stimulatory effects of FSH are mediated through another factor(s). FSH does however stimulate granulosa cells to express differentiated functions such as progesterone and oestradiol synthesis (Hsueh et al. 1984). FSH with or without hCG treatment in vivo stimulated differentiation and thereby increased the number of cells leaving the cell cycle. In other words, cells from increasingly mature follicles contain decreasing proportions of proliferating cells and increasing proportions of cells expressing differentiated functions. Similarly, in vitro gonadotrophin treatment increased the proportion of differentiated cells and inversely decreased the number of proliferating cells. Thus gonadotrophins do not seem to directly affect proliferation, but rather stimulate cells to differentiate and thereby indirectly decrease the proliferative activity of a cell population.

Androgens, as well as acting as an aromatase substrate for oestradiol synthesis enhance FSH stimulated progesterone and oestradiol synthesis in immature rat granulosa cells (Armstrong & Dorrington, 1976; Duleba et al. 1984) and mature rat granulosa cells (Zeleznik et al. 1979). These results are verified by our finding that testosterone enhances FSH-stimulated progesterone production by granulosa cells of all stages of maturity in vitro. These effects of androgens can be blocked with an anti-androgen (Zeleznik et al. 1979) and are therefore thought to be specific for the steroid. The mechanism of these effects of androgens is unknown, though enhanced cAMP production is involved (Hillier & deZwart, 1982). The fact that anti-androgen treatment blocks these androgen effects suggest that they are receptor-mediated, which lends support to the existence of an androgen receptor (Schreiber & Ross, 1976). Testosterone also enhances LH-responsive
Relative Roles of Gonadotrophins

granulosa cell steroid production in vitro consistent with previous findings (Nimrod & Lindner, 1976). It is however unclear why the presence of testosterone should have blocked any LH-responsive decrease in proliferation without affecting LH-induced progesterone production by the same cells (Figs. 2.2 and 2.3). It appears that the enhancement of LH-responsiveness in immature granulosa cells by DES in vivo is negated by the effects of testosterone in vitro. The parallel situation occurs in vivo where testosterone treatment blocks DES-stimulated ovarian weight gain in hypophysectomised immature female rats (Hillier & Ross, 1979). The significance of these observations, if any, is unclear.

Oestrogens increase ovarian weight in hypophysectomised rats (Louvet et al. 1975) though there are few reports of the growth-promoting effects of oestrogens on granulosa cells in vitro. It has been reported that thecal/interstitial cells are required for oestradiol to induce granulosa cell DNA synthesis (Bley et al. 1991), thereby implicating factors of thecal/interstitial origin in the mediation of this effect of oestradiol (Bendell & Dorrington, 1991). In vivo, DES enhances granulosa cell proliferation as assessed by BrdU incorporation, which is consistent with a requirement for thecal/interstitial derived signalling for oestrogen effects on cellular proliferation. The demonstration that DES promotes the induction of LH-responsiveness in immature granulosa cells which normally are unable to respond to LH (Table 2.1) supports the findings of previous studies (Goldenburg et al. 1972; Richards et al. 1979). This induction of LH receptor occurs in the presence of only very low levels of gonadotrophins and therefore seems to be a gonadotrophin-independent effect. The physiological implications of this finding are difficult to understand.

The effects of FSH on proliferation seem likely to be mediated by local regulators since FSH in vivo is unable to stimulate proliferation as it does in vitro. Rat granulosa cells seem to be resistant to further stimulation of proliferation in vitro and thus there have been few published studies of the effects of locally produced factors on rat granulosa cell proliferation in vitro (Gospodarowicz & Bialecki, 1979; Orly et al. 1984) The reason for this resistance is not clear, but it is likely that the lack of a system capable of providing adequate support for rat cellular proliferation in vitro is the main cause. As stated above oestrogens enhance the responsiveness of granulosa
Relative Roles of Gonadotrophins

It seems that granulosa cell growth is a tightly regulated event requiring complex interactions between locally produced factors derived from both ovarian cell types. The effects of IGF-I, inhibin and activin on granulosa cell thymidine uptake observed in this study are most probably due to the induction of granulosa cell differentiated functions by these factors rather than direct effects on cell proliferation. This conclusion is emphasised by the fact that any significant effects of the factors on thymidine uptake occurred in immature cells that were presumably induced to differentiate in vitro by these factors. Likewise, IGF-I is known to stimulate granulosa cell proliferation in granulosa cells of other species (Adashi et al. 1985a), yet it failed to stimulate thymidine uptake in our system.

Interestingly, oestradiol production in vitro by cells of all stages of maturity was not affected by IGF-I or inhibin (Fig. 2.10). However, both these factors increased progesterone production by immature and preovulatory cells, but not differentiated granulosa cells (Fig. 2.9). This indicates that inhibin and IGF-I increase P450SCC selectively without affecting P450arom activity. Activin had only a small stimulatory effect on non-differentiated and differentiated granulosa cell oestradiol production in vitro (Fig. 2.10) while it only increased progesterone production by differentiated granulosa cells (Fig. 2.9). Treatment with FSH plus hCG did not significantly affect oestradiol production but progesterone production was decreased, though not to non-differentiated levels (Fig. 2.9). These data illustrate that aromatase activity and progesterone production are differentially regulated throughout follicular maturation and suggest that the complex regulation of steroidogenesis during development may involve one or more of these factors.

FSH enhances granulosa cell aromatase activity, resulting in increased conversion of androgens to oestrogens (Dorrington et al. 1975), but in the absence of androgen substrate granulosa cells are incapable of producing oestrogens (Fig. 2.4). Since aromatase substrate is produced in the thecal/interstitial cell under the influence of LH (Fortune & Armstrong, 1977), both FSH and LH are thought to be essential for oestrogen biosynthesis (Armstrong & Dorrington, 1979). This two-cell, two-gonadotrophin theory of oestrogen synthesis has been verified by the use of
recombinant gonadotrophins to dissect out the precise contributions of gonadotrophins to normal oestradiol production. By measuring steroid levels in the blood of immature hypophysectomised rats treated with recombinant gonadotrophins this study has shown that FSH is unable to increase blood oestradiol levels in the absence of LH. However in the presence of a fixed low dose of LH then FSH dose-dependently increases oestradiol. Likewise in the presence of a fixed low dose of FSH then LH dose-dependently increases oestradiol. Androgen accumulation in rat plasma follows a similar pattern of gonadotrophic stimulation. In fact FSH dose-dependently increases androgen production in the presence of LH. Since thecal/interstitial cells are the only ovarian cell type capable of producing androgens (Fortune & Armstrong, 1977), and granulosa cells the only ovarian cell type capable of responding directly to FSH (Dorrington et al. 1975), this data suggests that some communication occurs between FSH-stimulated granulosa cells and androgen producing thecal/interstitial cells.

FSH alone is capable of stimulating follicular growth as indicated by dose-dependent increases in ovarian weight. It was also demonstrated that in the presence of a fixed dose of FSH, LH is also capable of increasing ovarian weight, though this is most probably due to thecal cell hypertrophy rather than follicular development (Erickson et al. 1985), caused by the atretic actions of androgens (Louvet et al. 1975). In keeping with the two-cell theory of follicular oestrogen production, FSH alone is incapable of eliciting any increases in uterine weight (which correlates well with oestradiol measurements in rat serum), and therefore cannot induce oestradiol production alone. However, in the presence of a fixed dose of FSH, LH causes uteri to balloon and thus uterine weight increases in a dose-dependent manner. From these results it is evident that both FSH and LH are necessary for follicular oestrogen synthesis. Though recombinant FSH can only induce the potential for aromatisation, oFSH can actually increase oestradiol production alone (Fig. 2.4). This difference is undoubtably due to LH contamination which indicates that very low levels of LH are sufficient to support FSH-stimulated oestrogen synthesis. FSH therefore is the primary stimulus to follicular oestrogen synthesis since it stimulates aromatase activity, but LH is also essential to provide support for FSH by supplying aromatase substrate.
In conclusion, FSH is the main stimulus for follicular growth and differentiation of granulosa cells, though the actions of FSH are mediated by locally produced factors. These data also suggest that low levels of LH are adequate for normal follicular maturation and LH plays a facilitatory role in this process. The aim of the rest of this thesis is to investigate paracrine mechanisms that modulate gonadotrophin action on the ovary during normal follicular development and oestrogen synthesis.
Chapter 3. Validation of a Thecal/Interstitial Cell Culture System

1. Introduction

The previous chapter described a study designed to determine the precise contribution of FSH and LH to normal follicular development and steroidogenesis. Having gained an insight into the individual effects of FSH and LH during follicular development it was the aim of this project to determine how the effects of each gonadotrophin are modulated at the level of the gonad. In particular the aim was to determine how thecal cell function (androgen synthesis) is affected by gonadotrophins and locally modulated. This chapter describes the validation of a model culture system for the study of rat thecal/interstitial cell steroidogenesis in vitro based on an existing method developed by Magoffin and Erickson (Magoffin & Erickson, 1982b). Androgen production in response to LH was used as a marker for hormonal responsiveness during the validation of this system.

In all mammals, thecal/interstitial cells, under primary hormonal control by LH (Bogovich & Richards, 1982), are the site of synthesis of androgens, essential substrates for oestrogen biosynthesis (Erickson et al. 1985). The first convincing evidence that the ovary produced androgens was provided when it was discovered that ovaries grafted to the ears of male castrate mice stimulated atrophic seminal vesicles and prostates to return to their normal states (Hill, 1937; Parkes, 1950). These findings were interpreted as evidence that the ovary secreted a male hormone into the circulation. It was subsequently reported that the masculinisation effect was independent of the adrenal gland (Deanesly, 1938), and that androgenic activity appeared to be associated with the theca interna of the follicles. Over 20 years later, work with explants of prostatic tissue (used as a marker of androgen synthesis) and ovarian fragments in the anterior chamber of the eye of a rat demonstrated that the theca interna and interstitial cells were the source of ovarian androgens (Falck et al. 1962). The final proof was provided by the use of biochemical techniques (Ryan & Petro, 1966; Rice & Savard, 1966), to discover that the theca interna and secondary interstitial tissue synthesise
androgens de novo. Since the mid-sixties much research has been devoted to understanding the significance of ovarian androgen production and how this process is regulated. It is clear that the primary hormonal control of androgen biosynthesis is by LH (Bogovich & Richards, 1982), but different follicular populations of thecal/interstitial cells respond differently to the same LH stimulus and thus it is also clear that there must be a secondary intrafollicular level of regulation.

Despite the importance of ovarian androgens, as described above, and hence the central role of thecal/interstitial cells in reproductive processes, the hormonal regulation of thecal/interstitial cell differentiation and function is poorly understood. The primary reason for this is that thecal/interstitial cells are embedded in connective tissue which makes it difficult to isolate them from granulosa cells and other contaminants. This chapter describes the validation of a primary serum-free cell culture model for thecal/interstitial cells that are capable of being hormonally stimulated to express differentiated functions.

2. Materials and Methods

2.1 Animals

Intact or hypophysectomized 21-day old female rats were purchased from Charles River UK Limited (Margate, Kent). All animals were killed by carbon dioxide asphyxiation at 25 (intact) or 27 days (hypophysectomized) of age. Ovaries were immediately removed for isolation of thecal/interstitial cells.

2.2 Isolation and culture of thecal/interstitial cells

The isolation of thecal/interstitial cells was based on a previously reported method (Magoffin & Erickson, 1982b), with the main difference being that ovaries were depleted of granulosa cells before enzymic digestion to release thecal cells. All visible ovarian follicles were first punctured using a 27 G hypodermic syringe needle to eliminate as many granulosa cells as possible. Residual ovarian tissue was then rinsed in culture medium before
enzymic digestion by incubation for 30 min at 37°C in fresh medium containing 0.1% (w/v) collagenase Cl. histolyticum type II (Sigma) and 0.01% DNase (Sigma). The medium was Medium-199 (Gibco) containing Earle's salts, 25 mM HEPES buffer, penicillin (50 IU/ml), streptomycin (50 mg/ml) and additional (2.0 mM) L-glutamine, supplemented with 0.1% (w/v) BSA. Complete dispersal into a single-cell suspension was achieved by repeated pipetting at the end of this incubation. The cells were sedimented by centrifugation (5 min at 800 x g), resuspended in 2ml of fresh culture medium containing 5.0% (v/v) donor calf serum (Gibco) and counted in a haemocytometer. Cell viability, determined by staining with trypan blue, was consistently >90%.

Multiwell plastic culture dishes (Linbro Space Savers™ from Flow Laboratories, Rickmansworth, Herts, UK.) were inoculated with replicate 0.5 ml portions of thecal cell suspension (40,000 cells) in culture medium containing 5.0% donor calf serum (DCS). Pre-incubation in serum-containing medium to allow cell anchorage and recovery from the enzymic dispersal procedure was for 24 h at 37°C in a humidified atmosphere of 5% CO₂/95% air. After removing the serum-containing medium and washing the cell monolayers with 1.0 ml pre-warmed (37°C) PBS, each culture well received 0.5 ml serum-free medium with or without human LH (LER-1972, 5179 IU LH/mg, 2.5 IU FSH/mg) (generously donated by Dr. L.E.Reichert Jr.) at concentrations between 0.1 and 30 ng/ml. In some experiments, rh-inhibin-A (Genentech Inc), rDNA-human inhibin 90/522, rDNA-human inhibin 91/624, 1st International standard for porcine inhibin (86/690) (all at concentrations from 0.1 to 30 ng/ml); human follicular fluid (92/696), collected from patients undergoing oocyte removal after FSH stimulation, at a concentration of 5.5% (all from National Institute for Biological Standards and Control, Potters Bar, Hertfordshire); rh-IGF-I (0.1 to 30 ng/ml); rh-FSH at concentrations between 25 and 100 ng/ml, or rh-activin-A (30 ng/ml) were also added to the cultures. All treatments were done in triplicate. Incubation was normally for 48 h at 37°C, after which the medium was collected and stored frozen at -20°C for subsequent analysis of androgen content by RIA, as described below. During the time-course experiment incubation was for 24 h, 48 h or 72 h.
2.3 Discontinuous density centrifugation

In order to produce a population of thecal/interstitial cells devoid of granulosa cell contamination, residual ovary cell suspensions were purified by discontinuous density centrifugation based on a previously published method (Magoffin & Erickson, 1988). First, 1 ml of 44% Percoll (Sigma; d = 1.13 g/ml) in Medium-199 (containing Earle's salts, 25 mM HEPES buffer, penicillin (50 IU/ml), streptomycin (50 mg/ml) and additional (2.0 mM) L-glutamine, supplemented with 0.1% (w/v) BSA) was pipetted into a sterile 12 x 75 mm Falcon centrifuge tube (Becton Dickinson & Co, Lincoln Park, NJ). Two ml of a Percoll solution adjusted to a specific gravity of 1.055 were carefully layered on top of the 44% solution. Dispersed residual ovary cells (as described above) were carefully layered on top of the d = 1.055 Percoll solution. The cells were centrifuged at 400 x g for 20 min at 4°C. The thecal/interstitial cells were collected by aspiration from the d = 1.055 layer (Fig. 3.1). The cells were washed in Medium-199 and the pellet resuspended in 2ml of fresh culture medium containing 5.0% (v/v) donor

![Diagram](image_url)
calf serum (Gibco) and counted in a haemocytometer. Cell viability, determined by staining with trypan blue, was consistently >90%. Cells were cultured as described above. The equation used to calculate the dilutions necessary to achieve a final d of 1.055 is as follows:

$$V_o = \frac{V \cdot (d - 0.1d_x - 0.9)}{d_o - 1}$$

Where:
- $V_o$ = volume of Percoll (ml)
- $d_o$ = $\delta$ of Percoll (1.130 g/ml)
- $d_x$ = $\delta$ of Medium-199 (1.058 g/ml)
- $V$ = volume required for final working solution (ml)

and $d = \delta$ required for final working solution (g/ml).

2.4 Steroid Radioimmunoassays

2.4.1 Oestradiol Radioimmunoassay

Oestradiol-17$\beta$ was measured in culture media by specific RIA as described in Chapter 2, section 2.5.2.

2.4.2 Androstenedione Radioimmunoassay

Androstenedione in spent culture medium was determined by specific RIA as described in Chapter 2, section 2.5.3.

2.4.3 Androsterone Radioimmunoassay

Androsterone in spent culture medium was determined by specific RIA. The assay buffer was PBS/gelatin (0.05M sodium phosphate, 0.15M NaCl, 0.1% gelatin). The androsterone antiserum was rabbit anti-androsterone-3-hemisuccinyl-BSA (Hillier et al. 1981). Major cross-reactions were: androsterone 100%, 5$\alpha$-androstan-3,17-dione 175%; androstenedione
5%; and less than 0.5% for all other steroids tested. Tracer, $[9,11-^3H]$-Androsterone, was diluted in assay buffer to give $\sim$10,000 cpm/500 $\mu$l. A standard curve covering the range of 0.025 to 6.4 pmoles/tube androsterone (Sigma), made up in culture medium, was used. Samples (5-100 $\mu$l) or standards (in 200 $\mu$l) were placed in tubes, and the volume of samples was adjusted to 200 $\mu$l. Quality controls (0.08 and 2.4 pmoles/tube) were also included at the beginning and end of each assay. Antibody (300 $\mu$l) and tracer (500 $\mu$l) were added, and tubes were incubated at 37°C for 1 h then at 4°C for 1 h. Tubes were placed on ice before addition of 200 $\mu$l dextran charcoal (1.25% dextran, 1.25% charcoal in assay buffer) to separate bound and free steroid. Tubes were mixed and incubated for 10 min on ice, then centrifuged at 3000 rpm for 10 min at 4°C. Supernatants were decanted, 3 ml scintillation cocktail (RIALUMA) was added, and bound tracer was measured by liquid scintillation counting (RackBeta). Standard curves were calculated using a commercial computer program (AssayZap). Samples which were outside the detection limits were re-assayed at the appropriate volume. The detection limit of the assay was in the range 0.03-0.04 pmoles/tube. Inter- and intra-assay coefficients of variation were less than 15%. Steroid production rates are expressed as picomoles of androsterone per culture/48 h.

2.5 Statistics

Statistical analysis was carried out using commercial software (CLR ANOVA). Analysis of variance with the Newman-Keuls test was used to analyse differences between experimental and control observations. Differences assigned a $P$ value of $<0.05$ were regarded as statistically significant.
3. Results

3.1 LH-dependent androgen production by rat thecal/interstitial cells in vitro

Androgen production in response to hLH was used as a marker for thecal/interstitial cell hormonal responsiveness during the validation of the cell culture system. Treatment with hLH in vitro elicited a dose-related increase in androgen production by non-Percoll treated cultured thecal/interstitial cells (Fig. 3.2) with significant \( (P < 0.01) \) stimulation at a lowest dose of 1 ng/ml. Maximum response occurred in the presence of hLH at a concentration of 10 ng/ml, with an ED\(_{50} \) of \(~3\) ng/ml. Cultured thecal/interstitial cells produced roughly similar amounts of androstenedione and androsterone. Results were therefore combined and expressed as total androgen production.

Thecal/interstitial cells isolated from hypophysectomised animals produced similar amounts of androgen to cells from intact controls and showed similar sensitivity and responsiveness to hLH in vitro (Fig. 3.3).

3.2 Demonstration of thecal/interstitial cell purity

In order to demonstrate the purity of the thecal/interstitial cell cultures, residual ovary dispersates were further purified using discontinuous Percoll density gradient centrifugation. Thecal/interstitial cells isolated from intact animals and further purified using the Percoll gradient exhibited levels of basal and LH-stimulated androgen production similar to those produced by thecal/interstitial cells from intact animals that underwent no further purification (Fig. 3.4).

Treatment of cultured thecal/interstitial cells in vitro with hLH at concentrations up to 30 ng/ml did not significantly increase oestradiol production (Fig. 3.5) but did dose-dependently increase androgen production. Androgens are a substrate for granulosa cell oestradiol production (Erickson et al. 1985) but this experiment illustrates the lack of granulosa cell aromatase activity in the cultures.
Fig. 3.2. LH-responsive androgen production in vitro by thecal/interstitial cells from the ovaries of intact female rats. Thecal/interstitial cell cultures (40,000 viable cells per culture well) were incubated for 48 h in serum-free medium containing increasing doses of hLH (0.03 to 30 ng/ml). Androstenedione+androstosterone accumulation in spent culture medium was determined by RIA. Composite data from 5 experiments are expressed as mean ± SE.
Thecal/interstitial cells from the ovaries of intact female rats and hypophysectomized female rats. Thecal/interstitial cell cultures (40,000 viable cells per culture well) were incubated for 48 h in serum-free medium containing increasing doses of hLH (0.03 to 30 ng/ml). Androstenedione+androsterone accumulation in spent culture medium was determined by RIA. Composite data from 2 experiments are expressed as mean ± SE. There was no significant difference in LH-responsive androgen production by thecal/interstitial cells from the ovaries of intact or hypophysectomised animals.
Fig. 3.4. Effect of Percoll density gradient purification of thecal/interstitial cells from the ovaries of intact animals. Dispersed cells were either centrifuged through Percoll (δ = 1.055) and the thecal/interstitial cells were collected by aspiration, or dispersed thecal/interstitial cells were cultured directly. Thecal/interstitial cell cultures (40,000 viable cells per culture well) were incubated for 48 h in serum-free medium containing hLH (10 ng/ml). Androstenedione+androsterone accumulation in spent culture medium was determined by RIA. Data from a representative experiment are expressed as mean ± SE (n = 3). There was no significant difference in LH-responsive androgen production by thecal/interstitial cells from the ovaries of intact or hypophysectomised animals or Percoll purified thecal/interstitial cells.
Fig. 3.5. Lack of LH-responsive oestradiol production in vitro by thecal/interstitial cells from the ovaries of intact female rats. Thecal/interstitial cell cultures (40,000 viable cells per culture well) were incubated for 48 h in serum-free medium containing increasing doses of hLH (0.03 to 30 ng/ml). Androstenedione, androsterone and oestradiol accumulation in spent culture medium was determined by RIA. Composite data from 5 experiments are expressed as mean ± SE. There was no significant stimulation of oestradiol accumulation by LH.
Treatment of cultured thecal/interstitial cells *in vitro* with rh-FSH at concentrations up to 100 ng/ml did not significantly increase androgen production (Fig. 3.6), confirming the lack of intrinsic LH-like activity of the rh-FSH preparation as well as illustrating the lack of any FSH responsive androgen production *in vitro* due to stimulation by granulosa cell derived factors. Similarly FSH can stimulate granulosa cell production of progesterone (Hillier *et al.* 1978), a substrate for thecal/interstitial cell androgen production (Lishinsky & Armstrong, 1983).

3.3 Time course of androgen production

To establish the optimal culture period to study the control of androgen production, a time course of androgen accumulation by thecal/interstitial cell cultures was established. Thecal/interstitial cells cultured for up to 72 h in the absence of any treatment showed increasing androgen accumulation over the time period (~4-fold increase). In the presence of hLH at a concentration of 10 ng/ml the increase was more dramatic (~8-fold) during the total time period (Fig. 3.7). The highest rate of LH-stimulated androgen production was between 24 h and 48 h, after which the rate declined. Therefore 48 h was chosen as the best time period to study the control of androgen production by thecal/interstitial cells *in vitro*.

3.4 Effect of recombinant human inhibin on thecal/interstitial cell androgen synthesis

To test the influence of a putative paracrine factor on thecal/interstitial androgen synthesis in this system, cells were cultured with rh-inhibin-A in the presence and absence of hLH. Recombinant human inhibin-A (30 ng/ml) alone did not measurably affect the low basal androgen production by thecal/interstitial cells from intact animals. However rh-inhibin-A (30 ng/ml) augmented LH-stimulated androgen production ~3-fold (Fig. 3.8).
Fig. 3.6. Lack of FSH-responsive androgen production in vitro by thecal/interstitial cells from the ovaries of intact female rats. Thecal/interstitial cell cultures (40,000 viable cells per culture well) were incubated for 48 h in serum-free medium containing increasing doses of rh-FSH (25 to 100 ng/ml). Androstenedione+androsterone accumulation in spent culture medium was determined by RIA. Data from a representative experiment are expressed as mean ± SE (n=3). There was no significant stimulation of androgen accumulation by FSH.
Fig. 3.7. Time course of androgen accumulation *in vitro* by thecal/interstitial cells from the ovaries of intact female rats. Thecal/interstitial cell cultures (40,000 viable cells per culture well) were incubated for 24 h, 48 h or 72 h in serum-free medium containing hLH (10 ng/ml). Androstenedione+androsterone accumulation in spent culture medium was determined by RIA. Composite data from 2 experiments are expressed as mean ± SE.
**Fig. 3.8.** Effect of inhibin on LH-responsive androgen production *in vitro* by thecal/interstitial cells from the ovaries of intact female rats. Thecal/interstitial cell cultures (40,000 viable cells per culture well) were incubated for 48 h in serum-free medium with and without hLH (10 ng/ml) and/or rh-inhibin-A (30 ng/ml). Androstenedione+androsterone accumulation in spent culture medium was determined by RIA. Composite data from 6 experiments are expressed as mean ± SE. Significant differences due to inhibin are indicated (P < 0.01).
This culture system was also validated using five other inhibin preparations as part of an International study of rh-inhibin (Fig. 3.9). Recombinant DNA-human inhibin 90/522 (J) and a coded duplicate (M) (30 ng/ml) both produced a 7.2-fold increase in LH-stimulated androgen synthesis. Recombinant DNA-human inhibin 91/624 (K) elicited an 11.2-fold increase. A coded duplicate of this preparation (L) elicited a 10.8-fold increase in androgen synthesis. The 1st International standard for porcine inhibin (86/690; N) elicited a 5.4-fold increase in LH-stimulated androgen synthesis. Human follicular fluid (92/696; P) at a concentration of 5.5% (v/v) increased LH-stimulated androgen synthesis nearly 10-fold. A dose response to these inhibin preparations is shown in Fig. 3.10.

3.5 Effect of recombinant human IGF-I on thecal/interstitial cell androgen synthesis

Cells were cultured with rh-IGF-I in the presence and absence of hLH to test the influence of this factor on thecal/interstitial androgen synthesis in this system. Recombinant human IGF-I (30 ng/ml) alone approximately doubled the levels of basal androgen production by thecal/interstitial cells from intact animals. In the presence of hLH (10 ng/ml), rh-IGF-I (30 ng/ml) increased LH-stimulated androgen production by nearly 50% (Fig. 3.11).

3.6 Effect of recombinant human activin on thecal/interstitial cell androgen synthesis

Thecal/interstitial cells were cultured with rh-activin-A in the presence and absence of hLH to test the effect this factor has on androgen synthesis in the culture system. Recombinant human activin-A (30 ng/ml) alone did not measurably affect the low basal androgen production by thecal/interstitial cells from intact animals. Likewise rh-activin-A (30 ng/ml) did not measurably affect LH-stimulated androgen production (Fig. 3.12).
Fig. 3.9. Effect of inhibin on LH-responsive androgen production in vitro by thecal/interstitial cells from the ovaries of intact female rats. Thecal/interstitial cell cultures (40,000 viable cells per culture well) were incubated for 48 h in serum-free medium with and without hLH (10 ng/ml) and/or rh-inhibin-A. The inhibin preparations were: Genentech rh-inhibin-A; rDNA-human inhibin 90/522 (J and M); rDNA-human inhibin 91/624 (K and L); 1st international standard for porcine inhibin (86/690; N) and human follicular fluid (92/696; P). Androstenedione+androstenedione accumulation in spent culture medium was determined by RIA. Composite data from 2 experiments are expressed as mean ± SE. Significant differences due to inhibin are indicated (*P < 0.01).
Fig. 3.10. Effect of inhibin on LH-responsive androgen production in vitro by thecal/interstitial cells from the ovaries of intact female rats. Thecal/interstitial cell cultures (40,000 viable cells per culture well) were incubated for 48 h in serum-free medium with and without hLH (10 ng/ml) and/or rh-inhibin-A (3 - 100 ng/ml). The inhibin preparations were: Genentech rh-inhibin-A; rDNA-human inhibin 90/522 (J and M); rDNA-human inhibin 91/624 (K and L) and 1st International Standard for Porcine Inhibin (86/690; N). Androstenedione+androstosterone accumulation in spent culture medium was determined by RIA. Composite data from 2 experiments are expressed as mean ± SE.
Fig. 3.11. Effect of IGF-I on LH-responsive androgen production in vitro by thecal/interstitial cells from the ovaries of intact female rats. Thecal/interstitial cell cultures (40,000 viable cells per culture well) were incubated for 48 h in serum-free medium with and without hLH (10 ng/ml) and/or rh-IGF-I (30 ng/ml). Androstenedione +androstosterone accumulation in spent culture medium was determined by RIA. Composite data from 5 experiments are expressed as mean ± SE. Significant differences due to IGF-I are indicated (P < 0.01).
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Fig. 3.12. Effect of activin on LH-responsive androgen production in vitro by thecal/interstitial cells from the ovaries of intact female rats. Thecal/interstitial cell cultures (40,000 viable cells per culture well) were incubated for 48 h in serum-free medium with and without hLH (10 ng/ml) and/or rh-activin-A (30 ng/ml). Androstenedione + androsterone accumulation in spent culture medium was determined by RIA. Composite data from 2 experiments are expressed as mean ± SE. There were no significant differences due to activin.
4. Discussion

The immature rat ovary contains a population of active androgen-producing cells which are localised in the theca and the secondary interstitial gland (Falck et al. 1962; Fortune & Armstrong, 1977). The results reported in this chapter have yielded a simple and reproducible system for culturing these androgen-producing cells in a purified form. Importantly the cells are hormonally responsive in serum-free culture and express differentiated functions in response to physiological concentrations of LH.

The ovarian androgens are of major physiological importance for oestrogen biosynthesis (Dorrington et al. 1975), follicular progesterone production (Armstrong & Dorrington, 1976), atresia (Hillier & Ross, 1979) and the onset of puberty. This study shows that isolated thecal/interstitial cells from intact and hypophysectomised animals are able to dose-dependently respond to physiological concentrations of LH in serum-free medium with the expression of normal differentiated functions. The two major androgens produced by rat thecal/interstitial cells in this system are androstenedione (an aromatisable C19 androgen) and androsterone (a non-aromatisable 5α-reduced androgen). Both these androgens are detected in roughly equal amounts by RIA indicating similarly high levels of 5α-reductase and P45017α activities in this model culture system.

A potentially significant disadvantage of thecal/interstitial cell cultures is the presence of granulosa cell contamination. Granulosa cells would be expected to secrete a variety of steroid and peptide hormones that can stimulate or inhibit thecal cell functions and therefore their presence can make the interpretation of experimental results difficult. To ensure the thecal/interstitial cells used for culture were as pure as possible we adopted a discontinuous Percoll gradient to separate thecal/interstitial cells from contaminating granulosa cells. A solution of Percoll with the specific gravity adjusted to 1.055 g/ml allows the majority of thecal/interstitial cells to sediment, but at the same time the granulosa cells (which possibly contain more cholesteryl esters in their cytoplasm) and other less dense cell types would float. To remove the red blood cells a 44% Percoll layer was placed at the bottom of the tube. However, Percoll separation did not noticeably
increase the purity of the thecal/interstitial cell cultures as measured by androgen production and as such this method was discontinued. We demonstrated a lack of oestrogen production by rat thecal/interstitial cells in vitro in response to increasing doses of LH and androstenedione, an aromatase substrate (Erickson et al. 1985). In the same culture there was a concomitant dose-dependent increase in LH-responsive androgen production. It would be expected that FSH would increase granulosa cell progesterone accumulation (Hillier et al. 1978), a substrate for thecal/interstitial cell P45017α (Lishinsky & Armstrong, 1983), and therefore increase androgen production. Therefore the finding that there was no measurable change in basal androgen production in response to increasing doses of FSH was interpreted as a lack of significant granulosa cell contamination.

A consequence of culturing ovarian thecal/interstitial cells is that they may spontaneously luteinize in culture. An in vivo characteristic of rat thecal/interstitial cells is the loss of P45017α expression around the time of ovulation, resulting in a switch from androgen to progesterone production by thecal/interstitial cells (Bogovich et al. 1986; Richards et al. 1986). By pre-incubation in 5% DCS we avoid this hazard and are able to maintain LH responsive androgen production for at least 72 h. The greatest rate of androgen production occurred between 24 h and 48 h, after which androgen production began to decline. Therefore a 48 h culture period was chosen as a suitable time period for the study of the control of androgen production by thecal/interstitial cells.

It has been previously reported that peptide growth factors such as inhibin (Hsueh et al. 1987) and IGF-I (Adashi et al. 1985a) may have a modulatory role in the control of androgen synthesis by thecal/interstitial cells. In our studies inhibin has no effect alone but does enhance LH-stimulated androgen synthesis up to 3-fold. This effect was verified using 5 different inhibin preparations during an international collaborative study of the proposed international standard for recombinant DNA-derived human inhibin. IGF-I alone approximately doubled basal androgen synthesis and increased LH-stimulated androgen synthesis by up to 50%. These results show that thecal/interstitial cells in this culture system are capable of responding to putative local modulators of androgen synthesis.
In conclusion, this chapter describes the validation of a culture system employing rat thecal/interstitial cells that respond to LH in a manner similar to the *in vivo* situation where androgen synthesis is increased in response to LH. The results presented in this chapter indicate that the culture of immature rat thecal/interstitial cells is suitable for use as an *in vitro* bioassay for rh-inhibin-A. In particular the cell culture system is a powerful tool for the study of paracrine modulation of thecal/interstitial cell androgen production. The next chapter utilises this culture system to investigate the regulation of thecal/interstitial cell function by potential paracrine factors of granulosa cell origin.
Chapter 4. Regulation of Androgen Synthesis

1. Introduction

In the previous chapter a thecal/interstitial cell culture was validated for the study of the control of androgen production. This chapter describes an investigation of the intrafollicular regulation of LH-stimulated thecal/interstitial cell androgen synthesis utilising the cell culture system and Northern hybridisation. Thecal/interstitial cells, under the endocrine control of LH, are major sites of androgen biosynthesis in the ovary. Cytochrome P450C17α (17-hydroxylase/C17-20 lyase) in these cells is the LH-responsive steroidogenic enzyme vital for androgen synthesis (Bogovich & Richards, 1982), and hence the provision of aromatase substrates required for estrogen synthesis in FSH-stimulated granulosa cells (Erickson et al. 1985).

The principal function of the ovary is the regular production of fertilisable oocytes. In order to accomplish this, a continual supply of primordial follicles must be recruited to begin growth and development. During the process of follicle growth a fraction of the growing follicles are selected to become dominant preovulatory follicles; the remainder undergo atresia. As they grow to preovulatory size, the dominant follicles secrete increasing amounts of oestradiol, which at the appropriate time triggers the preovulatory surge of LH and ultimately ovulation. There is a large body of literature that supports the fact that thecal cells play an essential role in these processes of follicle growth, selection, atresia, and oestradiol biosynthesis. During follicular growth and development the presumptive theca cells differentiate in an orderly and tightly controlled manner resulting in the sequential acquisition and loss of functional capabilities. These changes require the expression of a characteristic pattern of genes unique to the theca cell in a development-related manner. As the primordial follicle develops through preantral stages the theca cells express the genes for LH receptors, cholesterol side chain cleavage and 3β-hydroxysteroid dehydrogenase resulting in the capacity to respond to LH and produce progesterone but not androgens (Bogovich & Richards, 1982). When a follicle becomes selected to ovulate, physiologically low concentrations of LH present in the follicular
phase of the cycle stimulate growth and development of small antral follicles to the preovulatory stage (Richards et al. 1980; Bogovich et al. 1981). As the antral follicles grow to preovulatory size the theca express functional P450C17α and shift from progesterone producing to primarily androgen producing cells (Bogovich & Richards, 1982; Richards & Hedin, 1988). After ovulation the theca cells stop expressing P450C17α and lose the ability to produce androgens (Bogovich et al. 1986). As well as endocrine regulation by LH, thecal/interstitial P450C17α activity is likely to be subject to paracrine regulation by factors produced by granulosa cells. In vitro studies implicate diverse steroidal (e.g. oestradiol) and non-steroidal regulatory factors (e.g. inhibins and activins) in such a system but direct experimental evidence is lacking that paracrine signalling operates in vivo (Hillier, 1991a; Hillier, 1991b).

Previous attempts to demonstrate paracrine signalling in the ovarian follicle in vivo have generally been hampered by the unavailability of suitably pure forms of exogenous FSH with which to experiment (Fevold, 1941). Most pituitary or urinary FSH preparations available to-date have contained finite amounts of LH such that their injection into experimental animals activates thecal cell function via direct stimulation of the LH receptors that are constitutively present on thecal/interstitial cells, possibly obscuring any FSH-activated paracrine signal emanating in granulosa cells. The recent availability of rh-FSH expressed in Chinese Hamster Ovary cells offers a unique opportunity to test the follicular paracrine hypothesis, since this form of FSH can be produced completely devoid of LH. This chapter presents direct evidence from the use of rh-FSH that thecal/interstitial androgen synthesis is subject to paracrine regulation by granulosa cells in vivo.

2. Materials and Methods

2.1 Animals and in vivo treatments

All animals and in vivo treatments have been previously described in Chapter 2, section 2.1. Some animals were also treated with rh-growth hormone (rh-GH; 4 IU in 20 mg mannitol, 1.0 mg sodium chloride and PBS)
(Serono Laboratories UK) given as four 12-hourly injections at a total dose of 400 mIU.

2.2 Analysis of mRNA

2.2.1 Extraction, Purification and Quantitation of Total Cellular RNA

Total RNA was extracted from cultured thecal/interstitial cells according to the method of Chomczynsky and Sacchi (Chomczynski & Sacchi, 1987). Medium was removed from thecal/interstitial cell cultures and cells were lysed on ice with "solution D" (4M guanidinium thiocyanate [Fluka, Glossop, Derbyshire], 25mM sodium citrate, 0.5% [w/v] sarcosyl, 100mM β-mercaptoethanol; 200 μl/well). The cell lysate was transferred to polypropylene tubes (Falcon 2059, Becton Dickinson), and an additional 100 μl of solution D was added to each well, and pooled with the initial lysate. Alternatively, frozen tissue (whole ovaries or isolated thecal/interstitial or isolated granulosa cells) was homogenised in ice-cold solution D in corex tubes (Corning, New Jersey, USA.). To the RNA-containing solutions were added one tenth of a volume of 2M sodium acetate (pH4), one volume of water-saturated phenol and one fifth of a volume of chloroform:isoamyl alcohol (49:1 v/v) tubes being capped and shaken between additions. Tubes were shaken vigorously for 10sec after the last addition, and placed on ice for 15 min. Tubes were then centrifuged at 10,000 x g for 20 min at 4°C to separate the phases. The aqueous (top) phases were transferred to fresh tubes, taking great care to leave behind the protein present at the interface, and an equal volume of cold isopropanol was added. Tubes were left at -20°C for at least 1h to allow the RNA to precipitate. Precipitated RNA was recovered by centrifugation at 10,000 x g for 20 min at 4°C. The supernatants were poured off, pellets were dissolved in 300 μl solution D, and transferred to microcentrifuge tubes. RNA was reprecipitated with an equal volume of isopropanol at -20°C for at least 1h, and then recovered by centrifugation at 13,000 x g for 10 min at 4°C. RNA pellets were washed with 70% (v/v) ethanol and allowed to dry by leaving tubes open at room temperature, before being dissolved in 30 μl of 0.5% (w/v) SDS.
Concentration and purity of RNA preparations were determined by spectrophotometry. A small aliquot of each preparation was diluted with water, and transferred to a spectrophotometer cuvette. The purity of an RNA sample was calculated from the ratio of the absorbance of the solution at 260 nm (A$_{260}$) to its absorbance at 280 nm (A$_{280}$). A ratio of 2.0 indicated optimal purity. Any samples with an A$_{260}$:A$_{280}$ ratio of less than 1.5 were re-extracted with an equal volume of phenol:chloroform and reprecipitated with 0.1 volume of 3M sodium acetate and 2.5 volumes of ethanol at -20°C for at least 1h, then recovered by centrifugation, taken up in 0.5% SDS as before, and scanned in the spectrophotometer once again. Concentration of RNA samples was calculated from the A$_{260}$. A solution containing 40 µg of RNA/ml had an A$_{260}$ of 1.0. A$_{260}$ values of samples were therefore multiplied by 40 µg/ml, and corrected for the dilution factor to give the concentration of RNA in the original sample. One tenth of a volume of 3M sodium acetate and 2.5 volumes of ethanol were added to the samples, and they were stored at -70°C until required.

2.2.2 Electrophoresis and Northern Blotting

Equal amounts of total RNA from each sample were centrifuged at 13,000 x g for 10 min, washed with 1ml 75% (v/v) ethanol, and pellets were allowed to dry at room temperature. RNA was redissolved in 4.4 µl 0.5% (w/v) SDS by heating to 65°C for 10 min. Buffer (15.6 µl) was added to a final concentration of 20mM 3-(N-morpholino) propanesulphonic acid (MOPS), 5 mM sodium acetate, 1 mM EDTA, 50% formamide, 18% formaldehyde, and 50 µg/ml ethidium bromide, and samples were heated to 60°C for a further 5 min, to denature RNA, and placed immediately on ice. Two microlitres of dye (25% [w/v] Ficoll 400 [Pharmacia], 0.25% bromophenol blue, 1mM EDTA) was added, samples were vortexed briefly, and centrifuged for 5 sec, to bring the samples to the bottom of the tubes. Samples were immediately loaded onto gels containing 1.5% (w/v) agarose, 6.6% (v/v) formaldehyde in running buffer containing 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA (pH7.0), and subjected to electrophoresis using a commercial horizontal gel electrophoresis tank (Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire), in running buffer. After electrophoresis, gels were
photographed under ultraviolet transillumination to check integrity and even loading of RNA, and to measure migration of RNA molecular weight standards and ribosomal RNA. RNA was partially hydrolysed by soaking gels in 0.05 M NaOH for 20 min, to improve RNA transfer. Gels were then soaked for 45 min in 20 x SSC (3M NaCl, 300 mM sodium citrate [pH 7.0]), before RNA was transferred overnight to uncharged nylon membranes (Hybond-N, Amersham) by capillary blotting. RNA was fixed to Northern blots by exposure to ultraviolet radiation (260 nm) for 5 min.

2.2.3 Preparation of Plasmids

Plasmid containing cDNA encoding the rat Cytochrome P450C17α (17-hydroxylase/Cl7-20 lyase) (Fevold et al. 1989) was generously donated by Dr. J. Ian Mason, University of Texas Southwestern Medical Center, Dallas, Texas.

Plasmid containing cDNA encoding the rat 18S ribosomal RNA was also used in this study (Erikson et al. 1981).

2.2.3.1 Transfection

Plasmids were transfected into E. coli strain JM109 (Promega), which were rendered competent for transfection by the following procedure. Cells were streaked on M-9 plates containing thiamine-HCl (42 mM Na₂HPO₄, 22 mM KH₂PO₄, 20 mM NH₄Cl, 9 mM NaCl, 2 mM MgSO₄, 1 mM thiamine-HCl, 100 μM CaCl₂, 1.5% [w/v] agarose, 0.2% [w/v] glucose) and incubated overnight at 37°C. A single colony was picked, and used to inoculate 25 ml of Luria-Bertani medium (LB; 1% [w/v] bacto-tryptone, 0.5% [w/v] bacto-yeast extract [both from Difco], 0.5% [w/v] NaCl) which was then shaken at 225 rpm overnight at 30°C. Five hundred millilitres of LB medium were inoculated with 5 ml of this culture and shaken at 150 rpm until the absorbance of the culture at 600 nm reached 0.5. The culture was placed on ice water for 2 h, and the cells collected by centrifugation at 2500 x g for 15 min at 4°C. The cells were gently resuspended in 20 ml of ice-cold trituration buffer (100 mM CaCl₂, 70 mM MgCl₂, 40 mM sodium acetate [pH 5.5]) and the volume was made up to 500 ml with the same solution. The cells were then incubated on ice for 45 min before being centrifuged at 1800 x g for 10
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min and gently resuspended in 50 ml of ice-cold trituration buffer. 80% (v/v) glycerol was added to the suspension, dropwise with gentle swirling, to a final concentration of 15%. The cells were divided into 1ml aliquots, snap-frozen in an ethanol-dry ice bath and stored at -70°C.

Purified plasmids were introduced into the cells by a heat-shock transfection method. Aliquots of competent cells were thawed on ice and 100 μl of cell suspension were pipetted on ice into pre-chilled Falcon 2059 15ml polypropylene tubes. Purified plasmids (10 ng) were added to the cells on ice, moving the pipette tip through the cell suspension to mix, and tubes left on ice for 30 min. Tubes were heated to 42°C for 45 sec, and then placed on ice again for 2 min. 900 μl of SOC medium (2% [w/v] bactotryptone, 0.5% [w/v] bacto-yeast extract, 10 mM NaCl, 10 mM MgSO₄, 10 mM MgCl₂, 2.5 mM KCl, 20 mM glucose) was added and tubes placed at 37°C and shaken at 225 rpm for 1h. 100 μl of transformed cells were spread on LB agar (as LB medium, containing 1.5% [w/v] agar [Difco]) plates, containing 100 μg/ml ampicillin, 40 μg/ml X-Gal and 500 μM IPTG. Plates were incubated overnight at 37°C. White recombinant colonies were picked using a flamed inoculating loop and used to inoculate 5 ml aliquots of LB medium containing 50 μg/ml ampicillin in sterile Falcon 2059 tubes. The tubes were incubated for at least 6h at 37°C with shaking at 225 rpm. Cells were recovered from 1.5 ml of these cultures by centrifugation at 13,000 x g for 30 sec, and the supernatants discarded. Plasmids were recovered from the bacteria by resuspension in 110 μl STETL (50 mM Tris [pH8.0], 50 mM EDTA, 8% [w/v] sucrose, 5% [v/v] Triton X-100, 0.5 mg/ml lysozyme), and incubated at room temperature for 10 min. The suspensions were boiled for 2 min, and then centrifuged at 13,000 x g for 15 min. The gelatinous pellets were then removed with sterile pipette tips, and 110 μl isopropanol was added to the supernatants at room temperature, and the samples were immediately centrifuged at 13,000 x g for 15 min. The pellets of DNA were then washed with 70% ethanol, and allowed to dry. After dissolving the pellets in 30 μl TE buffer (10 mM Tris [pH7.5], 1 mM EDTA), the plasmids were stored at -20°C. In order to confirm that clones contained the required plasmid, plasmid preparations were incubated with the appropriate restriction enzymes to liberate inserts, and plasmids and restriction digests were subjected to electrophoresis in 0.8% [w/v] agarose gels containing 1
\[ \text{\(\mu g/ml\)} \text{ ethidium bromide, using TAE (40 mM Tris-acetate, 1 mM EDTA) as the electrode buffer, and visualised under ultraviolet light.} \]

2.2.3.2 Amplification

Positive clones were then grown in large-scale culture. Cells transformed with plasmids of interest were used to inoculate 25 ml cultures of LB medium containing 50 \(\mu g/ml\) ampicillin, which were incubated overnight at 37°C with shaking at 200 rpm. Overnight cultures were used to inoculate 400 ml cultures of LB medium containing 50 \(\mu g/ml\) ampicillin, which were incubated at 37°C with shaking at 150 rpm until the absorbance at 600nm reached 0.8. Chloramphenicol was added to a final concentration of 170 \(\mu g/ml\), and cultures were incubated overnight at 37°C with shaking at 150 rpm. Bacteria were pelleted at 3000 x g for 10 min, and plasmids were purified by an alkaline lysis method, using a commercial kit (Qiagen Maxi Prep kit, Hybaid). Bacterial pellets were resuspended in 10 ml of a buffer containing 50 mM Tris, 10 mM EDTA, 100 \(\mu g/ml\) RNase A. Ten ml of 200 mM NaOH containing 1% [w/v] SDS were added, and the suspensions incubated at room temperature for 5 min. Ten ml of 2.55 M potassium acetate (pH4.8) were added, suspensions were immediately gently mixed and centrifuged at 20,000 x g for 30 min at 4°C. Supernatants were removed promptly, and centrifuged again at 20,000 x g for 10 min at 4°C, to obtain a clear lysate. Plasmids were purified from lysates by ion exchange chromatography. Columns (Qiagen tip 500) were equilibrated with 10 ml of a buffer containing 50 mM MOPS (pH7.0), 750 mM NaCl, 0.15% (v/v) Triton X-100, 15% (v/v) ethanol, and the lysates applied by gravity. Columns were washed with 3 x 10 ml of a buffer containing 50 mM MOPS (pH7.0), 1M NaCl and 15% (v/v) ethanol, to remove RNA, protein and other impurities. Plasmids were eluted from the resin in 15 ml of a buffer containing 50 mM MOPS (pH8.2), 1.25 M NaCl and 15% (v/v) ethanol, and recovered by precipitation with 0.7 volumes of isopropanol and centrifugation at 10,000 x g for 30 min at room temperature. Pellets of plasmid DNA were washed with 10 ml 70% (v/v) ethanol, allowed to dry, and redissolved in TE buffer. Concentration of plasmids was assessed by spectrophotometry as described for RNA above, except that for the calculation of concentration, an \(A_{260}\) of 1.0
was assumed to indicate a concentration of 50 µg/ml double-stranded DNA. Plasmids were stored at -20°C in TE buffer, at a concentration of 5 mg/ml.

2.2.4 Preparation of $^{32}$P-labelled cDNA Probes

cDNA inserts were liberated from purified plasmids by incubation with restriction enzymes.

The rat P45017α cDNA was restricted with Sst I and Kpn I to generate fragments of 0.30, 1.142, 1.375 and 3.567 kb. The reaction mixture was electrophoresed in a 0.8% low melting point agarose TAE gel, and the 1.142 kb fragment was excised from the gel. The excised agarose was weighed and 3 times volume of sterile distilled water was added. The mixture was boiled for 5 min and then vortexed. The plasmid was stored in 50 ng aliquots at -20°C.

The rat 18S rRNA cDNA was restricted with Bam HI to yield a 1.4 kb fragment which was excised from the gel and treated as described above.

cDNA probes were labelled by random priming using a commercial kit (Megaprime Kit, Amersham), based on the method of Feinberg and Vogelstein (1983). Fifty nanograms of purified cDNA was denatured by boiling for 2 min, and placed on ice. Water was added to give a final reaction volume of 50 µl, and 10 µl of labelling buffer (unlabelled dGTP, dATP and dTTP in a buffer containing Tris [pH7.8], MgCl$_2$ and β-mercaptoethanol), 5 µl random sequence hexamer primers (in a buffer containing BSA) and 50 µCi of $[^{32}$P]dCTP (specific activity ~3000 Ci/mmol, Amersham) were added on ice in order. Klenow DNA polymerase (2 U) was added, the components were mixed by gentle passage through a pipette tip, and centrifuged briefly to the bottom of the tube, and the reaction mixture was incubated at 37°C for 30 min. Labelled cDNA was separated from unincorporated nucleotides by chromatography on Sephadex G-50 columns (Nick Columns, Pharmacia) in TE buffer, and yield of probe was measured by scintillation counting. Probe was denatured by boiling for 5 min and then placing on ice. Hybridisation buffer was added to give ~10$^6$ cpm/ml antisense DNA probe.
2.2.5 *Northern Hybridisation with* $^{32}$P-*labelled Probes*

Northern blots were prehybridised by incubation for at least 1h in hybridisation buffer (5 x SSPE [0.9 M NaCl, 0.05 M sodium phosphate, 0.005 EDTA], 5 x Denhardts [0.1% BSA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone], 0.5% SDS (w/v), 50% formamide, 10 mg salmon sperm DNA) using a commercial hybridisation oven (Hybaid). Prehybridisation buffer was replaced with probe solution. Prehybridisation and hybridisation were carried out at 42°C. Blots were washed twice for 5 min at room temperature in a large volume of 2 x SSC, twice for 30 min at 65°C in a large volume of 1 x SSC and 1% SDS, once for 30 min at room temperature in a large volume of 0.5 x SSC and 1% SDS. Blots were then wrapped in cling film (Saran Wrap, GRI, Dunmow, Essex), and exposed to X-ray film (Kodak X-Omat AR-5, Sigma), using two intensifying screens (GRI). Abundance of the $\sim$2.0 kb P45017α transcript was quantified by video densitometry and images were analysed using the Magiskan computer system (Joyce Loebl, UK). Results are expressed as the ratio of the P45017α and 18S rRNA autoradiographic signals in each sample.

2.3 Preparation of Granulosa Cell Conditioned Medium

Granulosa cells from preantral and early antral follicles were harvested in culture medium as described in chapter 2 section 2.2. The culture wells were inoculated with replicate 250 μl portions of cell suspension containing 100,000 viable granulosa cells. The granulosa cells were incubated for 48 h in the presence or absence of 1 IU/ml rh-FSH. The spent culture medium (granulosa cell conditioned medium) was collected and stored frozen at −20°C.

2.4 Isolation and culture of thecal/interstitial cells

Thecal/interstitial cell preparations were prepared using the procedure described in chapter 3, section 2.2. *In vitro* treatments included hLH (0.1-30 ng/ml), rh-IGF-I (0.1-30 ng/ml) and rh-inhibin-A (0.1-30 ng/ml).
2.5 Isolation and culture of granulosa cells

Granulosa cells from preantral and early antral follicles were harvested in culture medium as described in chapter 2 section 2.2 and cultured for 6 h in the presence of hFSH (30 ng/ml) and 1.0 μM testosterone (aromatase substrate).

2.6 Steroid Radioimmunoassays

Oestradiol, androstenedione and androsterone in spent culture medium were determined by radioimmunoassays as described in Chapter 3, sections 2.4.1, 2.4.2 and 2.4.3.

2.7 Statistics

Statistical analysis was carried out using commercial software (CLR ANOVA). Analysis of variance with the Newman-Keuls test was used to analyse differences between experimental and control observations. Differences assigned a P value of <0.05 were regarded as statistically significant.

3. Results

3.1 Effect of rh-FSH administration in vivo on whole ovarian P450C17α mRNA expression

Treatment of intact animals with rh-FSH (total dose 72 IU) increased the abundance of the ~2 kb-sized P450C17α mRNA transcript in ovarian total RNA approximately 5-fold, similar to the positive-control response (15 IU PMSG) in four of four experiments (Fig. 4.1). Only in over-exposed autoradiograms could the P450C17α mRNA signal of vehicle-treated control tissue be discerned (Fig. 4.2). In hypophysectomised animals, P450C17α mRNA abundance was even lower and the response to rh-FSH was either weak (<20% of that to 15 IU PMSG, in one of three experiments; Fig. 4.3) or absent (two of three experiments; Fig. 4.4).
The dose-dependence of rh-FSH action on ovarian P450C17α mRNA expression was established in experiments on intact animals (Fig. 4.5). By Northern analysis using P450C17α mRNA:18S rRNA signal ratios assessed by video scanning densitometry to account for variable sample loading, a 1.7-fold increase in P450C17α mRNA abundance occurred in response to the lowest rh-FSH total dose (16 IU), with 1.61-, 2.02- and 4.93-fold increases in response to 32, 48 and 72 IU rh-FSH, respectively.

3.2 Relative effect of rh-FSH administration in vivo on the expression of P450C17α mRNA in granulosa and thecal/interstitial cells

To assess the relative effect of treatment with rh-FSH on expression of P450C17α mRNA in granulosa and thecal/interstitial cells, Northern analysis was carried out on RNA extracted from cells isolated from the ovaries of intact animals 48 h after initiating treatment with 72 IU rh-FSH. As shown in Fig. 4.6, granulosa cells from control animals did not display a visible autoradiographic signal corresponding to P450C17α mRNA and treatment with rh-FSH barely elicited a response. On the other hand, P450C17α mRNA abundance was high in control thecal/interstitial cells and increased 4-6-fold by rh-FSH, resulting in a P450C17α mRNA signal ~14 times greater than in rh-FSH-stimulated granulosa cells.

3.3 Effect of r-LH and rh-FSH administration in vivo on whole ovarian P450C17α mRNA expression

Treatment of hypophysectomised animals with r-LH (total dose 10 IU) increased the abundance of the ~2 kb-sized P450C17α mRNA transcript in ovarian total RNA (Fig. 4.7). Consistent with previous observations, treatment with rh-FSH (total dose 72 IU) or injection vehicle alone did not noticeably increase the abundance of the P450C17α mRNA transcript, while PMSG treatment increased the abundance of the transcript. In the presence of a fixed dose of rh-FSH (30 IU), r-LH (total dose 1-30 IU) dose-dependently increased the abundance of the P450C17α mRNA transcript (Fig. 4.7). The
FIG. 4.1. Effect of rh-FSH administration in vivo on ovarian P450C17α mRNA expression in intact female rats. The animals received four 12-hourly SC injections of rh-FSH (total dose 72 IU); negative controls received injection vehicle alone; positive controls received a single injection (15 IU) of pregnant mare's serum gonadotropin. Ovaries were removed 48 h after the first FSH injection. Total ovarian RNA was size-fractionated (20 μg/track) by electrophoresis on a 1.2% agarose-formaldehyde gel and blotted on to a nylon membrane. Upper panel Northern analysis using a 32P-labelled (random priming) bovine cytochrome P450C17α cDNA. Exposure of the autoradiogram to Kodak XAR-5 film was overnight at -70°C using an intensifying screen. The ~2.0 kb-sized cytochrome P450C17α transcript is arrowed. Lower panel Ethidium bromide stained 18S rRNA, demonstrating uniformity of sample loading.
FIG. 4.2. Overexposed autoradiogram demonstrating the effect of rh-FSH administration *in vivo* on ovarian P450C17α mRNA expression in intact female rats. See Fig. 2.1 for experimental details. Exposure of the autoradiogram to Kodak XAR-5 film was for 3 days at -70°C using an intensifying screen. The ~2.0 kb-sized cytochrome P450C17α transcript is arrowed. *Lower panel* Ethidium bromide stained 18S rRNA, demonstrating uniformity of sample loading.
FIG. 4.3. Effect of rh-FSH administration in vivo on ovarian P450C17α mRNA expression in hypophysectomised female rats. The animals received four 12-hourly SC injections of rh-FSH (total dose 72 IU); negative controls received injection vehicle alone; positive controls received a single injection (15 IU) of pregnant mare’s serum gonadotropin. Ovaries were removed 48 h after the first FSH injection. Total ovarian RNA was size-fractionated (20 μg/track) by electrophoresis on a 1.2% agarose-formaldehyde gel and blotted on to a nylon membrane. Upper panel Northern analysis using a 32P-labelled (random priming) bovine cytochrome P450C17α cDNA. Exposure of the autoradiogram to Kodak XAR-5 film was overnight at -70 °C using an intensifying screen. The ~2.0 kb-sized cytochrome P450C17α transcript is arrowed. Lower panel Ethidium bromide stained 18S rRNA, demonstrating uniformity of sample loading.
FIG. 4.4. Effect of rh-FSH administration in vivo on ovarian P450C17α mRNA expression in hypophysectomised female rats. Experimental details were as described in Fig. 2.3. The ~2.0 kb-sized cytochrome P450C17α transcript is arrowed. Lower panel Ethidium bromide stained 18S rRNA, demonstrating uniformity of sample loading.
FIG. 4.5. Dose-dependent effect of rh-FSH administration *in vivo* on ovarian P450C17α mRNA expression in intact female rats. The animals received four 12-hourly SC injections of rh-FSH (total dose 16 to 72 IU); negative controls received injection vehicle alone; positive controls received a single injection (15 IU) of pregnant mare’s serum gonadotropin. Ovaries were removed 48 h after the first FSH injection. Total ovarian RNA was size-fractionated (20 µg/track) by electrophoresis on a 1.2% agarose-formaldehyde gel and blotted on to a nylon membrane. *Upper panel* Northern analysis using a $^{32}$P-labelled (random priming) bovine cytochrome P450C17α cDNA. Exposure of the autoradiogram to Kodak XAR-5 film was overnight at -70°C using an intensifying screen. The ~2.0 kb-sized cytochrome P450C17α transcript is arrowed. *Lower panel* Northern analysis using a $^{32}$P-labelled (random priming) rat 18S rRNA cDNA, to compensate for nonuniform sample loading. Exposure of the autoradiogram to Kodak XAR-5 film was for 2h at -70°C using an intensifying screen.
FIG. 4.6. Relative effect of rh-FSH administration *in vivo* on granulosa and thecal/interstitial cell expression of P450C17α mRNA. Intact, immature female rats received four 12-hourly SC injections of rh-FSH (total dose 72 IU); controls received injection vehicle alone. Ovaries were removed 48 h after the first FSH injection for isolation of granulosa and thecal/interstitial cells. Total ovarian RNA was size-fractionated (20 μg/track) by electrophoresis on a 1.2% agarose-formaldehyde gel and blotted on to a nylon membrane. *Upper panel* Northern analysis using a $^{32}$P-labelled (random priming) bovine cytochrome P450C17α cDNA. Exposure of the autoradiogram to Kodak XAR-5 film was overnight at -70 °C using an intensifying screen. The ~2.0 kb-sized cytochrome P450C17α transcript is arrowed. *Lower panel* Ethidium bromide stained 18S rRNA, demonstrating uniformity of sample loading.
FIG. 4.7. Effect of r-LH administration in vivo in the presence of a fixed dose of rh-FSH on ovarian P450C17α mRNA expression in hypophysectomised female rats. The animals received four 12-hourly SC injections of rh-FSH (total dose 30 IU) and/or r-LH (total dose 1, 10 or 30 IU); negative controls received injection vehicle alone; positive controls received a single injection (15 IU) of pregnant mare’s serum gonadotropin. Ovaries were removed 48 h after the first FSH injection. Total ovarian RNA was size-fractionated (20 μg/track) by electrophoresis on a 1.2% agarose-formaldehyde gel and blotted on to a nylon membrane. Upper panel Northern analysis using a 32P-labelled (random priming) bovine cytochrome P450C17α cDNA. Exposure of the autoradiogram to Kodak XAR-5 film was overnight at -70 °C using an intensifying screen. The ~2.0 kb-sized cytochrome P450C17α transcript is arrowed. Lower panel Ethidium bromide stained 18S rRNA, illustrating sample loading.
interpretation of this experiment must take account of the uneven sample loading and poor quality of mRNA used. To establish whether rh-FSH increased LH-stimulated P45017α mRNA expression hypophysectomised animals were treated with different combinations of rh-FSH (0, 30 or 72 IU) and r-LH (0, 1 or 10 IU). Treatment with rh-FSH alone or injection vehicle alone did not noticeably increase the abundance of the P450C17α mRNA transcript, while PMSG treatment increased the abundance of the transcript (Fig. 4.8). Recombinant LH alone at the lowest dose tested (1 IU) increased P45017α mRNA expression. In the presence of this low dose of LH, rh-FSH (72 IU) increased the abundance of the P45017α mRNA transcript (Fig. 4.8). Recombinant LH alone at a dose of 10 IU also increased P45017α mRNA expression. In the presence of this dose of LH, rh-FSH (72 IU) decreased the abundance of the P45017α mRNA transcript (Fig. 4.8). However, again the interpretation of this experiment was very difficult due to the uneven sample loading and poor quality of mRNA used.

3.4 Effect of rh-FSH administration in vivo on thecal/interstitial cell response to hLH in vitro

Cultured thecal/interstitial cells from the ovaries of intact animals produced roughly similar amounts of androstenedione and androsterone. Results were therefore combined and expressed as total androgen production.

Treatment with hLH in vitro elicited a dose-related increase in androgen production by cultured thecal/interstitial cells. Maximum response occurred in the presence of hLH at a concentration of 10 ng/ml, with an ED50 of ~3 ng/ml. Pretreatment in vivo with rh-FSH (total dose 72 IU) increased the maximal response to hLH approximately 2-fold without significantly altering the ED50 (Fig. 4.9A). Composite data from four experiments on cultured thecal/interstitial cells from the ovaries of intact animals are shown in Fig. 4.10 A.

Thecal/interstitial cells isolated from control hypophysectomised animals produced similar amounts of androgen to cells from intact controls and showed similar sensitivity and responsiveness to hLH in vitro (compare
FIG. 4.8. Effect of rh-FSH administration in vivo in the presence of fixed doses of r-LH on ovarian P450C17α mRNA expression in hypophysectomised female rats. The animals received four 12-hourly SC injections of rh-FSH (total dose 0.30 or 72 IU) and/or r-LH (total dose 0.1 or 10 IU); negative controls received injection vehicle alone; positive controls received a single injection (15 IU) of pregnant mare's serum gonadotropin. Ovaries were removed 48 h after the first FSH injection. Total ovarian RNA was size-fractionated (20 µg/track) by electrophoresis on a 1.2% agarose-formaldehyde gel and blotted onto a nylon membrane. Upper panel Northern analysis using a $^{32}$P-labelled (random priming) bovine cytochrome P450C17α cDNA. Exposure of the autoradiogram to Kodak XAR-5 film was overnight at -70 °C using an intensifying screen. The ~2.0 kb-sized cytochrome P450C17α transcript is arrowed. Lower panel Ethidium bromide stained 18S rRNA, illustrating sample loading.
FIG. 4.9. Effect of rh-FSH administration in vivo on hLH-responsive androgen production in vitro by thecal/interstitial cells from the ovaries of A. intact female rats and B. hypophysectomised female rats. Thecal/interstitial cell cultures (40,000 viable cells per culture well) were incubated for 48 h in serum-free medium containing increasing doses of hLH (0.1 to 30 ng/ml). Androstenedione+androsterone accumulation in spent culture medium was determined by radioimmunoassay. Data from a representative experiment are expressed as mean ± SE (n = 3). Asterisk denotes significant stimulation by rh-FSH versus untreated control: *P < 0.01.
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FIG. 4.10. Effect of rh-FSH administration in vivo on hLH-responsive androgen production in vitro by thecal/interstitial cells from the ovaries of A. intact female rats and B. hypophysectomised female rats. Thecal/interstitial cell cultures (40,000 viable cells per well) were incubated for 48 hours in serum-free medium with and without hLH (10 ng/ml). Androstenedione+androsterone accumulation in the spent culture medium was determined by radioimmunoassay. Composite data from 4 experiments are expressed as mean ± SE.
Figs. 4.9A and 4.9B). However, pretreatment of hypophysectomised animals with rh-FSH (total dose 72 IU) in vivo did not significantly affect sensitivity or responsiveness to hLH in vitro (Fig. 4.9B). Composite data from four experiments on thecal/interstitial cells from the ovaries of hypophysectomised animals are shown in Fig. 4.10B.

Treatment of cultured thecal/interstitial cells in vitro with rh-FSH at concentrations up to 100 ng/ml did not significantly increase androgen production (Fig 3.6), confirming lack of intrinsic 'LH-like' activity.

3.5 Effect of granulosa cell conditioned medium on basal and hLH-responsive thecal/interstitial cell androgen synthesis in vitro

To obtain in vitro evidence for FSH-stimulated paracrine signalling, granulosa cells from intact animals were cultured for 48 h in serum-free medium, with and without rh-FSH (1 IU/ml), and the spent medium was collected and added to thecal/interstitial cell cultures. Incubation of thecal/interstitial cells from intact animals in undiluted medium from FSH-treated granulosa cultures significantly altered androgen production in response to exogenous hLH (Fig. 4.11), the response to high-dose (30 ng/ml) hLH being ~25% higher (P<0.01). Medium from granulosa cells not treated with rh-FSH tended to suppress the response to hLH, although its effect was not statistically significant.

3.6 Effect of inhibin on basal and hLH-responsive thecal/interstitial cell androgen synthesis and P450C17α mRNA expression in vitro

To confirm the influence of a putative paracrine factor on thecal/interstitial androgen synthesis, cells were cultured with recombinant inhibin in the presence and absence of hLH. Regardless of whether the cells were obtained from intact or hypophysectomised animals, inhibin (30 ng/ml) alone, had no significant effect on androgen production but enhanced the stimulatory effect of hLH (10 ng/ml) 2-3 fold. A typical dose-response is shown in Fig. 4.12.
FIG. 4.11. Effect of conditioned medium from rh-FSH-stimulated granulosa cells on LH-responsive androgen production \textit{in vitro} by thecal/interstitial cells from the ovaries of intact female rats. Granulosa cell cultures (100,000 viable cells per culture well) were incubated for 48 h in serum-free medium with or without rh-FSH (1 IU/ml) and the spent medium was collected. Thecal/interstitial cell cultures (40,000 viable cells per culture) were incubated in either serum-free medium, spent medium from untreated granulosa cell cultures, or spent medium from rh-FSH-treated granulosa cells. Cultures were for 48 h in the presence of increasing doses of hLH (0.1 to 30 ng/ml). Androstenedione+androsterone accumulation in spent thecal/interstitial cell culture medium was determined by radioimmunoassay. Data from a representative experiment are expressed as mean ± SE (n = 3).
FIG. 4.12. Effect of inhibin on LH-responsive androgen production in vitro by thecal/interstitial cells from the ovaries of intact female rats. Thecal/interstitial cell cultures (40,000 viable cells per culture well) were incubated for 48 h in serum-free medium with and without hLH (10 ng/ml) and/or inhibin-A (0.1-30 ng/ml). Androstenedione +androstosterone accumulation in spent culture medium was determined by radioimmunoassay. Data from a representative experiment are expressed as mean ± SE (n = 3).
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To test the influence of inhibin on P45017α mRNA expression, thecal/interstitial cells from intact animals were cultured with recombinant inhibin in the presence and absence of hLH. Messenger RNA was then extracted and Northern hybridisation was carried out. In four out of ten experiments inhibin (30 ng/ml) alone, had no significant effect on P45017α mRNA expression but enhanced the stimulatory effect of hLH (10 ng/ml) (Fig. 4.13). In six out of ten experiments no clear pattern was observed (Fig. 4.13).

3.7 Effect of IGF-I on basal and hLH-responsive thecal/interstitial cell androgen synthesis in vitro

To confirm the influence of another putative paracrine factor on thecal/interstitial androgen synthesis, cells were cultured with recombinant IGF-I in the presence and absence of hLH. Regardless of whether the cells were obtained from intact or hypophysectomised animals, IGF-I (30 ng/ml) alone, only had a significant effect on androgen production at doses of 10 ng/ml or above. IGF-I also enhanced the stimulatory effect of hLH (10 ng/ml) by about 50%. A typical dose-response is shown in Fig. 4.14.

3.8 Effect of rh-GH administration in vivo on thecal/interstitial cell response to hLH in vitro

In an attempt to explain the differences in the effects of rh-FSH treatment in vivo on LH-responsive androgen production in vitro between cells from intact and hypophysectomised animals, hypophysectomised animals were treated in vivo with rh-GH (total dose 400 mIU). This treatment was sufficient to significantly augment a FSH-stimulated increase in ovarian weight Fig. 4.15) and increase granulosa cell aromatase activity in vitro, though this effect was not significant (Fig. 4.16). However, growth hormone treatment in vivo did not alter basal or LH-responsive androgen production in vitro by thecal/interstitial cells from untreated or rh-FSH treated hypophysectomised animals (Fig. 4.17).
FIG. 4.13. Effect of inhibin on LH-responsive androgen production and P45017α expression in vitro by thecal/interstitial cells from the ovaries of intact female rats. Thecal/interstitial cell cultures (40,000 viable cells per culture well) were incubated for 48 h in serum-free medium with and without hLH (10 ng/ml) and/or inhibin-A (30 ng/ml). Androstenedione +androsterone accumulation in spent culture medium was determined by radioimmunoassay. Data are expressed as mean ± SE. Thecal/interstitial cells were removed and total ovarian RNA was size-fractionated (20 μg/track) by electrophoresis on a 1.2% agarose-formaldehyde gel and blotted on to a nylon membrane. Upper panel Northern analysis using a $^{32}$P-labelled (random priming) bovine cytochrome P450C17α cDNA. Exposure of the autoradiogram to Kodak XAR-5 film was overnight at -70°C using an intensifying screen. The ~2.0 kb-sized cytochrome P450C17α transcript is shown. Autoradiographs are representative of six experiments. Lower panel Northern analysis using a $^{32}$P-labelled (random priming) bovine cytochrome P450C17α cDNA. Exposure of the autoradiogram to Kodak XAR-5 film was overnight at -70°C using an intensifying screen. The ~2.0 kb-sized cytochrome P450C17α transcript is shown. Autoradiographs are representative of four experiments.
FIG. 4.14. Effect of IGF-I on LH-responsive androgen production in vitro by thecal/interstitial cells from the ovaries of intact female rats. Thecal/interstitial cell cultures (40,000 viable cells per culture well) were incubated for 48 h in serum-free medium with and without hLH (10 ng/ml) and/or IGF-I (0.1-30 ng/ml). Androstenedione + androsterone accumulation in spent culture medium was determined by radioimmunoassay. Data from a representative experiment are expressed as mean ± SE (n = 3).
FIG. 4.15. Effect of GH on ovarian weight of hypophysectomised female rats. Animals were treated with rh-GH (total dose 400 mIU) and/or rh-FSH (total dose 72 IU) or injection vehicle alone (PBS in 0.1% BSA). Ovaries were removed after 48 h and all extraneous material was dissected. Results are expressed as mean weight of each ovarian pair ± SE. (n ≥ 6). a is significantly different from b and c (P <0.01), b is significantly different from c (P <0.01).
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FIG. 4.16. Effect of GH treatment *in vivo* on hFSH-responsive aromatase activity by rat granulosa cells *in vitro*. Granulosa cell cultures (40,000 viable cells per well) were incubated for 48 h in serum-free medium containing 1.0 μM testosterone (aromatase substrate) and hFSH (30 ng/ml). Oestradiol in spent medium was determined by RIA; values are expressed as mean ± SE (n=3). a is significantly different from b (P <0.01).
FIG. 4.17. Effect of growth hormone administration in vivo on hLH-responsive androgen production in vitro by thecal/interstitial cells from the ovaries of hypophysectomised female rats. Thecal/interstitial cell cultures (40,000 viable cells per culture well) were incubated for 48 h in serum-free medium containing hLH (10 ng/ml) and/or IGF-I (30 ng/ml). Androstenedione+androstereone accumulation in spent culture medium was determined by radioimmunoassay. Data are expressed as mean ± SE (n=3). There were no significant differences due to GH nor rh-FSH treatment.
3.9 Effect of rh-LH administration in vivo on thecal/interstitial cell response to hLH in vitro

In an attempt to explain the differences in the effects of rh-FSH treatment in vivo on LH-responsive androgen production in vitro between cells from intact and hypophysectomised animals, hypophysectomised animals were treated in vivo with r-LH (total dose 1-30 IU). Treatment of hypophysectomised animals in vivo with r-LH (total dose 10 IU) in combination with rh-FSH (total dose 72 IU) restored the ability of rh-FSH to increase thecal/interstitial responsiveness to LH in vitro (Fig. 4.18). However, hypophysectomised animals treated in vivo with Pergonal (40 IU of both LH and FSH) exhibited a lack of LH responsive androgen production (Fig. 4.19). Treatment in vitro with IGF-I did not affect the levels of basal nor LH-stimulated androgen production (Fig. 4.19).

To examine the effects of LH in vivo on androgen synthesis in vitro more closely, hypophysectomised animals were treated with increasing doses of r-LH (total dose 0, 1 or 30 IU) in the presence of a fixed dose of rh-FSH (total dose 30 IU). Co-treatment of rh-FSH treated animals with a low dose of r-LH (1 IU) in vivo increased LH-stimulated androgen production compared to cells from animals treated with rh-FSH alone (Fig. 4.20). However, androgen production in the presence of LH + inhibin (30 ng/ml) was unaffected by r-LH treatment in vivo. Co-treatment of rh-FSH treated animals with a high dose of r-LH (30 IU) in vivo decreased androgen production by LH- and LH + inhibin-treated cells to levels seen in unstimulated cells (Fig. 4.20).

3.10 Effect of DES administration in vivo on thecal/interstitial cell response to hLH in vitro

To test the influence of oestrogen, another candidate paracrine factor on thecal/interstitial androgen synthesis, cells from intact animals treated with DES in vivo were cultured with and without hLH (10 ng/ml). Treatment with DES in vivo decreased thecal/interstitial cell LH-responsiveness in vitro (Fig. 4.21). Treatment with DES in vivo also rendered thecal/interstitial cells unresponsive to rh-FSH treatment in vivo (Fig. 4.21).
Regulation of Androgen Synthesis

FIG. 4.18. Effect of rh-FSH administration \textit{in vivo} on hLH-responsive androgen production \textit{in vitro} by thecal/interstitial cells from the ovaries of hypophysectomised female rats treated \textit{in vivo} with A. injection vehicle alone and B. r-LH (1 IU). Thecal/interstitial cell cultures (40,000 viable cells per well) were incubated for 48 hours in serum-free medium with and without hLH (10 ng/ml). Androstenedione+androsterone accumulation in the spent culture medium was determined by radioimmunoassay. Data from a representative experiment are expressed as mean ± SE (n=3).
FIG. 4.19. Effect of rh-FSH (72 IU) and Pergonal (40 IU each of FSH and LH) administration in vivo on hLH-responsive androgen production in vitro by thecal/interstitial cells from the ovaries of hypophysectomised female rats. Thecal/interstitial cell cultures (40,000 viable cells per well) were incubated for 48 hours in serum-free medium with and without hLH (10 ng/ml) and or IGF-I (30 ng/ml). Androstenedione-androsterone accumulation in the spent culture medium was determined by radioimmunoassay. Data from a representative experiment are expressed as mean ± SE (n=3). Asterisk denotes significant difference due to Pergonal versus untreated control or rh-FSH: *P <0.01.
FIG. 4.20. Effect of r-LH administration in vivo on hLH-responsive androgen production in vitro by thecal/interstitial cells from the ovaries of rh-FSH treated hypophysectomised female rats. Thecal/interstitial cell cultures (40,000 viable cells per well) were incubated for 48 hours in serum-free medium with and without hLH (10 ng/ml) and/or IGF-I (30 ng/ml). Androstenedione-androsterone accumulation in the spent culture medium was determined by radioimmunoassay. Data from a representative experiment are expressed as mean ± SE (n=3). a denotes significant difference due to FSH 30 + LH 1 treatment compared to FSH 30 alone (P <0.01), b denotes significant difference due to FSH 30 + LH 30 treatment compared to FSH 30 alone or FSH 30 + LH 1 treatment (P <0.01).
FIG. 4.21. Effect of DES administration in vivo on hLH-responsive androgen production in vitro by thecal/interstitial cells from the ovaries of intact female rats treated in vivo with A. injection vehicle alone and B. rh-FSH. Thecal/interstitial cell cultures (40,000 viable cells per well) were incubated for 48 hours in serum-free medium with and without hLH (10 ng/ml). Androstenedione+androstosterone accumulation in the spent culture medium was determined by radioimmunoassay. Data from a representative experiment are expressed as mean ± SE (n=3).
4. Discussion

Granulosa cells but not thecal/interstitial cells possess FSH receptors, whereas P450C17α mRNA expression is far higher in thecal/interstitial cells than granulosa cells (Hedin et al. 1987), therefore the finding that treatment with rh-FSH in vivo enhances P450C17α mRNA expression and LH-responsive androgen synthesis in vitro can be interpreted as direct evidence that FSH activates granulosa-derived paracrine signalling in the ovary. Since P450C17α mRNA expression is almost exclusively localised to thecal/interstitial cells (see Chapter 5), then it can be inferred that any changes in P450C17α mRNA content of the ovary reflects changes in thecal/interstitial cell expression of P450C17α mRNA.

Human LH-responsive thecal/interstitial cell androgen synthesis in vitro was doubled by treatment with rh-FSH in vivo, but sensitivity to hLH was unchanged. Similarly, treatment of thecal/interstitial cells with hLH in the presence of conditioned medium from rh-FSH-stimulated granulosa cells augmented overall response without affecting sensitivity to hLH. The mechanism of this effect is not known but would be expected to involve increased activity of enzymes crucial to androgen synthesis. P450C17α and cholesterol side-chain cleavage (P450scc) are two such enzymes known to be regulated by LH. In cultured rat thecal/interstitial cells both enzymic activities are upregulated by insulin-like growth factor I (IGF-I), a granulosa-derived growth factor previously implicated in the paracrine regulation of androgen synthesis (Magoffin et al. 1990). As confirmed here, inhibin (another granulosa-derived protein) also enhances LH-responsive androgen synthesis (Hsueh et al. 1987). Our data also suggest that P45017α is a target of inhibin action, though the experiments were not always reproducible (see Fig. 4.13). Since FSH enhances granulosa cell production of IGF-I (Adashi et al. 1985a) and inhibin (Bicsak et al. 1986), both factors are potential intermediaries of FSH action on thecal/interstitial cells. Further work is required to determine if they actually affect LH-responsive androgen synthesis in vivo.

Hypophysectomy prevented the stimulatory action of FSH treatment in vivo on subsequent thecal/interstitial cell responsiveness to hLH in vitro. This suggests that simultaneous exposure to endogenous LH is necessary to
facilitate augmentation of thecal/interstitial function by FSH. In other words, paracrine signalling emanating in FSH-stimulated granulosa cells modulates the action of LH but cannot substitute for the endocrine role of LH per se. The finding in vitro that a candidate paracrine factor, inhibin, is unable to directly stimulate androgen synthesis whereas it potently enhances the action of hLH in vitro supports this interpretation. The finding that low dose LH treatment in vivo restored the stimulatory action of FSH treatment in vivo on subsequent thecal/interstitial cell responsiveness to hLH in vitro further supports the hypothesis that simultaneous LH exposure is necessary for FSH-stimulated augmentation of thecal/interstitial function.

Treatment with both FSH and LH is required to stimulate follicular oestrogen synthesis in hypophysectomised rats (Mannaerts et al. 1991) because androgens produced in LH-stimulated thecal/interstitial cells are essential aromatase substrates for estrogen synthesis in FSH-stimulated granulosa cells (Armstrong & Dorrington, 1979; Dorrington et al. 1975; Makris & Ryan, 1975; Fortune & Armstrong, 1977; Baird, 1977; Tonetta & diZerega, 1989). 17-Hydroxylase and C17-20 lyase activities of P450C17α are rate-limiting in androgen synthesis (Bogovich & Richards, 1982), and the finding that thecal/interstitial cell P450C17α mRNA expression is directly enhanced by treatment with PMSG in vivo is consistent with the fundamental role of LH in regulating these enzymic activities. Moreover, the reduction in P450C17α mRNA expression caused by hypophysectomy is explained by withdrawal of endogenous LH, since replacement therapy with r-LH in low doses restores P450C17α mRNA expression as well as the ability of rh-FSH in vivo to augment thecal/interstitial responsiveness to LH in vitro. However, a generalised metabolic disturbance subsequent to hypophysectomy would also be expected to influence gonadal steroidogenesis. In particular, adrenal collapse due to lack of ACTH or absence of circulating liver-derived IGF-I due to withdrawal of GH could negatively influence thecal androgen synthesis. Potentially important endocrine and metabolic effects of ACTH on androgen synthesis were not evaluated in this study. However, this study has shown that replacement therapy with rh-GH at a dose sufficient to significantly stimulate ovarian weight in hypophysectomised, immature female rats does not significantly alter basal or LH-responsive thecal/interstitial cell androgen production in vitro, effectively ruling out
both direct and indirect contributions of GH to ovarian androgen synthesis in this system.

If P450C17α mRNA expression is exclusive to the thecal/interstitial ovarian compartment, the finding that rh-FSH stimulates granulosa cell P450C17α mRNA expression as judged by Northern analysis is difficult to explain. The possibility that the granulosa cells from which RNA was isolated for Northern analysis were significantly contaminated with thecal/interstitial cells (see Fig. 4.6) cannot be ruled out. However, a more probable explanation is that every steroidogenic cell type expresses finite levels of all steroidogenic enzymes. The characteristic steroid secretory functions of endocrine glands reflect their histogenetic derivation and the trophic mechanisms to which specific cell types respond, consistent with the 'unified' concept of steroid hormone formation put forward by Ryan twenty years ago (Ryan, 1972). Thus FSH-stimulated granulosa cells, being richly endowed with aromatase, are functionally deficient in 17-hydroxylase and 17-20 lyase while the opposite is true of LH-stimulated thecal/interstitial cells. Either way, stimulation of granulosa cells with rh-FSH in vivo markedly enhances thecal/interstitial cell P450C17α mRNA expression which has implications for oestrogen synthesis as well as androgen synthesis.

The LH surge prior to ovulation leads to a drop in P450C17α mRNA and bioactivity (Hedin et al. 1987; Eckstein & Tsafriri, 1986). This is consistent with the finding that high doses of LH in vivo result in a decrease in P45017α mRNA expression and a loss of LH-responsive androgen production in vitro. Similarly DES treatment in vivo results in a reduction in LH-responsive androgen production in vitro consistent with the finding that oestrogens inhibit LH-stimulated androgen biosynthesis in rat thecal/interstitial cells (Erickson et al. 1985) by greater than 90%. The mechanism of oestrogen action in thecal/interstitial cells remains unclear. The inhibitory effects of oestradiol on thecal/interstitial cell androgen production occur without changes in LH receptors, LH-stimulated cAMP production or progesterone production (Erickson et al. 1985) and oestradiol does not cause a direct inhibition of P450C17α activity in ovarian homogenates (Johnson et al. 1984). These inhibitory effects of oestrogen may be the mechanism which mediates the decrease in P450C17α activity that occurs after the LH surge prior to ovulation in a wide variety of species (Erickson et al. 1985).
In conclusion, FSH is able to influence ovarian androgen synthesis via a paracrine mechanism that involves modulation of expression of thecal/interstitial P450C17α. Further research is required to determine which locally produced factor(s) mediates this action of FSH. It is likely that steroids produced by FSH-stimulated granulosa cells modulate androgen production by thecal/interstitial cells resulting in co-ordinated follicular steroidogenesis. Oestradiol is a potential candidate since it is able to regulate androgen synthesis by thecal/interstitial cells in vitro (Erickson et al. 1985). Other likely candidates are regulatory proteins produced by granulosa cells responding to direct stimulation by FSH. Such proteins include inhibin (Hsueh et al. 1987) and IGF-I (Adashi et al. 1985), which dose-dependently enhance LH-stimulated thecal/interstitial cell androgen synthesis in vitro and thereby qualify as potential paracrine modulators of androgen synthesis in vivo.
Chapter 5. Expression of Ovarian P45017α mRNA.

1. Introduction

The previous chapter reported an investigation of the control of ovarian thecal/interstitial cell function by gonadotrophins and paracrine factors. This chapter describes a study by in situ hybridisation of the regulation of P450C17α mRNA expression during follicular development. Cytochrome P450C17α (17-hydroxylase/C17-20 lyase) in thecal/interstitial cells is the LH-responsive steroidogenic enzyme involved in the conversion of progestogens to androgens (Bogovich & Richards, 1982), and hence the provision of aromatase substrates required for oestrogen synthesis in FSH-stimulated granulosa cells (reviewed by Erickson et al. 1985). To-date there has been no published report of the expression of P450C17α as studied by in situ hybridisation.

Thecal/interstitial cells are major sites of androgen biosynthesis in the ovary (Erickson et al. 1985). P450C17α is the single microsomal enzyme that catalyses steroid 17-hydroxylase and C17-20 lyase activities (Zuber et al. 1986). These activities reside in the thecal/interstitial cells of the ovary (Erickson et al. 1985) and are absent from granulosa cells (Richards et al. 1987). Granulosa cells are therefore incapable of de novo androgen synthesis and depend on the theca to supply extracellular androgen for use as oestrogen precursor consistent with the two-cell, two-gonadotrophin mechanism of oestrogen synthesis (Armstrong & Dorrington, 1979). Like P45017α activity, P450C17α mRNA and protein have been detected exclusively in the thecal/interstitial cells (Hedin et al. 1987; Eckstein & Tsafriri, 1986). LH promotes differentiation of thecal/interstitial cells and the induction of P450C17α in developing follicles (Bogovich et al. 1981). Throughout this process of follicular development P450C17α mRNA, protein content and bioactivity increase (Hedin et al. 1987). The LH surge leads to a drop in P450C17α mRNA, protein content and bioactivity to levels below those seen in small antral follicles (Hedin et al. 1987; Eckstein & Tsafriri, 1986).

As well as primary endocrine regulation by LH, thecal/interstitial P450C17α activity is likely to be subject to a secondary level of regulation by
factors produced by granulosa or thecal/interstitial cells. *In vitro* studies suggest many steroidal (e.g. oestradiol) and non-steroidal regulatory factors (e.g. inhibin and related peptides) could have a role in such a system. This study investigates the regulation of P450C17α mRNA expression by ‘pure’ (i.e. recombinant) gonadotrophins and intraovarian modulators of gonadotrophin action.

2. Materials and Methods

2.1 Animals and *in vivo* treatments

All animals and *in vivo* treatments have been described previously in Chapter 2, section 2.1.

2.2 Determination of stage of oestrous cycle

Four month old adult female Wistar rats were tracked by daily vaginal smearing to ascertain the stage of oestrous cycle that they had reached. The rat oestrous cycle was considered to consist of four stages; dioestrus 1, dioestrus 2, pro-oestrus and oestrus. A plastic stirring rod (Sarstedt, Beaumont Leys, Leicester) was dipped in saline and then a vaginal smear was taken and spread onto a glass slide containing a drop of saline solution. The smears were then viewed under phase contrast microscopy at a magnification of x 100. The animal was considered to be in dioestrus if the smear contained polymorpho-nucleot lymphocytes (PMNL) which are small, perfectly rounded cells. If the smear contained clumps of larger, round or oddly shaped cells then the animal was considered to be at the pro-oestrus stage of the cycle. The presence of clumps of angular cells in the complete absence of any PMNL was regarded as definitive for oestrus. Only animals that completed two regular 4 day cycles were used in the experiment.

2.3 Tissue preparation

Ovaries were fixed overnight at 4°C in 4% (w/v) paraformaldehyde (Sigma) dissolved in PBS. This was followed by sequential dehydration
treatments with PBS for 30 min at 4°C; 0.85% (w/v) NaCl dissolved in PBS for 30 min at 4°C; 0.85% (w/v) NaCl dissolved in PBS: ethanol (1:10) for 15 min at room temperature; 70% (v/v) ethanol for 30 sec at room temperature. The tissue was then automatically processed overnight (Shandon Scientific Ltd) through graded ethanol solutions, followed by xylene, 1:1 xylene-paraffin, then paraffin wax. Prepared tissues were then placed in moulds containing melted paraffin, which was then allowed to solidify. Tissue embedded in paraffin wax is stable for several years without loss of hybridisability of target RNAs or deterioration of morphology.

2.4 Slide preparation and tissue sectioning

Slides were cleaned and coated to help tissue section retention. Firstly slides were dipped in 10% (v/v) HCl in 70% (v/v) ethanol for 10 sec. This was followed by a 10 sec rinse in distilled water, then the slides were dipped in 95% (v/v) ethanol. The slides were placed in an oven at 150°C for 5 min. Once the slides had cooled they were twice dipped in a 2% (v/v) solution of TESPA in acetone for 10 sec, followed by two 10 sec washes in acetone. The slides were then rinsed in distilled water then baked dry at 42°C.

Using a standard rotary microtome (American Optical Corporation) tissue was sectioned at 5μm thickness to produce a ribbon of sections. This ribbon was floated on the surface of distilled water heated to 45°C in a water bath. Sections were mounted by inserting a coated slide into the water bath near the sections at a 45° angle to the water surface, touching an edge of the ribbon to the slide and slowly withdrawing the slide from the bath. Slides were placed in an oven overnight at 40°C to ensure sections had adhered to the slide.

2.5 Plasmid preparation

Plasmid containing cDNA encoding the rat Cytochrome P450C17α (17-hydroxylase/C17-20 lyase) (Fevold et al. 1989) was generously donated by Dr. J. Ian Mason, University of Texas Southwestern Medical Center, Dallas,
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From a clone containing a 1.88 kb insert, a 1.143 kb segment was excised (nucleotides 251-1394) using Kpn I and Sst I. This was then blunted with Klenow fragment of *E. coli* DNA polymerase I (Promega Ltd) and inserted into the Sma I site of pGEM-3Z vector (Promega Ltd). The orientation of the insert was such that SP6 RNA polymerase could be used to transcribe antisense RNA probes and T7 RNA polymerase to transcribe sense probes. Templates were prepared using Pvu II to generate an antisense template of 564 bp and Sca I to generate a sense template of 536 bp. A plasmid map is shown in Fig. 5.1.

Plasmid containing cDNA encoding the rat P450 aromatase (P450arom) (Hickey *et al.* 1990) was generously donated by Dr. J.S. Richards, Baylor College of Medicine, Houston, Texas. From a clone containing a 1047 bp insert the full insert was excised using Eco RI. This was blunted as above and inserted into the Sma I site of pGEM-3Z vector (Promega Ltd, UK). The orientation of the insert was such that SP6 RNA polymerase could be used to transcribe antisense RNA probes and T7 RNA polymerase to transcribe sense probes. Templates were prepared using Acc III to generate an antisense template of ~300 bp and Bam HI to generate a sense template of ~400 bp.

Plasmid containing cDNA encoding the rat LH receptor (LH-R) (LaPolt *et al.* 1990a) was generously donated by Dr. A.J.W. Hsueh, Stanford University School of Medicine, Stanford, California. The LH-R clone corresponding to bases 249 to 849 of the rat LH-R cDNA was subcloned into pGEM-3Z at Eco RI. The orientation of the insert was such that SP6 RNA polymerase could be used to transcribe sense RNA probes and T7 RNA polymerase to transcribe antisense probes. Templates were prepared using Bgl II to generate an antisense template of 408 bp and Bam HI to generate a sense template.

Plasmids were transfected and amplified, as described in chapter 4, sections 2.2.3.1 and 2.2.3.2.
FIG. 5.1. Restriction map of pGEM3Zf(+) rat P45017 alpha showing orientation of insert cDNA in relation to SP6 and T7 DNA polymerases.
2.6 Probe preparation and labelling

P45017α DNA templates were prepared by digestion of plasmid with Pvu II (to generate an antisense probe) or Sca I (to generate a sense probe to be used as a control for probe specificity). In order to ensure that plasmid was fully linearised, restriction digestion was carried out overnight at 37°C. A small aliquot of digested DNA was analysed by agarose gel electrophoresis and visualised under UV light. A single band of DNA was observed, indicating that plasmid was fully linearised, and its concentration was estimated by comparison with standards of known concentration.

Template DNA was precipitated with 0.1 volume of 3M sodium acetate (pH 7.0) and 2 volumes of ethanol at -20°C for at least 1h, and centrifuged at 13,000g for 10 min. The DNA pellet was redissolved in TE buffer to give a concentration of ~1 mg/ml. Transcription of RNA from the Pvu II template using SP6 RNA polymerase results in synthesis of RNA probes complementary to the portion of mRNA encoding the conserved P45017α and Ozols tridecapeptide regions of P45017α protein (Fevold et al. 1989). In vitro transcription was carried out using a commercial kit (Riboprobe Gemini System II, Promega). One microgram of template DNA was added to a tube containing 40 mM Tris (pH 7.5), 10 mM NaCl, 6 mM MgCl2, 2 mM spermidine, 26 mM DTT, 20 U ribonuclease inhibitor (RNasin, Promega), unlabelled ATP, GTP and CTP at 0.33 mM each. Fifty microcuries of 35S-labelled UTP (specific activity > 1000 Ci/mmol; final concentration, 6μM, Amersham), and 20 U SP6 (antisense) or T7 (sense) RNA polymerase were added, the components were mixed gently by passage through a pipette tip and brought to the bottom of the tube by brief centrifugation, and the reaction was allowed to take place for 30 min at 37°C. A further 20 U SP6 (antisense) or T7 (sense) RNA polymerase were added and the reaction was allowed to continue for 3 h at 37°C. Incorporation of labelled nucleotide and specific activity of probe were measured by adsorption of RNA to charged paper filters (DE81, Whatman). Duplicate 1μl aliquots of the reaction mixture were spotted onto DE81 filters and allowed to dry. One filter was washed four times for 5min in ~200ml 0.5M Na2HPO4 (pH 7.0), then rinsed in 70% ethanol and allowed to dry. Both filters were placed in scintillation vials with
4ml of scintillation fluid, and radioactivity bound to the filters was measured by liquid scintillation counting. Counts bound to the washed filter represented rUTP incorporated into RNA, and those on the unwashed filter represented the total input of rUTP. Incorporation and specific activity were calculated to be >70% and ~10⁶ cpm/μg respectively (assuming approximately equal proportions of all four nucleotides in the template sequence). Template DNA was removed by incubation with 2 U RNase-free DNase (RQ1 DNase, Promega) and 10μg/ml tRNA for 10 min at 37°C. This was followed by extraction with 1 volume of TE-saturated phenol, vortexing thoroughly and centrifugation at 12,000 x g for 2 min. The upper, aqueous phase was transferred to a fresh tube and 1 volume of phenol/chloroform was added. This mixture was vortexed and then centrifuged at 12,000 x g for 2 min. Again the upper, aqueous phase was transferred to a fresh tube and 1 volume of chloroform was added. This mixture was vortexed and then centrifuged at 12,000 x g for 2 min. Unincorporated nucleotides were removed from the labelled probe by ethanol precipitation; 0.2 volumes of 10 M ammonium acetate was added to the RNA sample and mixed. Three volumes of ethanol were added and the RNA was precipitated at -20°C for at least 1 h. The mixture was centrifuged at 12,000 x g for 20 min at 4°C and the supernatant was removed. The pellet was washed in 80% (v/v) ethanol and spun for 10 min at 12,000 x g. The supernatant was carefully removed and the pellet was vacuum dried. The pellet was then suspended in sufficient 50mM DTT to yield a final concentration of RNA of 5 ng/μl and stored at -70°C.

The DNA templates for LH-R and aromatase were prepared in the same way using the appropriate restriction enzymes as described above.

2.7 Prehybridisation

Tissue sections were deparaffinised and rehydrated by immersing slides in two changes of xylene for 10 min each time. The slides were then passed sequentially, for 2 min each, through 100%, 100%, 90% (v/v), 75% (v/v), 50% (v/v), and 30% (v/v) ethanol then 5 min in each of 0.85% (w/v) NaCl in PBS and PBS. This was followed by 20 min in 4% (w/v) paraformaldehyde in PBS, then two changes of PBS for 5 min each. Sections
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were digested in 20 μg/ml proteinase K in 50 mM Tris, 5 mM EDTA for 7.5 min. To stop the proteinase K reaction slides were dipped in PBS for 5 min, 4% (w/v) paraformaldehyde in PBS for 10 min, PBS for 5 min, sterile distilled water for 10 sec and triethanolamine (TEA) buffer for 30 min. To reduce electrostatic binding of probe sections were acetylated at their amino groups by 0.25% (v/v) acetic anhydride in TEA for two changes of 10 min each. The slides were then dehydrated through sequential solutions of PBS for 5 min, 0.85% (w/v) NaCl for 5 min then 1 min in each of 30% (v/v), 50% (v/v), 75% (v/v) and 90% (v/v) ethanol, followed by two 5 min periods in 100% ethanol. Slides were allowed to air dry for 2 h.

2.8 Hybridisation

Probe (5 ng/ml) was diluted to the required concentration (0.3 ng/ml/kb probe) using a hybridisation mix as diluent. The hybridisation mix consisted of 10 mM Na2HPO4 (pH 6.8), 5 mM EDTA (pH 8), 10 mM Tris-HCl (pH 7.5), 0.02% each BSA, Ficoll and polyvinyl pyrrolidine, 0.3 M NaCl, 50% formamide, 500 mg/ml yeast tRNA, 100 mM DTT and 50% dextran sulphate. The probe and hybridisation mix were mixed at 80°C for 2 min and then cooled on ice. The hybridisation mixture was then added to pretreated slides at a volume of 10-20 μl per slide. Sections were then covered with a coverslip to produce a thin and constant layer of probe over the sections. Slides were stored in a plastic box containing tissue soaked in 50% formamide, 5 x SSC to stop sections drying out. Closed boxes were sealed into a plastic bag containing soaked tissue and were stored overnight at 55°C to allow hybridisation.

Unhybridised probe was removed by sequential washes in 5 x SSC, 10 mM DTT for 30 min at 55°C; 50% formamide, 2 x SSC, 0.1 M DTT for 30 min at 65°C; and 0.5 M NaCl, 10 mM Tris, 5 mM EDTA (NTE buffer, pH 7.5) for three 10 min changes at 37°C. Non-specifically bound probe was hydrolysed with 40 μg/ml RNase in NTE for 30 min at 37°C. The slides were washed in NTE for 15 min at 37°C then 50% formamide, 2 x SSC, 10 mM β-mercaptoethanol for 20 min at 65°C. To increase the stringency of the washes the slides were washed in decreasing salt solutions; 2 x SSC for three changes of 10 min each at room temperature, then 0.1 x SSC for three changes of 10
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min each at room temperature. The sections were dehydrated by passing through successively increasing alcohol solutions containing 0.3 M ammonium acetate to prevent denaturation of in situ hybrids; 1 min in each of 30% (v/v), 50% (v/v), 75% (v/v) and 90% (v/v) ethanol, followed by two 5 min periods in 100% ethanol. Slides were allowed to air dry for 2 h.

2.9 Autoradiography

Kodak NTB-2 (Kodak) emulsion was melted in a 40°C water bath in a dark room illuminated by a safelight. Once liquefied the emulsion was diluted with an equal volume of distilled water and thoroughly mixed, then poured into a slide dipping chamber (Amersham). The emulsion was allowed to sit for 10-15 min to let any bubbles rise and then bubbles on the surface were removed by dipping several blank slides. Slides carrying hybridised sections were dipped in the emulsion twice, for about 2 sec each time. The bottom edges were blotted with a paper towel and the back of the slide was cleaned to remove the emulsion. Slides were placed on a foil-covered hot plate to dry. Once dry, the slides were placed in dessicant-filled, light-proof plastic boxes which were subsequently wrapped in foil and left at 4°C for 2 weeks.

2.10 Developing

The foil-wrapped box was allowed to stabilise at about 15°C while still sealed to avoid condensation on the slides which would reduce grain density. Slides were developed in D19 developer for 4 min. Development was stopped in distilled water and then the slides were fixed in Kodafix:distilled water (1:4) for 4 minutes and then rinsed in distilled water. All solutions were kept at the same temperature, 15°C, to prevent the emulsion cracking and flaking.

2.11 Staining and Mounting

Slides were stained by sequential dipping in haematoxylin, water, acid alcohol, water, Scotts tap water and water for optimal times to achieve
optimal visualisation. Slides were then dehydrated through successively increasing alcohol solutions; 1 min in each of 30% (v/v), 50% (v/v), 75% (v/v), 90% (v/v) and 100% ethanol followed by 5 min in xylene. Coverslips were mounted using Eukitt, and were then allowed to dry for 4 h.

2.12 Photography

Slides were examined under bright and darkfield optics, using a Zeiss photomicroscope and a yellow daylight filter at a magnification of x 10 or x 25. Kodakcolour II, 200 ASA film was used.

3. Results

3.1 In situ hybridisation of P450arom mRNA by hypophysectomised rat ovaries

The expression and localisation of P450arom mRNA was studied by in situ hybridisation. Control ovaries contained only small immature follicles in which P450arom mRNA was undetectable (Fig. 5.2A and B). Treatment with rh-FSH (total dose 72 IU) stimulated the development of antral follicles containing granulosa cells that clearly expressed P450arom (Fig. 5.2C and D). The P450arom mRNA signal was most intense in the mural cell layers and not discernible in any other cell type other than granulosa cells. HMG treatment (total dose 40 IU of both FSH and LH) also stimulated follicular growth and and granulosa cell P450arom mRNA formation (Fig. 5.2E and F). There was no evidence of P450arom mRNA expression above background levels (sense probe; Fig. 5.2G) in any other ovarian cell type.
FIG. 5.2. Bright- (left hand column) and darkfield (right hand column) views of *in situ* hybridisation with hypophysectomised rat ovariess using a P450arom cRNA probe. Bar = 100μm. A and B Section from an untreated rat ovary showing no hybridisation above background. C and D Section from a rh-FSH treated (total dose 72 IU) rat ovary showing intense hybridisation of the P450arom cRNA probe to predominately mural granulosa cells of antral follicles. E and F Section from a HMG treated (total dose 40 IU of both LH and FSH) rat ovary showing hybridisation to granulosa cells but not thecal/interstitial cells. G Sense cRNA probe illustrating specificity of antisense probe.
3.2 *In situ* hybridisation of LH receptor mRNA by hypophysectomised rat ovaries

The expression and localisation of LH receptor mRNA was studied by *in situ* hybridisation. LH receptor mRNA was detectable in control ovaries, but only in thecal/interstitial cells (Fig. 5.3A and B). The administration of rh-FSH (total dose 72 IU) induced LH receptor mRNA in the granulosa cells of large follicles without having any clear effect on thecal/interstitial cells (Fig. 5.3C and D). Granulosa cell LH receptor mRNA was also induced by HMG treatment (total dose 40 IU of both FSH and LH). This response to HMG was often more marked than that to rh-FSH but there was considerable follicle-to-follicle variation (Fig. 5.3E and F). The probe specificity was demonstrated by the use of a sense probe (Fig. 5.3G).

3.3 Evidence for thecal/interstitial cell-specific expression of P450C17α mRNA

The site of P45017α mRNA expression was studied by *in situ* hybridisation. *In-situ* hybridization with antisense 32P-labelled P450C17α cRNA demonstrated that P450C17α mRNA expression was confined to thecal cells at all stages of follicular development, persisting in secondary interstitial tissue within the stroma (Fig. 5.4). Expression beyond background levels (sense probe; Fig. 5.4G) was not observed in any other ovarian cell type.

3.4 Effect of diethylstilboestro1 treatment *in vivo* on expression of P450C17α mRNA by ovaries from intact animals

The effect of oestrogen *in vivo* on ovarian P45017α mRNA expression was tested by the use of DES implants for four days prior to removal of ovaries. P450C17α mRNA was detectable in control ovaries, but only in thecal/interstitial cells (Fig. 5.5A and B). The administration of rh-FSH (total dose 72 IU) had no clear effect on thecal/interstitial cell expression of P450C17α mRNA (Fig. 5.5E and F). The administration of
FIG. 5.3. Bright- (left hand column) and darkfield (right hand column) views of in situ hybridisation with hypophysectomised rat ovaries using a LH receptor cRNA probe. Bar = 100μm. A and B Section from an untreated rat ovary showing hybridisation is limited to thecal/interstitial cells. C and D Section from a rh-FSH treated (total dose 72 IU) rat ovary showing hybridisation of the LH receptor cRNA probe to granulosa cells as well as thecal/interstitial cells. E and F Section from a HMG treated (total dose 40 IU of both LH and FSH) rat ovary showing increased hybridisation signal intensity above that achieved with rh-FSH alone. G Sense cRNA probe illustrating specificity of antisense probe.
FIG. 5.4. Bright- (left hand column) and darkfield (right hand column) views of in situ hybridisation with adult rat ovaries at the proestrous stage of the oestrous cycle using a P450C17α cRNA probe. Bar = 100μm. A and B Section of a preovulatory follicle. The distribution of labelling indicates that P450C17α expression is predominately located in the thecal/interstitial cell layer. C and D Higher magnification of the same follicle section. The distribution of labelling indicates that P450C17α expression is predominately located in the thecal/interstitial cell layer and is not found in the granulosa cell layer. E and F Section of a corpus luteum showing little hybridisation above background levels. G Sense cRNA probe illustrating specificity of antisense probe.
FIG. 5.5. Bright- (left hand column) and darkfield (right hand column) views of in situ hybridisation with intact rat ovaries using a P450C17α cRNA probe. Bar = 100 μm. A and B Section from an untreated rat ovary showing hybridisation is limited to thecal/interstitial cells. C and D Section from a DES treated rat ovary showing decreased hybridisation of the P450C17α cRNA probe to thecal/interstitial cells.
FIG. 5.5. (contd.) Bright- (left hand column) and darkfield (right hand column) views of *in situ* hybridisation with intact rat ovaries using a P450C17α cRNA probe. Bar = 100μm. E and F Section from a rh-FSH treated (total dose 72 IU) rat ovary showing hybridisation is limited to thecal/interstitial cells at a similar intensity to that found in untreated ovaries. G and H Section from a DES and rh-FSH treated (total dose 72 IU) rat ovary showing decreased hybridisation of the P450C17α cRNA probe to thecal/interstitial cells relative to untreated ovaries or ovaries treated with rh-FSH or DES alone. I Sense cRNA probe illustrating specificity of antisense probe. For corresponding control, see Fig. 5.5 A and B.
DES treatment decreased intensity of P450C17α mRNA expression both in the presence and absence of rh-FSH (Fig. 5.5C, D, G and H). The probe specificity was demonstrated by the use of a sense probe (Fig. 5.5I).

3.5 *In situ* hybridisation of P450C17α mRNA by intact rat ovaries

The expression of ovarian P45017α mRNA in pituitary intact animals was studied by *in situ* hybridisation. P450C17α mRNA was detectable in control ovaries, but only in thecal/interstitial cells (Fig. 5.6A and B). Treatment with rh-FSH (total dose 72 IU) stimulated the development of antral follicles but showed no clear effect on thecal/interstitial cell expression of P450C17α mRNA (Fig. 5.6C and D). HMG treatment also stimulated follicular growth and increased thecal/interstitial cell expression of P450C17α mRNA (Fig. 5.6E and F) over that found in untreated ovaries. There was no evidence of P450C17α mRNA expression above background levels (sense probe; Fig. 5.6G) in any other ovarian cell type.

3.6 *In situ* hybridisation of P450C17α mRNA by hypophysectomised rat ovaries

The expression of ovarian P45017α mRNA in hypophysectomised animals was studied by *in situ* hybridisation. P450C17α mRNA was detectable in control ovaries, but only in thecal/interstitial cells (Fig. 5.7A and B). Treatment with rh-FSH (total dose 72 IU) stimulated the development of antral follicles and decreased the overall expression of P450C17α mRNA (Fig. 5.7C and D) though it was unclear whether this was due to a true decrease in levels of expression or the same levels of expression distributed over a larger area. HMG treatment (total dose 40 IU of both FSH and LH) also stimulated follicular growth and increased thecal/interstitial cell expression of P450C17α mRNA (Fig. 5.7E and F) over that found in untreated ovaries. There was no evidence of P450C17α mRNA expression above background levels (sense probe; Fig. 5.7G) in any other ovarian cell type.
FIG. 5.6. Bright- (left hand column) and darkfield (right hand column) views of *in situ* hybridisation with intact rat ovaries using a P450C17α cRNA probe. Bar = 100μm. **A and B** Section from an untreated rat ovary showing hybridisation is limited to thecal/interstitial cells. **C and D** Section from a rh-FSH treated (total dose 72 IU) rat ovary showing a similar intensity of hybridisation of the P450C17α cRNA probe to thecal/interstitial cells relative to untreated ovaries. **E and F** Section from a HMG treated (total dose 40 IU of both LH and FSH) rat ovary showing increased hybridisation signal intensity above that achieved with rh-FSH alone. **G** Sense cRNA probe illustrating specificity of antisense probe.
FIG. 5.7. Bright- (left hand column) and darkfield (right hand column) views of in situ hybridisation with hypophysectomised rat ovaries using a P450C17α cRNA probe. Bar = 100μm. A and B Section from an untreated rat ovary showing hybridisation is limited to thecal/interstitial cells. C and D Section from a rh-FSH treated (total dose 72 IU) rat ovary showing decreased intensity of hybridisation of the P450C17α cRNA probe to thecal/interstitial cells relative to untreated ovaries. E and F Section from a HMG treated (total dose 40 IU of both LH and FSH) rat ovary showing increased hybridisation signal intensity above that observed in untreated ovaries. G Sense cRNA probe illustrating specificity of antisense probe.
3.7 Establishment of dose-dependence of rh-FSH action on P450C17α mRNA expression by hypophysectomised rat ovaries

The dose-dependence of rh-FSH action on ovarian P450C17α mRNA expression was established in *in situ* hybridisation experiments on hypophysectomised animals (Fig. 5.8). P450C17α mRNA was detectable in control ovaries, but only in thecal/interstitial cells (Fig. 5.8A and B). The administration of a total dose of 3 IU rh-FSH (Fig. 5.8C and D) had no discernible effect on the expression of P450C17α mRNA. The administration of a total dose of 10 IU rh-FSH (Fig. 5.8E and F) or 30 IU rh-FSH (Fig. 5.8G and H) decreased expression of P450C17α mRNA compared to levels of expression found in untreated ovaries. The administration of a total dose of 100 IU rh-FSH (Fig. 5.8I and J) further decreased the intensity of P450C17α mRNA expression. HMG treatment (total dose 40 IU of both FSH and LH) increased thecal/interstitial cell expression of P450C17α mRNA (Fig. 5.8K and L) over that found in untreated ovaries. There was no evidence of P450C17α mRNA expression above background levels (sense probe; Fig. 5.8M) in any other ovarian cell type.

3.8 Effect of r-LH administration *in vivo* on expression of P450C17α mRNA by hypophysectomised rat ovaries

The effect of r-LH administration on expression of P450C17α mRNA expression in the presence and absence of rh-FSH was tested (Fig. 5.9). P450C17α mRNA was detectable in control ovaries, but only in thecal/interstitial cells (Fig. 5.9A and B). The administration of a total dose of 10 IU (Fig. 5.9N and O) increased intensity of expression of P450C17α mRNA over levels found in control ovaries. In the presence of a total dose of 30 IU rh-FSH the administration of a total dose of 1 IU r-LH (Fig. 5.9J and K) or 10 IU (Fig. 5.9P and Q) increased intensity of expression of P450C17α mRNA over levels found in ovaries treated with 30 IU rh-FSH alone (Fig. 5.9C and D). In the presence of a total dose of 72 IU rh-FSH the administration of a total dose of 1 IU r-LH (Fig 8L and M) or 10 IU (Fig. 5.9R and S) increased intensity of expression of P450C17α mRNA.
FIG. 5.8. Bright- (left hand column) and darkfield (right hand column) views of in situ hybridisation with hypophysectomised rat ovaries using a P450C17α cRNA probe. Bar = 100μm. A and B Section from an untreated rat ovary showing hybridisation is limited to thecal/interstitial cells. C and D Section from a rh-FSH treated (total dose 3 IU) rat ovary showing a similar intensity of hybridisation of the P450C17α cRNA probe to thecal/interstitial cells compared to untreated ovaries. E and F Section from a rh-FSH treated (total dose 10 IU) rat ovary showing decreased intensity of hybridisation of the P450C17α cRNA probe to thecal/interstitial cells relative to untreated ovaries.
FIG. 5.8. (contd.) Bright- (left hand column) and darkfield (right hand column) views of in situ hybridisation with hypophysectomised rat ovaries using a P450C17α cRNA probe. Bar = 100μm. G and H Section from a rh-FSH treated (total dose 30 IU) rat ovary showing further decreased intensity of hybridisation of the P450C17α cRNA probe to thecal/interstitial cells relative to untreated ovaries. I and J Section from a rh-FSH treated (total dose 100 IU) rat ovary showing further decreased intensity of hybridisation of the P450C17α cRNA probe to thecal/interstitial cells relative to untreated ovaries. K and L Section from a HMG treated (total dose 40 IU of both LH and FSH) rat ovary showing increased hybridisation signal intensity above that observed in untreated ovaries. M Sense cRNA probe illustrating specificity of antisense probe. For corresponding control, see Fig. 5.8 A and B.
FIG. 5.9. Bright- (left hand column) and darkfield (right hand column) views of *in situ* hybridisation with hypophysectomised rat ovaries using a P450C17α cRNA probe. Bar = 100 μm. 

A and B Section from an untreated rat ovary showing hybridisation is limited to thecal/interstitial cells. 

C and D Section from a rh-FSH (total dose 30 IU) and no r-LH treated rat ovary rat ovary showing decreased intensity of hybridisation of the P450C17α cRNA probe to thecal/interstitial cells relative to untreated ovaries. 

E and F Section from a rh-FSH (total dose 72 IU) and no r-LH treated rat ovary showing further decreased intensity of hybridisation of the P450C17α cRNA probe to thecal/interstitial cells relative to untreated ovaries. 

G Sense cRNA probe illustrating specificity of antisense probe.
FIG. 5.9. (contd.) Bright- (left hand column) and darkfield (right hand column) views of *in situ* hybridisation with hypophysectomised rat ovaries using a P450C17α cRNA probe. Bar = 100μm. **H** and **I** Section from a r-LH treated (total dose 1 IU) rat ovary showing amount of hybridisation is similar to untreated ovaries (for corresponding control, see Fig. 5.9 A and B). **J** and **K** Section from a rh-FSH (total dose 30 IU) and r-LH (total dose 1 IU) treated rat ovary rat ovary showing increased intensity of hybridisation of the P450C17α cRNA probe to thecal/interstitial cells relative to rh-FSH (total dose 30 IU) treated ovaries (for corresponding control, see Fig. 5.9 C and D). **L** and **M** Section from a rh-FSH (total dose 72 IU) and r-LH (total dose 1 IU) rat ovary showing increased intensity of hybridisation of the P450C17α cRNA probe to thecal/interstitial cells relative to rh-FSH (total dose 72 IU) treated ovaries (for corresponding control, see Fig. 5.9 E and F).
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FIG. 5.9. (contd.) Bright- (left hand column) and darkfield (right hand column) views of in situ hybridisation with hypophysectomised rat ovaries using a P450C17α cRNA probe. Bar = 100μm. N and O Section from a r-LH treated (total dose 10 IU) rat ovary showing increased hybridisation limited to thecal/interstitial cells relative to untreated ovaries (compare with 5.9 H and I). P and Q Section from a rh-FSH (total dose 30 IU) and r-LH (total dose 10 IU) treated rat ovary showing increased intensity of hybridisation of the P450C17α cRNA probe to thecal/interstitial cells relative to rh-FSH (total dose 30 IU) treated ovaries in the presence or absence of 1 IU total dose r-LH (compare with 5.9 J and K). R and S Section from a rh-FSH (total dose 72 IU) and r-LH (total dose 10 IU) rat ovary showing increased intensity of hybridisation of the P450C17α cRNA probe to thecal/interstitial cells relative to rh-FSH (total dose 72 IU) treated ovaries in the presence or absence of 1 IU total dose r-LH (compare with 5.9 L and M). T and U Section from a HMG treated (total dose 40 IU of both LH and FSH) rat ovary showing increased hybridisation signal intensity above that observed in untreated ovaries.
over levels found in ovaries treated with 72 IU rh-FSH alone (Fig. 5.9E and F). In the absence of r-LH the administration of 30 IU rh-FSH (Fig. 5.9C and D) or 72 IU rh-FSH (Fig. 5.9E and F) decreased intensity of P450C17α mRNA expression compared to levels of expression found in untreated ovaries (Fig. 5.9A and B). Interestingly, in the presence of 1 IU r-LH (Fig. 5.9H to M) or 10 IU r-LH (Fig. 5.9N to U) the administration of rh-FSH did not decrease levels of P450C17α mRNA expression and possibly increased the expression. There was no evidence of P450C17α mRNA expression above background levels (sense probe; Fig. 5.9G) in any other ovarian cell type.

4. Discussion

The finding that P450C17α mRNA expression is confined to thecal/interstitial cells confirms a basic requirement of the two cell, two gonadotrophin mechanism of follicular oestrogen synthesis (Whitelaw et al. 1992). This is the first time that P450C17α mRNA distribution in the ovary has been studied by in-situ hybridisation. The message is apparently absent from granulosa cells but abundantly expressed in thecal cells throughout preantral and antral follicular development, consistent with thecal cells being primary sites of androgen synthesis in the ovary. The significance of the observation that P45017α expression occurs in clusters or pockets of cells is not known. It is possible that this indicative of cell lineage. In other words, cells within the same pocket, whether it be labelled or unlabelled, could be derived from the same parent cell.

There has been no published in situ hybridisation study of P45017α mRNA expression in the ovary despite the fact that the technology has been available for some time. A possible explanation for this could be that there has been some difficulty due to the region of mRNA chosen that is complementary to the probe used. In this study the probes were complementary to the portion of the mRNA encoding the conserved P45017α sequence and Ozols tridecapeptide regions of the P45017α protein. It is possible that the probe must be complementary to one or both regions for adequate hybridisation to P45017α mRNA.
Using a combination of hypophysectomised rats and recombinant FSH this study presents a convincing demonstration that FSH acts directly in the absence of endogenous sex steroids and LH to induce granulosa cell aromatase expression in vivo. This induction occurred without a parallel increase in oestradiol secretion (Chapter 2, Table 2.2). This finding supports the two-cell, two-gonadotrophin hypothesis which requires simultaneous LH activation of androgen synthesis by thecal/interstitial cells for oestrogen synthesis (Armstrong & Dorrington, 1979). Aromatase mRNA expression was always confined to granulosa cell layers and absent from thecal/interstitial cells confirming another basic requirement of the two cell, two gonadotrophin mechanism of follicular oestrogen synthesis (Whitelaw et al. 1992).

In contrast to aromatase mRNA, LH receptor mRNA was expressed in the thecal/interstitial cells of control animals not treated with FSH. Treatment with FSH induced an increase in granulosa cell LH receptor mRNA expression, in parallel with its effect on aromatase expression. Again this induction occurred without a parallel increase in oestradiol secretion providing evidence that oestrogen is not required for the induction of LH receptor by FSH in contrast to a previous report (Richards et al. 1987). This finding however does not exclude the possibility that oestradiol is capable of modulating FSH action (Richards et al. 1987).

In untreated ovaries P450C17α mRNA is constitutively expressed whilst aromatase mRNA expression does not occur until it is induced by FSH. The observation that P450C17α mRNA in thecal/interstitial cells is expressed before aromatase mRNA in granulosa cells implies an interaction between these two cell types. Androgens induce rat granulosa cell aromatase in the presence of FSH by an androgen receptor mediated mechanism (Hillier & deZwart, 1982).

Oestrogens inhibit LH-stimulated androgen biosynthesis in rat thecal/interstitial cells (Erickson et al. 1985) by greater than 90%. The mechanism of oestrogen action in thecal/interstitial cells remains unclear. The inhibitory effects of oestradiol on thecal/interstitial cell androgen production occur without changes in LH receptors, LH-stimulated cAMP production or progesterone production (Erickson et al. 1985) and oestradiol does not cause a direct inhibition of P450C17α activity in ovarian
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homogenates (Johnson et al. 1984). These observations coupled with our finding that DES decreases P450C17α mRNA expression suggest a selective inhibitory block to transcription or translation of the P450C17α gene. These inhibitory effects of oestrogen may be the mechanism which mediates the decrease in P450C17α activity that occurs after ovulation in a wide variety of species (Erickson et al. 1985). P450C17α mRNA expression is found in low levels in the corpus luteum of the rat ovary. This finding is consistent with the low androgen levels, and consequently oestradiol, found in the corpus luteum which is primarily a progesterone-producing structure.

The complete withdrawal of gonadotrophins by hypophysectomy had no effect on the overall levels of P450C17α expression despite the high levels of atresia induced by such treatment. This suggests that P450C17α is expressed constitutively. Treatment of hypophysectomised animals with FSH resulted in a dose-dependent decrease in P450C17α mRNA expression. This decrease could be due to an expanding thecal cell layer caused by FSH-stimulated granulosa cell proliferation. The expanded thecal cell layer may express the same levels of P45017α mRNA, but the expression is spread over a greater area, giving the impression of decreased intensity. However, the same treatment in intact animals did not noticeably alter the level of P450C17α mRNA expression. These results suggest that FSH, in the absence of LH (i.e. hypophysectomised animal), is able to negatively regulate the expression of P450C17α mRNA. Since thecal/interstitial cells do not possess FSH receptors it is implied that this negative regulation is via a paracrine mechanism. Factors produced by FSH-stimulated granulosa cells include IGF-I (Hernandez et al. 1989), TGFβ (Kim & Schomberg, 1989), activin and inhibin (Meunier et al. 1988). TGFβ, in the presence of IGF-I, is capable of inhibiting basal androgen production by thecal/interstitial cells (Hernandez & Adashi, 1988). IGF-I and inhibin augment LH-stimulated androgen synthesis by thecal/interstitial cells but are unable to affect basal androgen synthesis by these cells (IGF-I: Adashi et al. 1985a; inhibin: Hsueh et al. 1987). Activin inhibits LH-stimulated androgen synthesis (Hsueh et al. 1987). It is therefore possible that the discrepancy between the effects of FSH on P450C17α mRNA expression in hypophysectomised and intact animals may be explained by the actions of these factors. It may be hypothesised that FSH treatment stimulates the production of IGF-I, TGFβ, activin and inhibin by
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granulosa cells. In the hypophysectomised animal the inhibin and activin would have no effect, due to the absence of LH, but TGFβ in the presence of IGF-I would decrease the expression of P450C17α mRNA. In the previous chapter it was shown that there is a requirement for LH to facilitate the actions of inhibin and IGF-I. In the intact animal there is endogenous LH present which therefore would enable inhibin or IGF-I produced by granulosa cells to overcome the combined effect of TGFβ and IGF-I and thereby increase LH-stimulated androgen production. If the granulosa cells are predominantly immature they will not be ready for aromatase substrate and will produce activin which inhibits LH-stimulated androgen production. Once the granulosa cells are mature enough for aromatisation they produce inhibin which augments LH-stimulated androgen production. These results are consistent with the physiological situation where early in development theca cells are progesterone producing and do not need to express P45017α activity. Thus, FSH may down regulate any thecal cell function until the granulosa cells are ready for aromatase substrate.

LH dose-dependently stimulated an increase in P450C17α mRNA expression in the absence of any other gonadotrophic stimulation and reversed the inhibitory effect of FSH alone on P450C17α mRNA expression. This finding supports the two-cell, two-gonadotrophin hypothesis which requires the presence of LH for activation of androgen synthesis by thecal/interstitial cells for normal oestrogen synthesis (Armstrong & Dorrington, 1979).

In conclusion this study has shown that FSH induces the expression of aromatase mRNA exclusively in granulosa cells. FSH also directly increases granulosa cell LH receptor mRNA but thecal/interstitial cell LH receptor mRNA is expressed constitutively. The induction of both these enzymes by FSH is independent of LH or locally produced oestrogen. These results provide evidence that P450C17α is expressed constitutively in a cell-specific manner and is subject to hormonal regulation by both FSH and LH and steroidal regulation by oestradiol. FSH is able to influence the expression of P450C17α mRNA by paracrine signalling. Further research is required to determine which locally produced factors mediate these actions of FSH. Candidates include factors produced by granulosa cells under the stimulation of FSH. Such proteins include inhibin, activin and IGF-I which
dose dependently enhance LH-stimulated thecal/interstitial cell androgen production and TGFβ which inhibits basal androgen production in the presence of IGF-I. These actions suggest paracrine roles for these factors in the modulation of P450C17α mRNA expression. The next chapter tests the hypothesis that inhibin is a granulosa cell-derived paracrine factor that enhances thecal cell androgen production.
Chapter 6. Rat Ovarian Follicle Culture

1. Introduction

The results of the previous chapters provide evidence for a FSH-stimulated paracrine enhancement of ovarian thecal/interstitial cell androgen synthesis. Thus factors produced by FSH-stimulated granulosa cells may act as paracrine signals in vivo. The results of the previous chapters also suggest that likely paracrine modulators include inhibin. This chapter describes a study of the intraovarian function of inhibin in follicular development using an in vitro rat ovarian follicle culture system.

Gonadal inhibin serves a classic endocrine function in the regulation of pituitary FSH secretion (see Ying, 1988 for a review). A local role for inhibin in the control of follicular oestrogen production has also been proposed, based on evidence that (1) inhibin is produced by FSH-stimulated rat (Bicsak et al. 1986) and human (Hillier et al. 1991a) granulosa cells, and (2) treatment with inhibin augments LH-stimulated androgen production by thecal/interstitial cells in vitro (Hsueh et al. 1987; Hillier et al. 1991c). Since thecal androgen is vital as an aromatase substrate in the two-cell, two-gonadotrophin mechanism of oestrogen synthesis (Armstrong & Dorrington, 1979), augmentation of LH-stimulated androgen synthesis by locally produced inhibin would therefore be expected to enhance follicular production of oestradiol (Hillier, 1991b).

Direct evidence that a substance exerts a paracrine function in vitro requires use of a physiological model in which normal follicular growth and differentiation can occur. Detailed investigations of the mechanisms of paracrine signalling within the ovarian follicle have been restricted by the lack of a defined culture system capable of sustaining normal follicular development. Monolayer culture systems of individual cell types have contributed greatly to the understanding of follicular development but they suffer from the disadvantage that follicular integrity is disrupted meaning that normal intra-follicular interactions cannot occur. Antral follicle cultures have mainly involved short-term incubation of large antral follicles for measuring steroid secretion (Bogovich & Richards, 1982; Laufer et al. 1982;
Carlsson et al. 1985; Tsafiri et al. 1989b). To test if inhibin has a physiologically significant paracrine action within the intact follicle we have turned to a whole follicle culture system initially developed for mouse ovarian follicles (Boland et al. 1993). Whole follicle culture has the advantage that normal communication between follicular cell types is maintained such that paracrine factors remain active at or near their site of production. Here we exploit this model to examine the consequence of immunoneutralisation of inhibin on follicular steroidogenesis.

Steroidogenesis is dependent upon the integrity of the follicle and cooperation of the two cell types found in the ovary. This interdependence of granulosa and thecal cells for rat ovarian follicular oestrogen production was clearly demonstrated by Falck in 1959 when it was shown that neither cell type could produce oestrogens alone. The “two-cell theory” (Short, 1962), based upon assays of endogenous steroid levels in the mare follicle and corpus luteum, proposed two types of cell in the ovary with different steroid producing capacities. In 1975, using a hamster ovarian model, Makris and Ryan established a clear-cut synergism of granulosa and thecal cells for oestrogen production, whereby the theca produced androgen which the granulosa aromatised to oestrogen. Dorrington et al. (1975) provided evidence that FSH regulates oestrogen biosynthesis in granulosa cells but not thecal cells. Fortune and Armstrong (1977) showed that the theca, under LH regulation, was the site of follicular androgen secretion by rat ovarian follicles and suggested that the theca provides the androgen precursor needed for follicular oestradiol synthesis. These results led to the proposal of the "two cell, two gonadotrophin" hypothesis (Armstrong and Dorrington, 1979). According to this model, LH stimulates the biosynthesis of androgen from cholesterol or granulosa-derived progesterone in the theca cell layer. Androgens diffuse across the lamina basalis and are converted to oestrogens by FSH-stimulated granulosa cells. In this study withdrawal of inhibin by immunoneutralisation was expected to suppress thecal androgen synthesis and hence granulosa cell oestrogen synthesis, thereby confirming the paracrine inhibin hypothesis.
2. Materials and Methods

2.1 Animals

Immature female Wistar rats were killed by CO\textsubscript{2} asphyxiation at 21 days of age and ovaries were aseptically removed for follicle isolation.

2.2 Follicle isolation

Ovaries were placed in Leibovitz L-15 medium (Gibco-BRL, Irvine, UK.) supplemented with 3mg/ml BSA fraction V (Sigma, Dorset, UK.) and immediately transported to the dissection laboratory. Initially, each ovary was teased into 4-8 pieces. Individual preantral follicles, 186 \mu m to 400 \mu m in diameter (excluding theca cell layer), were then micro-dissected from ovaries in watchglasses using two fine 28G needles attached to 1ml syringes (Steriseal, Worcester, UK.). Visualisation was at x40 magnification using a stereo dissecting microscope (Carl Zeiss Jena Ltd., Hertfordshire, UK.) fitted with a pre-calibrated ocular micrometer. Care was taken to leave a small clump of theca cells attached to the basement membrane, as the presence of this tissue has previously been shown to be vital to follicle development (Erickson et al. 1985). Follicles with a central, spherical oocyte and no signs of damage were cultured.

2.3 Preparation of culture dishes.

Ninety-six V-well, microtitre plates (Bibby Sterilin Ltd., Staffordshire, UK.) were silicone coated (Sambrook et al. 1989) by placing in a dessicator containing a beaker with 2ml of silicone (Repelcote\textsuperscript{TM}, Sigma). The dessicator was evacuated and left overnight. The plates were then rinsed in sterile water followed by ethanol the following day. Sterilisation was assured by ultra-violet irradiation for 5 h. The silicone coating prevented follicular cells from becoming attached and spreading on the V-well surface and thus an intact follicular structure was maintained throughout the culture period.
2.4 Follicle culture

Follicles were cultured individually in 20 μl droplets of α Minimum Essential Medium (α-MEM) (Gibco-BRL) under 50 μl mineral oil (Sigma; d=0.84g/ml) in 5% CO₂ in air at 37°C for 5 days. Medium was supplemented with 10 μg/ml human transferrin (Sigma), 1IU/ml human FSH (hFSH; biological potency = 1683 IU FSH/mg [2nd IRP HMG]; LH contamination 147 IU LH/mg [1st IRP 68/40]; generously donated by Dr. S. Raiti, National Hormone and Pituitary Program, Bethesda, MD., USA.) and 5% serum from immature rats. Serum was obtained from 21 day old rats and prepared by centrifugation after being allowed to clot at room temperature for about 10 min. The inhibin antiserum was raised in an ovariectomised Scottish Blackface ewe (McNeilly et al. 1989) using as the immunogen a peptide comprising the N-terminal 1-26 amino acid sequence of the α-subunit of porcine 32 kDa inhibin (Rivier et al. 1986) conjugated to ovalbumin. This antiserum was previously shown to neutralise the suppressive effect of inhibin on FSH production by dispersed sheep pituitary cells in vitro (Mann et al. 1989a). Non-immune sheep serum was obtained from an untreated Scottish Blackface ewe. Recombinant human inhibin-A (Mason, 1988) was from Genentech Inc. and androstenedione was from Sigma. All control and test treatments were done in replicate (n=12) and the main experiments were repeated at least twice, as summarised in Table 6.1.

After each successive 24 h period of culture, follicles were removed from their wells in a minimum volume of medium using a fine glass micropipette, and transferred to fresh culture medium. Follicle diameters were measured daily with a precalibrated ocular micrometer at x 40 magnification in medium at 37°C. Spent culture medium was stored at -20°C for steroid assay.
TABLE 1: Summary of Experiments.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>non-immune serum (1.0%)</td>
<td>inhibin antiserum (0.1%) inhibin antiserum (1.0%)</td>
</tr>
<tr>
<td>II</td>
<td>non-immune serum (1.0%)</td>
<td>inhibin antiserum (0.1%) inhibin antiserum (1.0%)</td>
</tr>
<tr>
<td>III</td>
<td>no serum</td>
<td>non-immune serum (1.0%)</td>
</tr>
<tr>
<td>IV</td>
<td>non-immune serum (1.0%)</td>
<td>inhibin antiserum (1.0%) inhibin antiserum (1.0%) + inhibin (50ng/ml)</td>
</tr>
<tr>
<td>V</td>
<td>no serum</td>
<td>inhibin antiserum (1.0%) inhibin antiserum (1.0%) + inhibin (50ng/ml)</td>
</tr>
<tr>
<td>VI</td>
<td>no serum</td>
<td>inhibin antiserum (1.0%) inhibin antiserum (1.0%) + androstenedione (1µM)</td>
</tr>
</tbody>
</table>

In every experiment, each control and test treatment comprised 12 follicles cultured individually.
2.5 Preparation of Follicle Sections

On day 5 of culture follicles were fixed overnight at 4°C in 4% (w/v) paraformaldehyde (Sigma) dissolved in PBS. This was followed by sequential dehydration treatments at room temperature with 85% (v/v) ethanol for 1 h; 95% (v/v) ethanol for 1 h; three treatments with 100% (v/v) ethanol for 1 h each; two treatments with xylene for 30 min each and then a third treatment with xylene overnight at room temperature. Tissue was then treated with paraffin wax for two successive 1 h periods at 60°C. Prepared tissues were then placed in molds containing melted paraffin, which was then allowed to solidify.

Slides were cleaned and coated to help tissue section retention as described previously (Chapter 5, section 2.4). Tissue was sectioned and mounted as described in Chapter 5, sections 2.4 and 2.11.

Slides were stained by sequential dipping in haematoxylin, water, acid alcohol, water, Scotts tap water, water, eosin and water for optimal times to achieve optimal visualisation. Slides were then dehydrated through successively increasing alcohol solutions; 1 min in each of 30% (v/v), 50% (v/v), 75% (v/v), 90% (v/v) and 100% ethanol followed by 5 min in xylene. Coverslips were mounted using Eukitt which was then allowed to dry for 4 h.

Slides were examined under brightfield optics, using a Zeiss photomicroscope and a yellow daylight filter at a magnification of x 10. Kodakcolour II, 200 ASA film was used.

2.6 Steroid Assays

Oestradiol, progesterone, androsterone and androstenedione in spent culture medium were determined by RIA using standard procedures and antisera as described previously (Chapter 2, sections 2.5.1, 2.5.2 and 2.5.3, and Chapter 3, section 2.4.3). The inter- and intraassay precision for each steroid was less than 15%. Steroid accumulation is expressed as picomoles steroid per follicle per 24 h.
2.7 Statistics

Statistical analysis was carried out using commercial software (CLR ANOVA). Analysis of variance with the Newman-Keuls test was used to analyse differences between experimental and control observations. Differences assigned a $P$ value of $<0.05$ were regarded as statistically significant.

3. Results

3.1 Follicle growth and morphology

3.1.1 Follicle size at the start of culture

Previous studies have shown in the mouse that factors influencing growth of follicles in vitro include how often medium is changed, number of follicles per well, species and age of serum donor and follicle size at the start of culture (Nayudu & Osborn, 1992). Thus follicles of varying starting diameters (186 μm to 400 μm) were compared for their growth in culture under optimal conditions (Fig. 6.1). The most rapidly growing follicles (240 μm) increased by approximately 45 μm a day to an average size of 470 μm which is comparable with sizes of 500 - 750 μm reported in vivo (Freeman, 1988). The growth profile of this starting size of follicle best represented the physiological growth of a primary follicle through to a preovulatory follicle. If the follicle starting size was too small the follicle was usually dissected without an intact theca layer and as such failed to proceed through antral development. Follicles too large were generally atretic due to the lack of circulating gonadotrophins in vivo to rescue them from this fate. All cultures described in the following results started with follicles of 244 ± 10 μm diameter.
FIG. 6.1. Growth rates of rat follicles of differing starting sizes cultured individually in vitro for 5 days in medium without inhibin antiserum. Results are expressed as mean.
3.1.2 Follicle growth and steroidogenesis in vitro

Preantral follicles cultured in medium containing FSH grew from a mean (± SE) diameter of 244 ± 10 μm to 470 ± 28 μm during the 5 day culture period (Fig. 6.2). Antrum formation began on Day 2. By the end of the culture period follicles had attained preovulatory size and Graafian morphology (Fig. 6.3).

Oestradiol production by follicles cultured in medium containing FSH was significantly elevated on Day 2 (Fig. 6.4), coincident with the onset of antrum formation. The rate of oestradiol production increased steadily throughout culture. Progesterone production also increased on Day 2 but peaked on Day 3 and then declined on Days 4 and 5 (Fig. 6.4). Androgen accumulation during the culture period was not measurably changed (Table 6.2).

<table>
<thead>
<tr>
<th>Treatment:</th>
<th>Control</th>
<th>Antiserum</th>
<th>Antiserum + Inhibin</th>
<th>Antiserum + Androstenedione</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAY 1</td>
<td>1.20 ± 0.56</td>
<td>0.88 ± 0.52</td>
<td>0.96 ± 0.24</td>
<td>0.88 ± 0.40</td>
</tr>
<tr>
<td>DAY 2</td>
<td>1.52 ± 0.68</td>
<td>0.88 ± 0.32</td>
<td>1.16 ± 0.48</td>
<td>0.96 ± 0.40</td>
</tr>
<tr>
<td>DAY 3</td>
<td>1.08 ± 0.68</td>
<td>0.84 ± 0.28</td>
<td>0.88 ± 0.28</td>
<td>1.24 ± 0.32</td>
</tr>
<tr>
<td>DAY 4</td>
<td>1.32 ± 0.64</td>
<td>1.20 ± 0.60</td>
<td>1.08 ± 0.52</td>
<td>0.84 ± 0.52</td>
</tr>
<tr>
<td>DAY 5</td>
<td>0.80 ± 0.56</td>
<td>0.64 ± 0.40</td>
<td>0.80 ± 0.16</td>
<td>0.96 ± 0.64</td>
</tr>
</tbody>
</table>

Table 6.2. Androsterone accumulation (pmol/follicle/24h) by follicles cultured individually in vitro for 5 days in medium containing homologous serum and FSH (1 IU/ml). Treatments were inhibin antiserum (1.0 %), inhibin (50 ng/ml) and androstenedione (1 μM). Where measured androstenedione accumulation was below the minimal detectable dose of the RIA (<0.025 pmol). Composite data from 3 experiments is expressed as mean ± SE.

3.2 Effect of addition of inhibin antiserum to follicle cultures

To test the effect of lack of inhibin during follicular development follicles were cultured in the presence of inhibin antiserum. Follicles cultured in the presence of the inhibin antiserum or non-immune serum
FIG. 6.2. Growth rates of rat follicles cultured individually *in vitro* for 5 days in medium with or without inhibin antiserum (0.1% and 1%). Composite data from six experiments (mean ± SE).
FIG. 6.3. Morphology of a preovulatory follicle stained with haemotoxylin and eosin. Follicles were cultured individually *in vitro* for 5 days in medium without inhibin antiserum. Follicle diameter at end of culture was 440\(\mu\)m.
FIG. 6.4. Steroid production \textit{in vitro} by individual rat follicles. Whole follicles were incubated for 5 days in culture medium without inhibin antiserum. Each 24 h of culture, follicles were removed from their wells using a fine glass micropipette, and transferred to fresh culture medium. Steroid accumulation in spent culture medium was determined by radioimmunoassay. Composite data from experiments III, V and VI (mean $\pm$ SE, n=36). *$P < 0.01$ compared with steroid accumulation on Day 1.
grew at a similar rate to follicles cultured in the absence of antiserum during the 5 day culture period (Fig. 6.2).

Treatment with antiserum at a concentration of 1.0% (v/v) reduced oestradiol production on Days 2-5 to levels that were not significantly higher than those found on the first day of culture (Fig. 6.5). Progesterone accumulation in the presence of the antiserum was significantly raised on Day 2 and increased steadily throughout the period of culture (Fig. 6.6). Treatment with non-immune serum did not significantly alter steroid production relative to untreated controls (Figs. 6.5 and 6.6). The effects of the inhibin antiserum on steroidogenesis were dose-dependent with significant suppression of oestradiol and enhancement of progesterone at a concentration of 0.1% (v/v) (Fig. 6.7). Androgen production was not measurably affected by the addition of inhibin antiserum (Table 6.2).

3.3 Effect of supplementing antiserum-treated culture medium with inhibin

To test the effect of replacing ‘neutralised’ inhibin antiserum-treated follicles were cultured in the presence of exogenous inhibin. Follicles cultured with 50 ng/ml recombinant human inhibin in the presence of the inhibin antiserum (1.0%) showed a significant increase in oestradiol levels on Day 1 over those seen in the presence of antiserum alone (Fig. 6.8a) or no treatment at all (Fig. 6.4). This higher level of oestradiol production was maintained throughout the period of culture. Progesterone production (Fig. 6.8b) was also initially increased by the addition of exogenous inhibin on Day 1, unaffected at Day 2, and remained suppressed relative to treatment with antiserum alone at Days 3-5.
FIG. 6.5. Effect of inhibin antiserum compared with A. non-immune serum and B. untreated follicles on oestradiol accumulation in vitro by rat follicles. Whole follicles were incubated for 5 days in medium with inhibin antiserum (1.0%) or non-immune serum (1.0%). Each 24 hours of culture, follicles were removed from their wells using a fine glass micropipette, and transferred to fresh culture medium. Steroid accumulation in spent culture medium was determined by radioimmunoassay. Composite data from experiments I and II (A) or V and VI (B) (mean ± SE, n=24). *P <0.01 compared with control.
FIG. 6.6. Effect of inhibin antiserum compared with A. non-immune serum and B. untreated follicles on progesterone accumulation in vitro by rat follicles. Whole follicles were incubated for 5 days in medium with inhibin antiserum (1.0%) or non-immune serum (1.0%). Each 24 hours of culture, follicles were removed from their wells using a fine glass micropipette, and transferred to fresh culture medium. Steroid accumulation in spent culture medium was determined by radioimmunoassay. Composite data from experiments I and II (A) or V and VI (B) (mean ± SE, n=24). *P <0.01 compared with control.
FIG. 6.7. Dose-dependent effect of inhibin antiserum on A. oestradiol and B. progesterone accumulation in vitro by rat follicles on Day 5 of culture. Whole follicles were incubated for 5 days in medium with inhibin antiserum (0.1% and 1.0%). Each 24 hours of culture, follicles were removed from their wells using a fine glass micropipette, and transferred to fresh culture medium. Steroid accumulation in spent culture medium was determined by radioimmunoassay. Composite data from experiments I and II (mean ± SE, n=24). *P* <0.01 compared with non-immune serum treated control.
FIG. 6.8. Effect of exogenous inhibin on A. oestradiol and B. progesterone accumulation in vitro by antiserum-treated rat follicles. Whole follicles were incubated for 5 days in medium with inhibin antiserum and 50 ng inhibin /ml. Every 24 hours in culture, follicles were removed from their wells using a fine glass micropipette, and transferred to fresh culture medium. Steroid accumulation in spent culture medium was determined by radioimmunoassay. Composite data from experiments IV and V (mean ± SE, n=24). *P <0.01 compared with antiserum treatment.
3.4 Effect of supplementing antiserum-treated culture medium with androstenedione

In an attempt to bypass the block to androgen synthesis caused by the removal of inhibin, antiserum-treated follicles were cultured in the presence of an aromatisable androgen, androstenedione. Oestradiol production in the presence of inhibin antiserum was significantly increased on Day 1 by the addition of androstenedione to antiserum-treated medium (Fig. 6.9a). Oestradiol production increased steadily throughout the culture and was greater than in the absence of any treatment (Fig. 6.4). Progesterone accumulation in the presence of androstenedione and antiserum was significantly raised on Day 1 (Fig. 6.9b) and increased steadily throughout the period of culture.

4. Discussion

These results represent the most direct evidence to-date of a paracrine function for inhibin in the regulation of follicular oestrogen synthesis. Treatment of intact follicles with an antiserum directed against the inhibin α-subunit markedly suppressed FSH-stimulated oestradiol secretion. Although androstenedione levels were not measurably affected, simultaneous treatment with exogenous androstenedione restored oestrogen secretion by anti-inhibin treated follicles. It therefore seems most likely that inhibin promotes follicular oestrogen formation via a paracrine mechanism involving increased production of aromatase precursor in thecal/interstitial cells.

The FSH preparation used in this study was significantly (8.7 %) contaminated with LH, which would explain its ability to promote follicular oestrogen synthesis in the absence of additional LH. Both the original "two-cell" theory of steroid synthesis (Short, 1962) and the contemporary "two-cell, two-gonadotrophin" model of follicular oestrogen synthesis (Armstrong & Dorrington, 1979) appear to operate in the rat follicle. Previously we showed that inhibin dose-dependently
FIG. 6.9. Effect of exogenous androstenedione on A. oestradiol and B. progesterone accumulation in vitro by antiserum-treated rat follicles. Whole follicles were incubated for 5 days in medium with inhibin antiserum and 1 mM androstenedione. Every 24 hours in culture, follicles were removed from their wells using a fine glass micropipette, and transferred to fresh culture medium. Steroid accumulation in spent culture medium was determined by radioimmunoassay. Data from experiment VI (mean ± SE; n=12). *P <0.01 compared with antiserum treatment. Data for antiserum + androstenedione on day 3 excludes one outlier (oestradiol = 51 nmol; progesterone = 1200 nmol) and therefore n=11.
enhances LH-stimulated thecal/interstitial cell androgen synthesis *in vitro* (Chapters 3 and 4). This led us to propose that FSH may influence ovarian androgen synthesis via a paracrine mechanism arising in FSH-stimulated granulosa cells. According to the Armstrong and Dorrington model, withdrawal of such FSH-dependent paracrine signaling would bring about decreased androgen synthesis, and hence reduced oestrogen production. Selective inhibition of androgen synthesis in this way would then be expected to cause the build up of granulosa-derived progesterone, which is a thecal androgen precursor (Short, 1962). Since treatment of isolated thecal:interstitial cells with inhibin potently stimulates androgen synthesis without affecting progesterone (Hillier *et al.*, 1991c), all the data point to an action at the level of Cytochrome P450C17α (17-hydroxylase/C17-20 lyase), which is the rate limiting steroidogenic enzyme in androgen synthesis (Bogovich & Richards, 1982).

To rule out non-specific effects of the inhibin antiserum we added exogenous inhibin or androstenedione to antiserum-treated follicle cultures. The addition of inhibin restored a measure of oestradiol production and decreased progesterone accumulation. This suggests that inhibin immunoneutralisation causes selective inhibition of androgen synthesis and is further consistent with action at the level of P450C17α. Addition of the aromatisable androgen androstenedione to antiserum-treated follicle cultures fully overcame the block to oestradiol synthesis, confirming the crucial role of thecal androgen as an aromatase precursor. The stimulatory effect of exogenous androstenedione on progesterone accumulation in antiserum-treated follicles agrees with the proposed paracrine role of androgen in augmenting C21 steroid synthesis in FSH-stimulated granulosa cells (Armstrong and Dorrington, 1976). Interestingly, oestradiol production by inhibin antiserum-treated follicles cultured with FSH in the presence of exogenous inhibin or androstenedione increased more rapidly than follicles treated with FSH alone. This observation suggests that the inductive action of FSH on granulosa cell inhibin production (hence androgen production) is a rate-limiting factor in initiation of oestrogen secretion by the preovulatory follicle.

Presumably androgen production and aromatase activity each limit the rate of oestrogen synthesis in the preovulatory follicle. As the aromatising
capacity of the preovulatory follicle increases a commensurate increase in thecal androgen synthesis is required to sustain the rising oestradiol production rate. The attendant rapid turnover of androgen to oestrogen is probably the main reason why androgen does not accumulate at a measurable level in cultures of FSH-treated whole follicles.

The dramatic effects on steroidogenesis of the inhibin antiserum treatment were surprising though it must be remembered that the system of follicle culture is a fragile one and thus any imbalance of the normal hormonal environment is likely to be detrimental to normal steroidogenesis. Nevertheless, previous observations in vitro (Chapters 3 and 4) have shown that inhibin is able to augment LH-stimulated androgen production by up to 4-fold. Since androgen accumulation appears to be the rate-limiting step in oestrogen synthesis in this culture system then it may be presumed that oestradiol production would be increased by a comparable amount due to this effect of inhibin. Therefore the selective removal of inhibin would be expected to decrease oestradiol production by up to 75% in the absence of any other intraovarian modulation of androgen synthesis. It should not be forgotten though that other factors such as IGF-I can augment LH-stimulated androgen synthesis 1.5-fold (Hernandez et al. 1988b; Magoffin & Erickson, 1988a) and thus mask some of the effects of inhibin immunoneutralisation. Therefore it was unexpected to observe that the selective removal of inhibin decreased oestradiol synthesis to 15% of levels seen in the absence of antiserum. This discrepancy may be explained by the negative effects of activin and possibly TGFβ which both inhibit LH-stimulated androgen synthesis (Activin: Hsueh et al. 1987; TGFβ: Magoffin et al. 1989) and are produced by FSH-stimulated granulosa cells (Activin: Woodruff et al. 1987; TGFβ: Kim & Schomberg, 1989). It has also been reported that inhibin can neutralise the inhibitory effects of activin on LH-induced androgen synthesis (Hillier et al. 1991b). It may be postulated that the removal of inhibin increases the activin:inhibin ratio and therefore decreases LH-stimulated androgen production, and hence oestradiol synthesis. Activin also increases progesterone production by GC (Miro et al. 1991). TGFβ selectively decreases P45017α activity (Hernandez et al. 1990a) and therefore androgen production, as well as increasing progesterone production by FSH-stimulated granulosa cells (Dodson & Schomberg, 1987; Knecht et al. 1987).
and LH-stimulated theca cells (Magoffin et al. 1989; Hernandez et al. 1990a).
These effects of activin and TGFβ are consistent with our finding that inhibin immunoneutralisation decreases oestradiol synthesis and increases progesterone accumulation. Follistatin is known to decrease FSH-stimulated aromatase activity, and increase granulosa cell progesterone production (Xiao et al. 1990; Xiao & Findlay, 1991). It is expressed in increasing amounts with development of the follicle and thus may be also contributing to the enhanced progesterone and decreased oestradiol production rates.

It has been previously reported that an intrabursal injection of inhibin influences folliculogenesis by causing growth of follicles into the recruited (350 - 500 µm) size class (Woodruff et al. 1990). Follicular growth was also enhanced by intrabursal gonadotrophin treatment, and inhibin was capable of augmenting this effect. However, removal of inhibin by antiserum treatment does not significantly affect growth rate nor alter follicular diameter after 5 days in culture. The combined results of both studies suggest that FSH exerts the major control over follicular growth, whilst inhibin though not essential, can augment FSH-stimulated folliculogenesis.

In conclusion, we have used a whole follicle culture system to evaluate the influence of inhibin on gonadotrophin stimulated growth and oestrogen secretion. Immunoneutralisation of inhibin selectively suppressed FSH-stimulated oestrogen synthesis without retarding follicular growth. The inhibitory action of inhibin withdrawal on oestrogen synthesis was fully compensated by replacement therapy with exogenous inhibin or androstenedione. These results support our hypothesis that locally produced inhibin exerts a physiologically significant paracrine function in the regulation of follicular oestrogen synthesis in vivo.
Chapter 7. General Discussion

1. Introduction

Paracrine control within the endocrine system involves local cellular production of hormones, which diffuse out of the cell and exert their actions on neighbouring target cells. The term "paracrine" was first applied to the local production of gut peptides that mediate cell-cell interactions in the digestive tract (Van Noorden & Polak, 1979). The significance of paracrine control to the coordination of ovarian follicular development has attracted a great deal of interest in recent years.

The 'two-cell, two-gonadotrophin' theory of preovulatory oestrogen synthesis provides a model for paracrine signalling within the ovarian follicle (Armstrong & Dorrington, 1979). This model requires that granulosa cells exclusively express receptors for FSH, while thecal cells express LH receptors (Hsueh et al. 1984). FSH action is accomplished by receptor-mediated cAMP intracellular signalling mechanisms that increase the expression of differentiated functions, such as LH receptor and aromatase (Chapter 5), the enzyme crucial for oestrogen synthesis. LH receptors are also coupled to steroidogenesis via cAMP, the major steroidogenic enzyme under LH control being P45017α (Chapters 4 and 5). This enzyme is crucial for follicular androgen synthesis, and hence the provision of aromatase substrate for FSH-stimulated granulosa cells. Granulosa cells do not express significant amounts of P45017α (Chapters 4 and 5), whereas thecal cells do not express aromatase (Chapter 5). While every follicle is exposed to the same gonadotrophin profile during the ovarian cycle, they all react differently due to local modulation of the actions of gonadotrophins.

2. Evidence for Paracrine Signalling in the Ovary

The overall objective of the studies described in this thesis was to investigate the local modulation of gonadotrophin action in the ovary. Firstly, the relative contributions of FSH and LH to follicular development were investigated. It was found that FSH is required for normal follicular
development, however this is unlikely to be entirely a direct effect of the hormone. This study demonstrates that FSH is capable of stimulating the rate of granulosa cell proliferation in vivo, but not in vitro (Chapter 2). This suggests that the growth stimulating effects of FSH are mediated through another factor(s). Factors that potentially mediate this effect of FSH include oestrogens, TGFα and IGF-I. Oestrogens increase ovarian weight in hypophysectomised rats (Louvet et al. 1975), although it has been reported that thecal/interstitial cells are required to induce granulosa cell DNA synthesis (Bley et al. 1991), thereby implicating factors of thecal cell origin in the mediation of this effect (Bendell & Dorrington, 1991). TGFα, of thecal cell origin, promotes granulosa cell proliferation, but inhibits granulosa cell differentiation and thecal cell androgen production (May & Schomberg, 1989). IGF-I augments the responses of ovarian cells to gonadotrophins (Chapters 2, 3 and 4) and is believed to be involved in the stimulation of granulosa cell proliferation (Giudice, 1992). These factors therefore qualify as potential paracrine mediators of the growth stimulatory effects of FSH.

As well as promoting follicular growth, FSH was shown to stimulate granulosa cell expression of differentiated functions, such as progesterone and oestradiol synthesis, both in vivo and in vitro (Chapter 2). Androgens of thecal cell origin, as well as acting as a substrate for aromatase, enhance FSH-stimulated progesterone and oestradiol synthesis, but block oestradiol enhancement of LH-responsiveness in granulosa cells (Chapter 2). These results show that FSH can exert a direct action upon granulosa cell maturation, but also that androgens, of thecal cell origin, are capable of augmenting these effects of FSH. Therefore androgens can also act as paracrine signals during follicular maturation.

According to the 'two-cell, two-gonadotrophin' theory, both FSH and LH are required for normal oestrogen synthesis. This study shows that FSH alone is incapable of eliciting an increase in plasma levels of either oestradiol or androgen in the complete absence of LH (Chapter 2). However, once LH was restored, FSH dose-dependently increased plasma levels of both steroids. This observation implies that cell-cell communication is necessary for normal follicular oestrogen synthesis, and verifies the 'two-cell, two-gonadotrophin' theory.
In summary, these results suggest that FSH is the primary stimulus to oestradiol synthesis and follicular growth, but that LH is essential to support these actions of FSH. Paracrine communication is important for mediating the actions of FSH and LH to ensure coordinated follicular development.

Administration of FSH \textit{in vivo} enhances P45017α mRNA expression and LH-responsive androgen synthesis \textit{in vitro} (Chapter 4). Granulosa cells but not thecal/interstitial cells possess FSH receptors, whereas P45017α expression, and hence the capability for androgen synthesis, is far higher in thecal/interstitial cells than granulosa cells (Chapter 4). Therefore, these findings can be interpreted as direct evidence that FSH activates granulosa-derived paracrine signalling in the ovary. Hypophysectomy impedes this stimulatory action of FSH, but replacement therapy with LH restores the ability of FSH to enhance thecal cell function. Therefore simultaneous exposure to endogenous LH is necessary to facilitate augmentation of thecal/interstitial function by FSH. It is likely that factors produced by FSH-stimulated granulosa cells modulate androgen production by thecal/interstitial cells resulting in coordinated follicular steroidogenesis. A candidate for this role is inhibin, which dose-dependently enhances LH-stimulated thecal/interstitial cell androgen synthesis \textit{in vitro} (Chapters 3 and 4).

To test the hypothesis that inhibin exerts a physiologically significant paracrine function within the intact follicle, a whole follicle culture system was used (Chapter 6). Withdrawal of inhibin by immunoneutralisation during culture of individual follicles \textit{in vitro} provided the most direct evidence to-date of a paracrine function for inhibin in the regulation of follicular oestrogen synthesis. Immunoneutralisation of inhibin selectively suppressed FSH-stimulated oestrogen synthesis without affecting follicular growth. These results support the hypothesis that inhibin promotes follicular oestrogen synthesis via a paracrine mechanism involving increased production of aromatase precursor in thecal/interstitial cells.

In summary this thesis has shown that FSH exerts primary control over follicular development while LH supports and augments the regulatory function of FSH during folliculogenesis. It also provides direct evidence that FSH can influence LH-responsive thecal/interstitial androgen production \textit{in vivo} and suggests similar potentials for inhibin, IGF-I and oestradiol of
granulosa origin in mediating this action of FSH. However, while potential roles have been demonstrated for these factors, the physiological relevance of these findings has not yet been discussed.

3. Physiological relevance of paracrine signalling

For normal follicular function, the ovary must be exposed to the correct gonadotrophin stimulus. The central control of ovarian development is via the hypothalamic control of pituitary gonadotrophin secretion (Knobil, 1980), but the precise control and coordination cannot be exercised by gonadotrophins alone. There are several events in ovarian follicular development which occur without adequate changes in gonadotrophin levels to explain their occurrence. Such events include recruitment, selection and dominance of follicles, and atresia. It is highly probable that these events are mediated by the local modulation of gonadotrophin action. Indeed, local communication is necessary for the functional coordination of ovarian cell types to ensure normal follicular development and oestrogen synthesis. This section will attempt to assign some physiological relevance to the findings of this thesis.

Once the selection of antral follicles from the preantral pool has occurred, the ovary must choose which one(s) will attain dominance and eventually ovulate. This dominance is exerted despite a decrease in gonadotrophin support, and therefore an important consideration in the selection of a dominant follicle is a high rate of oestradiol and inhibin synthesis for negative feedback to decrease pituitary FSH production. Equally important is the role of local intraovarian factors in modifying the response to gonadotrophins to favour the dominant follicle. The FSH-induced acquisition of LH receptors on mature granulosa cells enables these cells to respond to tonic levels of LH in the same way that they would respond to FSH. In this way LH can stimulate intracellular cAMP accumulation and substitute for the lowered levels of FSH. Therefore follicles that have a low threshold for FSH due to a high aromatase activity and acquisition of LH responsiveness are more likely to survive the lower FSH levels and are preferentially selected.
Factors implicated in the attainment of dominance include inhibin and IGF-I. Both factors are of granulosa cell origin and augment LH-stimulated androgen production by thecal cells (Chapters 3 and 4). This enables a high level of oestrogen secretion because of the provision of aromatase substrate and the augmentative action of androgen on FSH-stimulated oestrogen synthesis. Oestradiol in rats increases inhibin-α and -β (inhibin) subunit mRNA expression but does not affect inhibin-β (activin) subunit mRNA expression (Turner et al. 1989). In this way oestradiol can modulate the relative rates of granulosa cell inhibin/activin synthesis by favouring inhibin synthesis later in follicular development.

These actions are potentially important in the attainment of dominance during the mid-follicular phase when aromatase activity, LH receptor content of granulosa cells, and IGF-I and inhibin production reach critical levels. The LH receptor, being coupled to cAMP-mediated post-receptor signalling, like the FSH receptor, stimulates granulosa cells to produce inhibin and IGF-I and thus sensitises thecal cells to LH. Similarly thecal cell androgen increases aromatase activity, and inhibin and IGF-I production. Since both these factors increase LH-stimulated androgen production by thecal cells, there is a reciprocal mechanism to increase oestradiol synthesis. Thus in the situation of declining FSH stimulation, the non-dominant follicles are unable to survive as growth is restricted. The dominant follicle(s) survives because inhibin and IGF-I increase the sensitivity of follicles to both FSH and LH, and thereby compensate for the low FSH levels. In this way the high levels of oestradiol synthesis that are normally associated with the dominant preovulatory follicle are facilitated. Therefore, the modulation of LH action by FSH-stimulated granulosa cell paracrine signalling can determine the fate of the follicle.

4. Future prospects

Undoubtedly there are many other processes in ovarian follicular development where paracrine communication is important. For instance it has been proposed that the prevention of oocyte maturation potentially involves inhibin (O et al. 1989), whereas TGFβ stimulates meiotic maturation (Tsafriti et al. 1989b). However, immature follicles with arrested oocytes have
no inhibin, and the development-related expression of TGFβ has yet to be determined. Thus the situation is obviously very complex and there are probably a combination of factors involved in this process.

The hypothesis that inhibin and IGF-I exert a physiologically significant role in ovarian follicular development is based solely on the demonstration of potential actions of these factors on ovarian cells. However, many of these actions still need to be verified in vivo and the hypothesis tested and confirmed. It is important to remember that we should not assign status to any one factor and ignore the others. The emerging situation is one whereby modulators of gonadotrophin action form complex interactions during follicular development, and variations in the availability of one or more factors at various stages of development can alter the response of follicles to the same gonadotrophin stimulus.

In order for a candidate paracrine factor to be considered for a potential action it must satisfy certain standard criteria (Franchimont, 1986). It must be produced locally, be received locally and exert its action locally. All the factors mentioned in this thesis satisfy these basic criteria, but once they are met there must be rigorous testing to demonstrate a more meaningful role in vivo. Specifically, final proof of in vivo relevance requires evidence of indispensability by means of selective neutralisation of the candidate factor. Although this study has provided some evidence of the indispensability of inhibin by immunoneutralisation, a more thorough experiment would involve transgenic technology. By this technique the potential relevance of the intraovarian actions of inhibin, as assessed by its dispensability to ovarian function, could be determined. This would overcome the problem that by their very nature, paracrine factors are found in small quantities and act within a discrete area, and thus are difficult to block in vivo by immunoneutralisation. However, it must be remembered that since local modulation of gonadotrophin action involves complex interactions, then the removal of one factor may upset the normal balance resulting in an unnatural hormonal environment. The fact that some paracrine factors have other roles in ovarian physiology must also be considered. For example, inhibin exerts an endocrine control over pituitary function (Ying, 1988), and thus the removal of inhibin will upset the feedback regulation of gonadotrophin secretion. Similarly, it has been shown recently that the
selective removal of the inhibin-α gene in transgenic mice leads to the development of gonadal stromal tumours (Matzuk et al. 1992). The gene for inhibin-α is thought to be a tumour suppressor gene, and inhibin a tumour suppressor protein, as well as exerting its other endocrine and paracrine actions. It therefore seems that in the future a major challenge to ovarian physiologists will be to demonstrate meaningful intraovarian mechanisms regulating gonadotrophin action by approaches that do not upset the normal hormonal balance. These will give important new insights into alterations of normal physiology that cause syndromes such as infertility and polycystic ovary syndrome.


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