CHANGES IN GENE EXPRESSION DURING BOVINE GRANULOSA CELL LUTEINIZATION

by

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DECLARATION

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DEDICATION

To my parents with thanks.

Abstract

The corpus luteum develops from the remnants of the ovulatory follicle and rapidly becomes, for a short time, the most active of the steroidogenic tissues. Both the granulosa and thecal cells of the follicle differentiate into luteal cells although it is likely that they show different activities within the corpus luteum. Studies described in this thesis were designed to examine some of the changes which take place in the luteinizing granulosa cells using both corpora lutea of known ages and by following changes in cell activity following luteinization in culture. In particular, changes in expression of two cell-surface receptors, the follicle-stimulating hormone (FSH) receptor and the putative high density lipoprotein (HDL) receptor SR-BI.

The first studies were designed to characterise changes in granulosa cells during luteinization in culture and to compare to changes *in vivo* during normal luteal development. Investigation of culture conditions showed that it was necessary to serum-treat culture dishes to obtain cell-attachment. Comparison of serum-containing and serum-free culture medium showed that granulosa cell number, progesterone production and cytochrome P450 side chain cleavage (P450scc) activity only increased markedly in the absence of serum. Levels of mRNA encoding P450scc increased in culture and *in vivo* during luteinization while levels of cytochrome P450 aromatase decreased markedly at the same time. There was little change in levels of the transcription factor SF-1. These studies showed that changes *in vitro* were similar to changes *in vivo*.

It is uncertain whether bovine granulosa cells retain FSH-receptors after luteinization and whether there is a change in receptor splicing at this time. Using reverse-transcription and the polymerase chain reaction (RT-PCR) and Southern blotting expression of the FSH-receptor was measured in granulosa cells before and after luteinization *in vivo* and *in vitro*. Before luteinization full-length transcripts and two major alternate transcripts were observed. The alternate transcripts lacked exons 4 and 5 or exon 9. After luteinization *in vitro* or *in vivo* no full-length receptor transcripts were detected but shortened transcripts, lacking the transmembrane domain of the receptor, were present. Results show that after luteinization there is a decrease in the level of expression and a change in the pattern of receptor splicing. This is similar, in reverse, to changes which occur during gonadal development.

Steroidogenesis by the developing corpus luteum is dependent upon provision of the substrate cholesterol by circulating lipoproteins. In cattle high density lipoprotein (HDL) is the major source of cholesterol. Granulosa cells are exposed to HDL prior to ovulation and it was postulated that levels of HDL-receptor expression might limit granulosa cell steroidogenesis. At the start of these studies the only putative HDL-receptor was an HDL-binding protein (HBP) described in humans and rats. Using PCR a bovine partial cDNA was isolated and sequenced and by semi-quantitative RT-PCR levels of HBP were measured during luteinization. Levels of HBP mRNA increased, though not markedly, *in vivo* although changes in culture were less clear-cut. Subsequently, a second putative HDL-receptor (known as SR-BI) was described in the literature which fulfilled all of the requirements of a physiologically-relevant receptor. Using the RACE technique the full-length sequence of the bovine SR-BI HDL-receptor was determined and shown to have close homology to sequences from other species. Examination of tissue distribution showed high levels in adrenal cortex and corpus luteum with lower levels in liver and spleen. Using RT-PCR levels of SR-BI mRNA were shown to increase 7-fold during corpus luteum development and 5-fold during granulosa cell luteinization. Results show that luteinization is associated with an increase in HDL-receptor expression which, along with changes in steroidogenic enzyme expression, is likely to explain the marked increase in steroidogenic capacity associated with luteinization.

Publications

The following full publications were produced during the period of studies described in this thesis:

O'Shaughnessy PJ, Dudley K, Rajapaksha WRAKJS, 1996. Expression of follicle stimulating hormone-receptor mRNA during gonadal development. Mol Cell Endocrinol 125:169-175

Rajapaksha WRAKJS, Robertson L, O'Shaughnessy PJ, 1996. Expression of follicle-stimulating hormone-receptor mRNA alternate transcripts in bovine granulosa cells during luteinization in vivo and in vitro. Mol Cell Endocrinol120:25-30

Rajapaksha WRAKJS, McBride M, Robertson L, O'Shaughnessy PJ, 1997. Sequence of the bovine HDL-receptor (SR-BI) cDNA and changes in receptor mRNA expression during granulosa cell luteinization in vivo and in vitro. Mol Cell Endocrinol 134:59-67

Sha JA, Dudley K, Rajapaksha WRAKJS, O'Shaughnessy PJ, 1997. Sequence of mouse 17β-hydroxysteroid dehydrogenase type 3 cDNA and tissue distribution of the type 1 and type 3 isoform mRNAs. J Steroid Biochem Mol Biol 60:19-24

Abbreviations

3βHSD	3β-hydroxysteroid dehydrogenase
BCS	Bovine calf serum
bp	Base pairs
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
dbcAMP	Dibutyryl cyclic adenosine monophosphate
CG	Chorionic gonadotrophin
CSCC	Cholesterol side-chain cleavage
DMEM/F12	Dulbecco's minimum essential medium and Ham's F12
DNA	Deoxyribonucleic acid
ddNTP	Dideoxynucleotide triphospate
dNTP	deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
FGF	fibroblast growth factor
FSH	Follicle-stimulating hormone
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDF-9	Growth differentiation factor
GSP	Gene-specific primer
GTP	Guanine tri phospate
HBP	High density lipoprotein binding protein
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
kb	Kilobases
LDL	Low density lipoprrotein
LH	Luteinizing hormone
mRNA	Messenger ribonucleic acid
P450arom	Cytochrome P450 aromatase
P450c17	Cytochrome P450 17α-hydroxylase
P450scc	Cytochrome P450 side chain cleavage
PCR	Polymerase chain reaction
RIA	Radioimmunoassay
RACE	Rapid amplification of complementary ends
RNA	Ribonucleic acid
RT	Reverse transcription
SDS	Sodium dodecyl sulphate
SF-1	Steroidogenic factor-1
SLF	Steel factor
SR-BI	Class B scavenger receptor
StAR	Steroidogenic acute regulatory
TBE	Tris-borate-EDTA
TIMP	tissue inhibitors of metaloproteinases
TSH	Thyroid stimulating hormone
VIP	Vasoactive intestinal peptide

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Chapter 1

Introduction

1.1 Introduction

The mammalian ovaries are paired organs situated within the abdominal cavity and covered by a single layer of surface epithelium. The essential functions of the ovaries are to produce oocytes and to develop and maintain other aspects of the reproductive tract. The identification of the ovarian follicles, first mistaken as the eggs themselves occurred in 1672 by Regnier de Graaf (see Asdell, 1969). This led to mature pre-ovulatory follicles becoming known as Graafian follicles. It was not until 155 years later, in 1827, that Karl Ernst Von Baer discovered that the mammalian egg, unlike larger avian and amphibian eggs, was only a small part of the entire follicular structure. Since then three primary structures of the follicle, the oocyte, the membrana granulosa and the theca have been identified. In addition the corpus luteum has been shown to form from the ruptured pre-ovulatory follicle. In the early 1900s the gonads were recognised as organs of internal secretions and the female reproductive system has subsequently been identified as a complex endocrine system in which several hormones interact to promote oocyte maturation, utero-tubular function and maintenance of pregnancy. Formation of the corpus luteum is fundamental to maintenance of pregnancy and studies described in this thesis are aimed at improving our understanding of events which occur during the early stages of corpus luteum development.

1.2 Development of the ovarian follicle

The ovarian follicles are unique structures due to their diversified functional characteristics. They play the dominant role in the dual functions of the ovary. These functions are (a) oocyte maturation and release and (b) production of the steroids necessary for

regulating both follicular growth and development of the reproductive tract. In addition they give rise to the corpus luteum which is essential in most species for maintenance of early pregnancy and in many species for maintenance of pregnancy up to term. The follicles attain their functional competence following a series of developmental changes and growth of the follicle is a continuous process going on at all times and at all ages (Peters *et al*, 1975).

Follicular development in different species of mammals has been extensively investigated. I include here a general description of folliculogenesis which is applicable to most mammals and, below a more detailed description of ovarian and follicular development in the bovine.

There remains some uncertainty over the origins of the follicular cells (which will be developing into the granulosa cells). It has been suggested that follicular cells originate a) from stromal cells (Peters & Pederson, 1967; Peters, 1969), b) from the epithelium of the mesonephric tubules and rete ovarii (Byskov & Linternmoore, 1973, Byskov, 1975) or c) from the cells of the germinal epithelium (Everett, 1943). Current opinion appears to suggest that the potential granulosa cells may arise from both the germinal epithelium and the mesonephric tubules (Boland & Gosden, 1994).

In all species, the earliest recognisable follicles are the primordial follicles which consist of an oocyte surrounded by a single layer of squamous follicular cells which sit on a basement membrane. Development of these follicles to the primary type is associated with growth of the oocyte and enlargement of follicular cells to become cuboidal granulosa cells. This growth in size is, presumably, associated with increased activity of these granulosa cells. Further development of the follicles to the secretory stage is associated with proliferation of the granulosa cell layer so that it appears to become several layers thick. All the granulosa cells maintain contact with the basement membrane, at least in some species, and the granulosa cell

layer should be more accurately described as pseudostratified (Pineda, 1989). During the period of primary follicle development a unique extracellular matrix, the zona pellucida, appears between the granulosa cell layer and the oocyte. It is now clear that it is the oocyte which synthesises the zona pellucida proteins (Philpott *et al*, 1987;Wasserman, 1990). Also during the secondary phase a new type of cell, the thecal cell, becomes morphologically distinguishable surrounding the outer side of the basement membrane. The origin of these cells is unknown and is discussed further below.

Factors which regulate development of primordial follicles and primary follicles remain unclear although a number of recent studies have identified factors which may be involved. Follicular cells in primordial follicles secrete Steel factor (SLF) which acts on c-kit ligand in oocytes (Motro *et al*, 1991). Blockage of this interaction prevents primordial follicular growth suggesting an essential role for SLF (Yoshidaa *et al*, 1997). Further development of the primary follicle, similarly, requires growth differentiation factor-9 (GDF-9) since in mice lacking GDF-9 follicle growth does not proceed beyond the primary stage (Dong *et al*, 1996). The initiation of primordial follicle growth is not gonadotrophin-dependent since primordial follicles develop in the hypophysectomised animal and *in vitro* (Fortune & Eppig, 1979). The neuropeptide vasoactive intestinal peptide (VIP) may, however, be involved in stimulating early follicle development (Mayerhofer *et al*, 1997). In those follicles which do not undergo atresia the granulosa cells continue to proliferate and secrete a fluid which collects to form the antrum. The oocytes remain surrounded by granulosa cells (the corona radiata) and associated with the main body of granulosa cells by stalk of cells (the cumulus oophorus). Further growth of the antral follicle towards ovulation is associated with continual proliferation of the granulosa and thecal cells and secretion of antral fluid to form the pre-ovulatory follicle.

There is some controversy as to when thecal cells arise during follicular growth as these cells cannot be distinguished from ovarian interstitial and stromal cells on the basis of morphology alone. In early studies, Brambell *et al* (1927) suggested that the theca interna begins to differentiate when the follicle has about two layers of granulosa cells. More recently Hirshfield (1991) has suggested that thecal cells may be present at the very outset of follicular growth. It is possible, therefore, that all components of a follicle may be present at the outset of follicular development. Other studies by Boland & Gosden (1994) in which the origin of the cells from mouse chimeras was studied also support the idea that thecal cells may be present from the early formation of the follicles.

In cattle, there are around 150,000 primordial follicles present in the ovary at birth and the structure of the bovine primordial follicle has been described in some detail (van Wezel & Rodgers, 1996). Follicle growth and development is very similar to that described above. Large antral follicles are found pre-pubertally in cattle but pre-ovulatory follicles are not found until after puberty. During the oestrus cycle of the cow, several cohorts of antral follicles emerge at one time and one of these becomes the dominant follicle which suppresses growth of the other follicles. The dominant follicle will eventually become atretic if there is no ovulatory surge of LH.

1.3 Formation of the corpus luteum

Ovulation of the pre-ovulatory follicle leads to formation of the corpus luteum which is a remarkable structure. It develops very quickly from the remains of the ruptured follicle and in most species becomes the most active steroid-producing structure in the body. If the animal is not pregnant then, again in most species, the corpus luteum degenerates very rapidly at the end of the luteal phase to allow ovulation to occur again. The corpus luteum itself is a fairly

straightforward structure made up of large and small luteal cells with fibroblasts and numerous blood vessels.

The earliest description of corpus luteum formation is by Corner (1919) who showed that both granulosa and thecal cells were involved in formation of the corpus luteum in the sow. Both thecal and granulosa cells also appear to be involved in corpus luteum formation in many other mammals including the cow (Donaldson & Hansel, 1965; Alila & Hansel, 1984). The fate of these cells in the developing corpus luteum has been more controversial but there is now a general belief that the thecal cells form small luteal cells while the granulosa cells form large luteal cells (Donaldson & Hansel, 1965; McClellan *et al*, 1977; O'Shea *et al*, 1980; Alila & Hansel, 1984). The large and small luteal cells have been shown to be functionally distinct. In most species tested, including the cow, small luteal cells appear to be responsible for increased progesterone secretion in response to LH whereas large luteal cells contribute to high basal progesterone levels but show little or no response to LH (Lemon & Loir, 1977; Urseley & Leymarie, 1979; O'Shea *et al*, 1980; Hoyer *et al*, 1986; Weber *et al*, 1987).

Control of corpus luteum formation remains unclear although there is mounting evidence that the oocyte is normally involved in inhibiting luteinization of the granulosa cells layers. It is known (see Chapter 3) that granulosa cells put in culture show changes which are very similar to those occurring at luteinization (Henderson & Swanston, 1978; Henderson & Moon, 1979; Geenen *et al*, 1985; Luck *et al*, 1990). In addition, it has been shown in this laboratory that ablating large follicles using an ultrasound-guided needle can lead to apparent formation of a normal corpus luteum (Robertson *et al*, unpublished). Most recently it has been reported that in mice which are unable to form gap junctions between oocyte and surrounding granulosa cells there is inappropriate and spontaneous luteinization of the granulosa cells of large antral follicles (Simon *et al*, 1997). This accumulating data suggests that the granulosa

cells reach a stage at which they will luteinize spontaneously but are inhibited by an oocyte-derived factor.

Against this, however, is recent evidence that granulosa cells will not luteinize spontaneously in serum-free culture (Gutierrez *et al*, 1997) although this may just indicate that some serum-derived factors are required for luteinization to proceed.

1.4 Pituitary control of gonadal function

Pituitary control of gonadal function was first demonstrated by Smith (1926; 1927 a & b; 1930) who showed that following hypophysectomy there was regression in the weight of the sex organs which could be partially prevented by anterior pituitary transplantation. It was further shown that anterior pituitary transplants caused an increase in the mass of secondary sexual characteristics (Smith & Engle, 1927). In 1931 Fevold, Hisaw & Leonard reported that the anterior lobe of the pituitary contains two active fractions, one causing follicular growth and the other luteal growth. This work was confirmed by Wallen-Lawrence in 1934 and the two active factors became known as follicle stimulating hormone (FSH) and luteinizing hormone (LH).

More recently the structure of the gonadotrophins has become known. Both LH and FSH are made up of two glycosylated peptide chains designated as the α and β subunits. The α subunit is common to LH, FSH, thyroid stimulating hormone (TSH) and chorionic gonadotrophin (CG) while the β subunit confers immuno- and bio-specificity to the hormone. The two subunits are expressed by separate genes and are then linked by disulphides bond. Oligosaccharides, composed of galactose, mannose, fucose, acetyl glycosamine and sialate (Green *et al*, 1985 a & b) are attached post-translationally and these determine conformation and charge distribution across the molecule. The type and number of radicals on the sialate

terminal group vary and this determines charge heterogeneity (Green *et al*, 1985a). Variation of glycosylation (within and between species) is a major factor in determining biopotency.

Since the 1970s the primary amino acid sequences of the glycoproteins have been available and in the late 1970s and 1980s the gene structures were determined. The α-subunit gene was first isolated and characterised using human material (Fiddes & Goodman, 1981). The gene was shown to exist as a single copy containing four exons spanning approximately 9.4 kb. The molecular structures of the LH and FSH β-subunits have also been determined and shown to be about 1.45 kb in length with two exons (Boorstein *et al* 1982; Maurer, 1987). The organisation of the β-subunit is sufficiently different from that of the α-subunit to suggest that they do not have a common evolutionary origin. Most recently, the crystalline structure of hCG has been determined (Lapthorn *et al*, 1994) which has shed light on possible mechanisms of gonadotrophic hormone-receptor interactions.

1.5 Gonadotrophin regulation of ovarian function

The role of the gonadotrophins in regulating ovarian function has been the subject of intense investigation since the gonadotrophins were first identified. The requirement for gonadotrophins to allow follicle development was first seen experimentally in hypophysectomised animals and, more recently, in mutant and transgenic mice in which circulating gonadotrophins are low or completely absent (Jones & Krohn, 1961; Cattanach *et al*, 1977; Kendall *et al*, 1995). In these animals folliculogenesis usually reaches the late secondary and, occasionally, early antral stage with no further development (Halpin *et al*, 1986). This suggests that gonadotrophins are not required for early follicle development although whether these follicles are functionally normal in the absence of gonadotrophins is not known. A number of studies have shown that development of late secondary follicles is

dependent upon stimulation by FSH and oestradiol (Goldenberg et al, 1972; Richards, 1980). Effects of these hormones are to increase granulosa cell proliferation, antral fluid secretion and aromatase activity. At this stage LH is also required for stimulation of thecal cells to produce androgen substrate for aromatisation by the developing granulosa cell layer (Moon *et al,* 1978). As the antral follicle approaches the pre-ovulatory stage LH-receptors are synthesised by the granulosa cell layer (Zeleznick *et al,* 1974; Richards & Midgley, 1976) and the LH surge induces ovulation through release of a proteolytic enzyme cocktail (Espey, 1974; Lipner, 1988). Recent interesting data have shown clearly that progesterone is also required at this time to allow ovulation to occur (Lydon *et al,* 1995).

To this point gonadotrophin regulation of follicle development is fairly uniform across species although some species require copulation to induce the LH surge. Hormonal regulation of corpus luteum development is, however, more species dependent. In cattle LH is the major luteotrophin, as it is in most species. The corpus luteum also contains receptors for prolactin and prolactin may stimulate progesterone production suggesting that it is also a luteotrophic hormone (Bartosik *et al,* 1967; Scott *et al,* 1992) although others have questioned any role for prolactin (Bevers *et al,* 1988).

1.5i Gonadotrophin receptors

It is now known that the biochemical actions of gonadotrophins are expressed through their interaction with cell surface receptors which, with the exception of the LH/CG- receptor, are specific for each of the hormones. Receptors for FSH and LH have been shown to be present in the testis and ovary (Heckert & Griswold, 1991). In the testis the FSH receptor is present only on the Sertoli cells while, in the female, FSH receptors are located in the granulosa cells of the developing ovarian follicles (Uilenbroek & Richards, 1979; Uilenbroek & van der

Linden, 1983; Camp *et al*, 1991; Kliesch *et al*, 1992; Rannikki *et al*, 1995, Tisdall *et al*, 1995) FSH action is required in the female for normal folliculogenesis and oocyte maturation. In the testis, LH receptors are present in the Leydig cells and LH action is required for normal steroidogenesis in these cells (Scott *et al*, 1990). In the female, LH receptors are present in the interstitial cells, thecal cells, granulosa cells of large antral follicles and in the luteal cells of most species (Zeleznick *et al*, 1974; McFarland *et al*, 1989; Camp *et al*, 1991). In addition, it has also been shown that the LH receptor has a more widespread distribution and is also found in non-gonadal tissues (Reshef *et al*, 1990; Reiter *et al*, 1995).

Signal transduction within the cell is by a second messenger system and the gonadotrophins have been shown to affect intracellular cyclic adenosine monophosphate (cAMP) via an adenylate cyclase system (Dufau *et al*, 1980 & 1988) and calcium levels (Davis *et al*, 1987; Sullivan & Cooke 1986) and protein kinase C activity (Winter & Dufau, 1984). Cells respond in a variety of ways to gonadotrophin stimulation including protein and steroid synthesis and cellular proliferation.

1.5ii Structure of gonadotrophin receptors

Studies on the genetic organization of the LH- and FSH-receptors show that they have strong structural similarities. Both are members of the Guaninetriphospate(GTP)-binding protein superfamily forming a distinct subgroup with the TSH-receptor. This group is characterised by the presence of a large extracellular domain encoded by the first nine or ten exons of the gene (McFarland *et al*, 1989; Parmentier *et al*, 1989; Sprengel *et al*, 1990; Gross *et al*, 1991; Koo *et al*, 1991; Heckert *et al*, 1992). The FSH receptor has 10 exons while the LH receptor has 11 exons (Fig. 1.1). In both, the last exon is very large and encodes the transmembrane spanning and cytoplasmic domains. The first 9 or 10 exons encode the

extracellular domain which confers hormone specificity to the receptor. The transmembrane domain encodes the C-terminal region containing seven transmembrane helices, three extracellular loops and the cytoplasmic tail (McFarland *et al*, 1989). Recent studies on hormone-receptor interactions between gonadotrophins and their receptors have used site-directed mutagenesis and modelling studies and have suggested that the interaction of the hormone with the receptor leads to a conformational change in the receptor which may involve reorientation of the helices of the transmembrane domain (Bhowmick *et al*, 1996; Puett *et al*, 1996)

The molecular sequence of the LH receptor has been determined in the pig, rat, human, mouse, cow, (Loosefelt, *et al*, 1989; McFarland *et al*, 1989; Minegishi *et al*, 1990; Gudermann *et al*, 1992, Lussier *et al*, 1995) while the FSH receptor cDNA sequence has been determined in the rat, cow, mouse, human, sheep and donkey (Heckert, *et al*, 1992; Kelton *et al*, 1992; Khan *et al*, 1993; Houde *et al*, 1994; O'Shaughnessy *et al*, 1994; Richard *et al*, 1997).

1.5iii Alternate splicing of gonadotrophin receptors

Molecular studies have shown that various forms of the LH and FSH arise as a result of alternate splicing. Known alternate transcripts of the FSH-receptor in different species are shown in Fig.1.2. This diagram is drawn from results by Gromoll *et al* (1992), Kelton *et al* (1992), Khan *et al* (1993), O'Shaughnessy *et al* (1994), Themmen *et al* (1994) and Tisdall *et al* (1995). As can be seen, most transcripts arise as a result of exon deletion in the extra-cellular domain. There is considerable variation in the nature of transcripts between species and it is difficult from this figure to discern a common theme. The identified alternate transcripts are not, however, exhaustive in any one species and there may be transcripts common to all species.

There is good evidence, for example, that a shortened transcript of the mouse FSH receptor exists lacking exon 10 (O'Shaughnessy *et al*, 1997), similar to those in the rat and sheep. Surprisingly little is known about possible functions of these potential receptor isoforms or whether most of them are translated. Recently a couple of extra alternate transcripts of the sheep FSH-receptor have been described (Sairam *et al*, 1996; Sairam *et al*, 1997) which may have specific functions. A transcript of the receptor which is 25 residues short at the carboxy terminus has been shown to act as a dominant negative form of the receptor inhibiting FSH action (Sairam *et al*,1996). Another form, which has only the first eight exons and a modified carboxy terminus which acts as a transmembrane domain, will bind FSH and has structural properties typical of a growth factor receptor (Sairam *et al*, 1997). It is possible, therefore that alternate splicing of the FSH receptor could regulate sensitivity of a cell to FSH and the type of response mediated by the hormone.

1.5iv Regulation of gonadotrophin-receptor expression through alternate splicing

It is now clear that there are developmental changes in the splicing of both the LH- and FSH-receptor transcripts. The first demonstration was by Sokka *et al* (1992) who showed that the first detectable LH-receptor mRNA expressed in the rat ovary during development is a shortened transcript expressing only the extracellular domain (or part of it) of the receptor. Expression of the extracellular domain was evident from as early as embryonic day 17 while full-length transcripts could not be measured until about day 7 after birth. Similarly, FSH-receptor transcripts encoding the extracellular domain of the receptor only appear before full-length transcripts in the rat ovary (Rannikki *et al*, 1995). More recent studies on mouse ovary development has shown a similar sequence of events with shortened, extracellular transcripts expressed before full-length transcripts appear (O'Shaughnessy *et al*, 1997). Similar

developmental mechanisms for both gonadotrophin-receptors occur in the testis (Rannikki *et al*, 1995; Zhang *et al*, 1994). Responsiveness of the gonads in the rat and mouse to LH and FSH coincides with expression of the full-length receptor suggesting that development of sensitivity to the hormone is dependent upon a change in receptor splicing (Sokka *et al*, 1992; O'Shaughnessy *et al*, 1997). It appears that in gonadal cells expression of the gonadotrophin-receptors is constitutive but that sensitivity of the tissue to the hormone is regulated by alternate splicing of the receptor primary transcript. During early development only shortened transcripts are present which are highly unlikely to transduce hormonal stimulation to the cell. As development occurs splicing changes and full-length active receptors appear. Other alternate transcripts of the FSH-receptor have been described for ovine testes which may have specific roles in regulating responsiveness to the hormone but there is little evidence for similar transcripts in other species and these may be specific to the sheep (Sairam *et al*, 1997; Yarney *et al*, 1997).

1.5v FSH-receptors during folliculogenesis and luteinization

From studies in the developing mouse and from *in situ* hybridisation work in the sheep ovary it is likely that significant levels of full-length FSH-receptor mRNA are first expressed in the follicle when it reaches the primary stage (O'Shaughnessy *et al*, 1994; Tisdall *et al*, 1995; O'Shaughnessy *et al*, 1997). Normal expression of some granulosa-specific products such as cytochrome P450 aromatase (P450arom) become gonadotrophin-dependent around this time (Gray *et al*, 1995). Studies using hypophysectomised or mutant animals (described above) indicate that the follicle can still grow in the absence of gonadotrophins at this stage but once they reach the early secondary stage growth slows down markedly (Halpin *et al*, 1986). Thus,

there is an absolute requirement for FSH once the follicle has reached the secondary stage of development.

Studies using *in situ* hybridisation and topical autoradiography have shown that FSH-receptors are present on growing follicles at all stages past the primary stage (Uilenbroek & van der Linden, 1983; Roy *et al*, 1987; Tisdall *et al*, 1995). Currently there is a lack of precise data showing changes in FSH-receptor number during folliculogenesis although FSH-binding does increase as follicles develop from pre-antral to antral stages (Yamoto *et al*, 1992) and mRNA levels increase during pre-ovulatory follicle growth (Lapolt *et al*, 1992). As the follicle develops under stimulation by FSH the granulosa cells start to express LH-receptors (Zeleznick *et al*, 1974; Segaloff *et al*, 1990). The effects of LH are to induce ovulation and allow follicle luteinization. In the developing corpus luteum of most species LH-receptors continue to be expressed since LH is the luteotrophic hormone. The fate of the FSH-receptors is unclear, however. In the bovine direct studies have not been able to show any effect of FSH on luteal steroidogenesis *in vivo* or *in vitro* (Hansel *et al*, 1973) despite the reported presence of FSH binding sites (Manns *et al*, 1984).

1.6 Steroidogenic enzymes

One of the important functions of the ovary is to produce the steroid hormones, oestrogens (C18 steroid), progestagens (C21 steroids) and androgens (C19 steroids). These classes of steroid hormones are structurally similar and are produced through common pathways. Biosynthesis of these hormones takes place within different compartments of the ovary with oestrogens produced mainly by granulosa cells of antral follicles, progestagens produced mainly in the corpus luteum and androgens produced mainly in the interstitial and thecal cells. It should be noted, however, that there is species variation in this arrangement.

Steroidogenesis involves the formation of steroid from cholesterol and this requires a large number of enzymes. Studies using radioactive steroids as substrates and measuring the products formed from incubation with steroidogenic tissues have led to the elucidation of general steroidogenic pathways and the most common steroidogenic pathways in the gonads are shown in Fig 1.3.

Three of the important enzymes which catalyse formation of different steroids are cytochrome P450 enzymes which catalyse distinct mixed-function oxidase reactions. The term cytochrome P450 is used to describe a large number of oxidative enzymes all of which are about 500 amino acids long and contain a single heme group. The name arises because they all show characteristic spectral shifts at 450 nm when binding to carbon monoxide. All the enzymes reduce atmospheric oxygen with electrons from NADPH and are involved in hydroxylation and cleavage reactions.

Most P450 enzymes are found in the smooth endoplasmic reticulum of the liver where they metabolise a huge number of exogenous and endogenous toxins, drugs etc. The three P450 enzymes involved in ovarian steroidogenesis are cholesterol side chain cleavage (CSCC), 17α-hydroxylase (P450c17) and aromatase (P450arom) each of which is described further below along with the other major non-P450 steroidogenic enzyme 3β-hydroxysteroid dehydrogenase.

1.6i Cholesterol side chain cleavage

The cytochrome P450 side chain cleavage enzyme (P450scc) catalyzes the formation of pregnenolone from cholesterol. This is the first step in production of all steroid hormones, it is also the rate-limiting step and is the step which is controlled acutely by gonadotrophin stimulation.

P450 scc catalyzes the removal of the side chain C22-27 of cholesterol forming an unstable intermediate, isocaproaldehyde, which is oxidized to isocaproic acid (4-hydroxyl, 4-methylpentanoic acid) and the C21 steroid pregnenolone. The enzyme is located in the inner mitochondrial membrane and the pregnenolone which is formed diffuses along the mitochondrial membrane and then undergoes further metabolism by enzymes associated with smooth endoplasmic reticulum (Fig 1.3). During acute periods of trophic stimulation, it is the transport of cholesterol to the inner mitochondrial membrane which is the limiting step in pregnenolone production and thus steroid production (Iida *et al*, 1989).

In the process of steroid biosynthesis from cholesterol three distinct chemical reactions are involved. These are 20α-hydroxylation, 22R-hydroxylation and scission of the cholesterol side chain at the bond between carbon atoms 20 and 22 to yield pregnenolone and isocaproic acid. Since the three intermediates in the conversion of cholesterol to pregnenolone can be isolated it was originally thought that three separate enzymes might be involved, 20-hydroxylase, 22-hydroxylase and 20,22-lyase (see review by Lieberman *et al.* 1984). It was subsequently shown, however, that a single protein was responsible for all the steps between cholesterol and pregnenolone (Shikita & Hall, 1973).

As described above P450scc is the rate-limiting enzyme in steroid synthesis and the enzyme affected, acutely, by hormonal stimulation. For these reasons efforts to clone the steroidogenic enzymes were directed first at P450scc and a 1821 bp clone was isolated and sequenced in 1984 from a bovine adrenal cDNA library (Morohashi *et al*, 1984). Subsequently, P450scc cDNA sequence has also been obtained from human (Chung *et al*, 1986), rat (Oonk *et al*, 1989), mouse (O'Shaughnessy & Mannan, 1994), pig (Mulheron *et al*, 1989), rabbit (Yang *et al*, 1993) and sheep (Pestell *et al*, 1993). The deduced amino acid sequences contain a heme binding region common to the P450 gene superfamily and a specific 25 amino acid

region of high homology between species which may act on the P450scc binding region (Morohashi *et al*, 1984; Mulheron *et al*, 1989). The genes for human and rat P450scc have also been cloned (Morohashi *et al*, 1987; Oonk *et al*, 1990) and shown to be about 12 kb in length with 9 exons.

The localisation of P450scc in the ovary has been studied immunologically using material from the rat. Luteal cells from cyclic, pregnant and pseudopregnant animals were immunostained and P450scc was also expressed in the interstitial cells of prepubertal and cyclic adult rats and in the thecal lining of the preovulatory follicles (Farkash *et al*, 1986; Goascogne *et al*, 1989). The granulosa of ovarian follicles, whatever their stage of development, including preovulatory follicles, were not labelled. Other studies (Goldring *et al* 1987) have shown that P450scc is present in rat granulosa cells and failure of Farkash *et al*, (1986) to detect P450 scc in granulosa cells may be related to the methods used. Similarly, in humans P450scc was confined to the theca interna of well-developed follicles and to the corpus luteum (Sasano *et al*, 1989). It should be noted, however, that granulosa cells from the cow can synthesise progesterone and that these cells must, therefore, contain P450scc (O'Shaughnessy *et al*, 1990; Luck *et al*, 1990; Langhout *et al*, 1991; Gutierrez *et al*, 1997) despite the inability of previous workers to detect P450scc mRNA by Northern blotting (Rodgers *et al*, 1987)

1.6ii 3β-hydroxysteroid dehydrogenase/isomerase

Pregnenolone formed from cholesterol may undergo 17αhydroxylation to 17α-hydroxy-pregnenolone or it may he converted to progesterone (see diagram below), both reactions taking place in the smooth endoplasmic reticulum (Fig 1.2).

The enzyme(s) catalysing the 3β-hydroxysteroid dehydrogenation and isomerization of the double bond from the B ring to the A ring have proved to be complex. An obvious

question since early studies has been whether separate enzymes exist to catalyse the dehydrogenation and isomerisation reactions. In addition, early enzymatic studies suggested that two or three different isozymes may exist (Ewald *et al*, 1964a, b). Recent studies using molecular biology techniques have resolved these issues. A cDNA corresponding to 3β-hydroxysteroid dehydrogenase (3βHSD) was first isolated from human placenta (Luu *et al*, 1989) and it was shown by a separate group that expression of a cDNA to 3βHSD in COS cells conferred both dehydrogenase and isomerase activity on the cells (Lorence *et al*, 1990). This showed that both enzyme activities are present in a single protein. Subsequent studies have shown that there are multiple forms of 3βHSD with at least two present in the human (Rheaume *et al*, 1991), four in the rat (Simard *et al*, 1993) and six in the mouse (Bain *et al*, 1991; O'Shaughnessy & Payne, 1996). Within a given species the different forms share significant identity but differ from each other by 5-10% in the coding regions.

Using immunocytochemical and *in situ* hybridisation methods Dupont *et al* (1990) have reported that 3βHSD is localised in the corpus luteum and the theca interna and that granulosa cells lack activity. This confirms earlier histochemical reports that 3βHSD is absent in granulosa cells (Fischer & Kahn, 1972). A layer of 3βHSD-positive granulosa cells has been identified in the guinea pig ovary, however, showing that there are species differences in the localisation of this enzyme (Dupont *et al*, 1990). Similarly, as discussed above, in cattle the granulosa cells produce progesterone in culture and the cells must, therefore, contain 3βHSD activity.

1.6iii 17α-hydroxylase

The enzyme cytochrome P450 17α-hydroxylase (P450c17) catalyses the hydroxylation of pregnenolone and progesterone to form 17α-hydroxypregnenolone and 17α-hydroxy-progesterone (Fig 1.2).

17α-Hydroxypregnenolone and 17α-hydroxyprogesterone may be further metabolised by cleavage between the C17 and C20 sites to form dehydroepiandrosterone and androstenedione. Until the early 1980s it was thought that 17α-hydroxylase and C17-20 lyase were separate enzymes since the two activities could be modulated independently. Studies by Nakajin *et al* (1981), however, showed that both enzyme activities are on a single protein. This was further confirmed when it was shown that a bovine cDNA for P450c17, when expressed in COS cells, encoded a protein with both 17α-hydroxylase activity and C17-20 lyase activity (Zuber *et al*, 1986). Since identification of the bovine cDNA for P450c17 the sequence of human, rat, mouse, bovine, pig and sheep P450c17, have been reported (Zuber *et al*, 1986; Chung *et al*, 1987; Fevold *et al*, 1989; Youngblood *et al*, 1991; Conley *et al*, 1992).

The P450c17 enzyme has been localised immunocytochemically to the theca interna alone in human, porcine and bovine ovaries (Rodgers *et al*, 1986; Sasano *et al*, 1989).

1.6iv Aromatase

The enzymatic pathways for biosynthesis of the aromatic oestrogen remained a mystery for many years. It was known that aromatic compounds are not usually synthesized in higher vertebrate species and substances like the aromatic amino acid phenylalanine must be obtained via the diet. Nevertheless, the aromatase enzyme has now been extensively studied and it has been shown to be a member of the cytochrome P450 family (P450arom). The enzyme causes three hydroxylations of C19 androgens; two at the C19 methyl group and one at the C2 position resulting in loss of C19 and aromatization of the A ring of the steroid.

The human P450arom cDNA was first cloned and sequenced in 1987 (Simpson *et al*, 1987) and shown to be 2.4 kb in length and encoding 419 amino acids. More recently, cDNAs for chicken, rat, bovine and mouse P450arom have been reported (McPhaul *et al*, 1988; Hickey *et al*, 1990; Terashima *et al*, 1991; Hinshelwood *et al*, 1993). The human gene for P450arom

has been characterised and been shown to contain 9 coding exons (Means *et al*, 1989; Toda *et al*, 1990). In addition to these 9 coding exons at least 6 non-coding exons have been identified (Toda *et al*, 1990; Means *et al*, 1991; Mahendroo *et al*, 1991). Transcripts isolated from different tissues have been shown to contain different 5'-termini arising from the use of tissue-specific promoters and alternate splicing mechanisms (Means *et al*, 1991; Mahendroo *et al*, 1991).

In the ovary aromatase activity is generally thought to be confined to the granulosa cells. Immunocytochemical studies by Sasano *et al* (1989), on human ovaries have tended to support this idea. They reported that P450arom was only present in the granulosa cells of the developing follicle while P450scc and P450c17 were confined to the theca interna. This was confirmed in rats and mice by the studies of Ishimura *et al* (1989) who reported that P450arom was detectable only in granulosa cells of large preovulatory follicles. More recently, immunocytochemical studies by El-Massarany *et al* (1991) have suggested that P450arom is not restricted to the granulosa cells in the rat ovary. Their studies suggested that the enzyme is present in other tissues including the stromal tissue. This confusion may arise from differing sensitivities of the methods used to measure aromatase activity, related to the different antibodies used. In addition there appears to be species dependency in the localisation of aromatase in the ovary with good evidence in the sow and rhesus monkey that thecal tissue contains significant activity (Stoklosawa *et al*, 1982; Vernon *et al*, 1983). Studies using *in situ* hybridisation have, again, suggested that aromatase is confined to the granulosa cells in the rat although the method may not have been sensitive enough to pick up the enzyme at lower amounts in other tissues (Telfer *et al*, 1992).

1.6v Control of enzyme activity

Steroidogenic enzyme activity in the ovary is regulated principally by the

gonadotrophins. The acute effect of gonadotrophins on steroidogenic activity can be looked on as consisting of two separate phases. The first phase depends on increased transport of cholesterol substrate to the P450scc enzyme (Crivello & Jeffcoate, 1980) via the cytoskeleton which appears to link lipid droplets and the mitochondria (Almahbobi & Hall, 1990). The second phase consists of transfer of the cholesterol from the outer to the inner mitochondrial membrane (Liscum & Dahl, 1992). This second phase is thought to be facilitated by the steroidogenic acute regulatory (StAR) protein (Clark *et al*, 1994; Clark *et al*, 1995).

The longer term effects of gonadotrophins are mediated by increased levels of enzyme activity which in turn is associated with increased levels of mRNA for the appropriate enzyme. Regulation of early follicular steroidogenesis has used developmental studies, largely in laboratory species, and has shown that gonadotrophins are essential for P450arom expression at the primary stage of folliculogenesis (Gray *et al*, 1995). Expression of P450scc and P450c17 expression, in contrast, becomes gonadotrophin-dependent later in development around the secondary and early antral stages (O'Shaughnessy & Mannan, 1994; Gray *et al*, 1996). Larger follicles have been studied using isolated follicles to examine enzyme activity or mRNA levels. Goldring *et al* (1987) have shown that protein and mRNA levels of P450scc are regulated by FSH in rat granulosa cells while thecal levels of mRNA are regulated by LH (Richards *et al*, 1987). Similarly, levels of 17α-hydroxylase enzyme activity, protein and mRNA appear to be regulated by LH in thecal tissue (Richards *et al*, 1987). Aromatase activity and mRNA levels are regulated by FSH in granulosa cells from small antral follicles (Hsueh *et al*, 1984; Fitzpatrick & Richards, 1991). The levels of mRNA are also regulated by synergy between FSH and various steroid hormones including oestrogen (Fitzpatrick & Richards, 1991). Interestingly, high levels of FSH will inhibit P450arom mRNA expression although enzyme activity is unaffected. The physiological significance of this is unclear.

All three of the cytochrome P450 enzymes involved in ovarian steroidogenesis show characteristic changes during follicular development which are, presumably, related to hormonal stimulation. Thus, P450scc mRNA levels are low in granulosa and thecal cells of pre-antral follicles but increase markedly as antral follicles develop (Goldring *et al*, 1987; O'Shaughnessy & Mannan, 1994). P450arom levels start to rise in primary follicles but are fairly low until the antral follicle stage is reached while P450c17 levels remain extremely low until the early antral stage develops (Richards *et al*, 1987; Hickey *et al*, 1988; Gray *et al*, 1995; Gray *et al*, 1996).

In addition to the gonadotrophins a number of other factors have been described which regulate steroidogenic activity. VIP increases P450scc protein levels in cultured granulosa cells and increases aromatase activity in the neonatal rat ovary (Trzeciak *et al*, 1986; George & Ojeda, 1987; Mayerhofer *et al*, 1997). Transforming growth hormone-β and tumour necrosis factor-α both inhibit 17α-hydroxylase activity (Hernandez *et al*, 1990; Andreani *et al*, 1991) while β-adrenergic agents will stimulate thecal androgen production (Hernandez *et al*, 1988). Other factors such as Steel Factor and GDF-9 are required for normal follicle development but it is not clear if they affect steroidogenesis directly (Dong *et al*, 1996; Yoshida *et al*, 1997). It is likely that these factors and others have modulatory roles in controlling ovarian steroidogenesis but it is certain that overall control must come from the pituitary-derived gonadotrophins.

1.6 va *Steroidogenic factor-1*

Steroidogenic factor-1 (SF-1) is an orphan nuclear receptor shown to be required for development of steroidogenic organs (Lala *et al*, 1992; Honda *et al*, 1993, Luo *et al*, 1994). More recently it has started to become clear that SF-1 is involved in almost every area of reproduction from GnRH secretion to luteal function. SF-1 was first isolated from bovine

adrenal cortex (Morohashi *et al*, 1992) and this was followed by isolation of the corresponding cDNA (Honda *et al*, 1993). The nucleotide sequence of SF-1 carries a Zn-finger protein and functional analysis of SF-1 showed that the nucleotide sequences recognised by SF-1 were all present in the promoter sequences of the steroidogenic P450 enzymes (Morohashi *et al*, 1992). Subsequently, it was shown that SF-1 would induce P450 enzyme transcription in cell lines transfected with an SF-1 expression vector (Morohashi *et al*, 1993).

In mice SF-1 has been shown to be expressed in the adrenal gland, testis and ovary and it is thought to regulate expression of the cytochrome P450 steroid-hydroxylase enzymes in these tissues (Ikeda *et al*, 1993; Honda *et al*, 1993). In the embryo SF-1 is found in adrenal and gonads from the earliest stages and also in tissues which give rise to the hypothalamus. The vital role of SF-1 in regulation and development was seen with the generation of mice with disruption in the *Ftz-F1* gene which codes for SF-1. These animals lacked adrenal glands and gonads (Luo *et al*, 1994) showing that SF-1 is an essential regulator of gonadal and adrenal development.

In the ovary there is evidence that SF-1 is involved in granulosa cell development and the follicle growth from small to large antral and, in particular, with expression of P450arom (Shapiro *et al*, 1996). Additionally, it has been suggested that SF-1 may be involved in granulosa cell luteinization from evidence that SF-1 is expressed in antral follicles just prior to luteinization but at only low levels in the corpus luteum (Wehrenberg *et al*, 1997). Other experiments using synchronised immature rats treated with hCG to induce ovulation saw a decline in SF-1 expression after the injection of hCG which might suggest that SF-1 is not involved in granulosa cell luteinization . This would seem unlikely, however, from the role of SF-1 in development of all other steroidogenic tissues and from the clear involvement of SF-1 in maintaining P450scc levels in the corpus luteum (Liu & Simpson, 1997).

1.7 Steroid production by the ovary

The main steroids produced by the ovaries in all mammals are progesterone and oestrogen which regulate the reproductive tract (including the ovary) and other parts of the body. Progesterone is produced by the corpus luteum during the luteal phase and, perhaps, by the granulosa cells in the follicular phase. Oestrogens are produced in the granulosa cells of all animals studied and in thecal and luteal cells of some species. Oestrogen production requires formation, first, of C19 steroids which, in turn, arise from pregnenolone or progesterone by the action of 17α-hydroxylase (Fig 1.3). As described above, this enzyme has been shown to be lacking in granulosa cells as assessed both histologically and biochemically (Sasano *et al*, 1990). The thecal cells, in contrast, have been shown to contain all the enzymes necessary for androgen production (Fortune & Armstrong, 1977). To account for ovarian oestrogen production a two-cell theory has been proposed whereby androgens produced by the thecal cells are converted by the granulosa cells to oestrogens (Falck, 1959; Moore, 1977). The role of the other steroidogenic cells, the interstitial cells, in steroid production by the ovary is less clear although they may produce progesterone or contribute androgens for granulosa-derived oestrogen production (Erickson *et al*, 1985).

1.7i Steroidogenesis in the bovine ovary

Steroid production by bovine follicles follows the general model described above. Granulosa cells can synthesise progesterone but lack P450c17 and depend upon thecal androgens for substrate for aromatisation (Rodgers *et al*, 1986; O'Shaughnessy *et al*, 1990; Gutierrez *et al*, 1997). Following luteinization in cattle there is a rapid loss of 17α-hydroxylase and aromatase activity and mRNA encoding P450c17 and P450arom so that both androgens and oestrogens are not produced by the bovine corpus luteum (Henderson & Moon, 1979; Rodgers *et al*, 1986; Rodgers *et al*, 1987; Lauber *et al*, 1991; Voss & Fortune, 1993a,b).

Lauber *et al* (1991) have shown that the loss of P450c17 mRNA in the bovine corpus luteum is due to the complete absence of positive transcription factors. A similar mechanism seems likely for loss of P450arom mRNA although it has not been measured directly.

1.8 Lipoproteins and steroidogenesis

Ovarian steroids are produced from cholesterol derived from one of the three possible sources (a) preformed cholesterol taken up from the blood primarily in the form of lipoproteins (b) preformed cholesterol stored within ovarian cells either as free cholesterol, a constituent of cell membranes, or liberated from cholesterol esters stored within cytoplasmic lipid droplets and (c) cholesterol synthesized *de novo* in the ovarian cell from 2-carbon compounds (Helig *et al*, 1970; Kovanan *et al*, 1978; Carr *et al*, 1980; Schuler *et al*, 1981; Carr *et al*, 1982). Most steroidogenic cells use preformed cholesterol (source b) but the extent to which *de novo* sysnthesis or lipoprotein uptake is used to supply these stores varies with animal species and with cell type involved (O'Shaughnessy & Wathes, 1985). In all steroidogenic cells *de novo* cholesterol synthesis will provide a basal level of substrate for steroidogenesis although in some cells, such as the Leydig cell, most of the cholesterol used for testosterone synthesis appears to arise by *de novo* synthesis, at least in the rat (Quinn *et al*, 1981).

Most steroidogenic cells, however, derive cholesterol for steroidogenesis from the lipoproteins low density lipoprotein (LDL) and high density lipoprotein (HDL). These lipoproteins have a core of esterified cholesterol surrounded by a lipid monolayer containing one or more proteins which are important in mediating binding of the lipoprotein to the cell-surface receptor. The major difference between HDL and LDL is in the type of protein associated with the lipid monolayer (Mahley, 1981). Species differences occur in the relative importance of the two classes of lipoproteins in the provision of cholesterol to steroidogenic tissues. HDL is the major circulating lipoprotein in most species (Mills & Taylaur, 1971) and

in those species in which it has been tested it appears, with a few exceptions, to be the major lipoprotein form supplying the steroidogenic tissues (McNamara *et al*, 1981; Pate & Condon, 1982; Strauss *et al*, 1982; McLean & Miller, 1986; Carroll *et al*, 1992; Bao *et al*, 1995). The most notable exception to this is man in which LDL is the major form of lipoprotein supplying the steroidogenic cells (Carr *et al*, 1982).

1.8i Uptake of cholesterol from lipoproteins

1.8ia *LDL*

The pathway for LDL uptake by cells was determined early (in the late seventies) since the LDL receptor and faulty LDL uptake are associated with cardiovascular pathology. Under normal circumstances, when cells need cholesterol for membrane synthesis or, in some cases, steroidogenesis, they synthesize receptor protein for LDL. Once in the plasma membrane the LDL receptors diffuse until they associate in coated pits that are in the process of forming. Since coated pits pinch off constantly to form coated vesicles, any LDL particle bound to LDL receptors in the coated vesicles are rapidly internalized. After shedding the clathrin coats, the contents of the endocytotic vesicles are passed to the endosomes. In endosomes the LDL particles and their receptors dissociate: the receptors are returned to the plasma membrane, while the LDL ends up in the lysosomes. In the lysosome the cholesteryl esters in the LDL particles are hydrolysed to free cholesterol which thereby become available to the cell (Brown & Goldstein, 1979; Brown & Goldstein, 1984; Goldstein *et al*, 1985). The free cholesterol in the cell may also be esterified and stored in the cell in lipid droplets. The cDNA encoding the LDL receptor was isolated by Yamamoto *et al* in 1984 and the protein shown to contain 5 domains, mutations in which often lead to dysfunction in the receptor and familial hypercholesterolemia

1.8ib *HDL*

Compared to LDL our knowledge of cholesterol uptake from HDL was relatively scarce until recently. In most tissues HDL appears to serve to remove excess cholesterol (Oram, 1986) but in the steroidogenic tissues HDL can act like LDL and supply cholesterol as substrate for steroidogenesis. It is likely that HDL can be internalised in the same way as LDL (Paavola & Strauss, 1983; Rahim *et al*, 1991) in some cells but uptake of HDL cholesterol can also occur in a more selective manner. In certain cells HDL "docks" with a cell-surface receptor and delivers some cholesterol to the cell before dissociating again from the cell surface (Glass, *et al*, 1983; Leitersdorf *et al*, 1984; Glass *et al*, 1985; Pittman *et al*, 1987). The internalised cholesterol ester may then either be hydrolysed or stored within lipid droplets (Paavola & Strauss, 1983).

Considerable effort has been put into isolation and identification of a physiologically active HDL-receptor. A single, high affinity binding protein for HDL was reported to be present in rat luteal tissue by Ferreri & Menon (1990) and in 1992 McKnight *et al* reported isolation of an HDL-binding protein (HBP) from human fibroblasts which had properties associated with an HDL-receptor. The mRNA encoding HBP was up-regulated by increased intracellular concentrations of cholesterol and it was postulated that HBP may be involved in movement of cholesterol out of fibroblasts. This led to the hypothesis that HBP was involved in maintaining cholesterol homeostasis in luteal tissue although the predicted structure did not suggest that HBP acted as a classic plasma membrane receptor. In 1994 the rat HBP mRNA was isolated and sequenced (Chen & Menon, 1994) and was shown to be expressed in the rat ovary in most cell types except the stroma and connective tissue. Levels of HBP mRNA were regulated by hCG and the authors concluded that HBP may play a physiological role in the ovary although it was not clear if it could act as the receptor mediating HDL uptake by the cells.

More recently, after work on this thesis was started, a cDNA was isolated and shown to fulfill all of the physiologically relevant criteria of an HDL-receptor (Acton *et al*, 1994; Acton *et al*, 1996). This cDNA was initially isolated as a scavenger receptor using degenerate PCR (Acton *et al*, 1994) and named SR-BI (class B scavenger receptor). Scavenger receptors mediate the endocytosis of chemically modified lipoproteins and may be involved in atherosclerosis (Steinberg *et al*, 1989) and SR-BI was isolated based on its homology to CD36 which binds oxidised LDL. Subsequent expression studies showed that SR-BI would bind HDL and mediate selective cholesterol ester uptake in a transfected cell line (Acton *et al*, 1996). Furthermore, the receptor was expressed almost exclusively in liver, adrenal and ovary - those tissues in which selective uptake of HDL cholesterol has been shown previously. Further evidence that SR-BI is the physiological HDL-receptor has come from mouse knockout studies in which the SR-BI gene was inactivated (Rigotti *et al*, 1997). In these animals the plasma cholesterol concentration was increased 125% due, probably, to decreased selective cholesterol uptake by cells from the plasma. In addition, antibodies directed at SR-BI will inhibit cholesterol uptake into mouse adrenal cells by 70% (Temel *et al*, 1997). Current evidence is, therefore, overwhelmingly in favour of SR-BI being the physiological HDL-receptor. The gene structure of the SR-BI has recently been reported and it was shown, interestingly, that the promoter region contains a binding site for SF-1 and that this SF-1 binding site was essential for normal transcription of the gene (Cao *et al*, 1997).

1.8ii Lipoproteins and luteal function

In most mammalian species progesterone production by the ovary increases markedly after ovulation as the corpus luteum develops. This change in steroidogenic activity is due, largely, to a huge increase in progesterone production by the luteinizing granulosa cells (Carr *et al*, 1982; O'Shaughnessy *et al*, 1990; Smith *et al*, 1994). It has been proposed that the

increased progesterone production following ovulation in humans is due to vascularisation of the granulosa cell layer making cholesterol substrate available from circulating LDL (Carr *et al*, 1982). Prior to ovulation, the granulosa cell layer is avascular and LDL cannot pass the basement membrane of the follicle. Only the thecal cell layer is, therefore, exposed to this lipoprotein. After luteinization rapid angiogenesis occurs and blood vessels invade the luteinising granulosa cell layer supplying the circulating LDL and increased substrate to support the high levels of luteal steroidogenesis.

Bovine luteal cells, in contrast to the human, can utilise cholesterol from both LDL and HDL for progesterone synthesis with HDL more likely to be of importance in maintaining luteal steroidogenesis (Pate & Condon, 1982; Savion *et al*, 1982; O'Shaughnessy & Wathes, 1985; Carroll *et al*, 1992; Bao *et al*, 1995). HDL, in contrast to LDL, can pass the follicle basement membrane and concentrations of HDL are relatively high in bovine follicular fluid (Savion *et al*, 1982; Brantmeier *et al*, 1987). The granulosa cells are, therefore, exposed to lipoprotein before ovulation. There is, however, little or no response of bovine granulosa cells to HDL before luteinization despite the ability of the cells to respond to stimulation by 22R-hydroxycholesterol with a rise in progesterone levels (O'Shaughnessy *et al*, 1990). This suggests that HDL-receptors are at low levels or are absent in the pre-ovulatory follicle (O'Shaughnessy *et al*, 1990; Bao *et al*, 1995). The recent identification of the class B scavenger receptor SR-B1 as an HDL-receptor provides the opportunity to test this hypothesis by measuring levels of HDL-receptor mRNA.

1.9 Production of non-steroidogenic factors by the corpus luteum

In addition to progesterone, which is without doubt the most important secretion from the corpus luteum, the developing and mature corpus luteum produces a number of

proteins and other factors, some of which are secreted into the bloodstream and act elsewhere and some of which act locally.

1.9i Proteins

Luteinization of granulosa cells is associated with marked changes in protein synthesis and secretion. The best known of these changes is the marked rise in oxytocin synthesis associated with luteinization in cattle and sheep (Wathes *et al*, 1983; Swann *et al*, 1984; Wathes *et al*, 1986). Shortly after the start of luteinization, on about day 3, expression of oxytocin mRNA reaches a peak before declining over the rest of the luteal phase (Ivell *et al*, 1985). Despite high production and secretion of oxytocin during the luteal phase the exact physiological role of oxytocin is not certain. Oxytocin can affect progesterone secretion by the luteal cells (Miyamoto & Schams, 1991) although circulating progesterone levels are not affected by oxytocin treatment during the mid-luteal phase (Gilbert, *et al*, 1989; Lutz, *et al*, 1991). Oxytocin may be involved in stimulating smooth muscle contraction in the uterine tube and uterus to assist in gamete transport (Luck, 1989) and oxytocin receptors have been identified on these tissue (Sheldrick & Flint, 1985; Ayad *et al*, 1990). Lastly it has been suggested that oxytocin may be involved in luteolysis through stimulation of prostaglandin release (Auletta & Flint, 1988; Silvia *et al*, 1991) although others have challenged this model (Ottobre *et al*, 1984; Kotwica & Skarznski, 1993).

In contrast to oxytocin, inhibin secretion by granulosa cells is decreased by luteinization in cattle (Martin *et al*, 1991). Inhibin secretion by the corpus luteum of the higher primate is maintained, however (Groome *et al*, 1994), and is probably of importance in regulating circulating FSH levels during the luteal phase.

The process of follicle ovulation and corpus luteum formation requires marked changes in tissue remodelling with associated changes in the extracellular matrix and angiogenesis. Control of extracellular matrix turnover is dependent on two groups of enzymes - metaloproteinases and plasminogen activator/plasmin which interact to form active metaloproteinases which can cause extracellular matrix breakdown. In contrast, tissue inhibitors of metaloproteinases (TIMP) will inactivate metaloproteinases and regulate extracellular matrix changes. TIMP protein and mRNA have been shown to be present in corpora lutea (Freudenstein *et al*, 1990; Tanka *et al*, 1992; Smith *et al*, 1993). Nevertheless, the exact role of metaloproteinases and TIMP in luteal function is not clear although they may play a role in endothelial and steroidogenic cell migration during corpus luteum development (O'Shea *et al*, 1980; Mignatti *et al*, 1989).

Following luteinization of the ovulatory follicle there is rapid neovascularisation of the developing corpus luteum. The factors and cell types involved in this angiogenic process are largely unknown. One of the most-widely studied angiogenic factors is fibroblast growth factor (FGF) which is present in luteal extracts (Grazul-Bilsak *et al*, 1992) and which may be produced by luteinized bovine granulosa cells in culture (Neufeld *et al*, 1987). A number of other potential angiogenic factors have been described but their role in luteal angiogenesis is uncertain (see Smith *et al*, 1994).

1.9ii Prostaglandins

Prostaglandins are produced by granulosa cells prior to ovulation and during corpus luteum development and function. Inhibition of pre-ovulatory prostaglandin synthesis does not alter luteinization or subsequent luteal function in a number of species (Grinwich *et al*, 1972; Wallach *et al*, 1975; Ainsworth *et al*, 1979; Murdoch & Dunn, 1983) suggesting that

this does not have an important role in luteal formation. In contrast, mature luteal cells produce both stimulatory (PGE_2, PGI_2) and inhibitory ($PGF_{2\alpha}$) prostaglandins which can regulate progesterone secretion (Rodgers *et al*, 1988) via specific binding sites on large and small luteal cells (Chegini *et al*, 1991) indicating a possible paracrine role in luteal function.

1.10 Studies in this thesis

Studies outlined in this thesis were aimed at increasing understanding of the changes which occur in granulosa cell function during and after luteinization. In particular it was aimed to study changes in two cell-surface proteins, the FSH- and HDL-receptors, one of which appears to become redundant after luteinization and the other of which may be crucial in maintaining luteal steroidogenesis. To study changes in these receptors two systems were used; an *in vivo* system in which corpora lutea were collected from animals at known stages of the luteal phase and a cell culture system using granulosa cells luteinising *in vitro*. Other studies described in this thesis are aimed, therefore, at development of the cell culture system and characterisation of changes in granulosa cell activity during luteinization *in vivo* and *in vitro*.

Bovine cells have been used for these studies because a) luteinization of bovine granulosa cells is well-characterised both *in vivo* and *in vitro* b) tissue was available from animals in the Veterinary School and from a local abattoir and c) the cow is a relevant domestic species in which embryo loss is known to occur as a result of an inadequate corpus luteum.

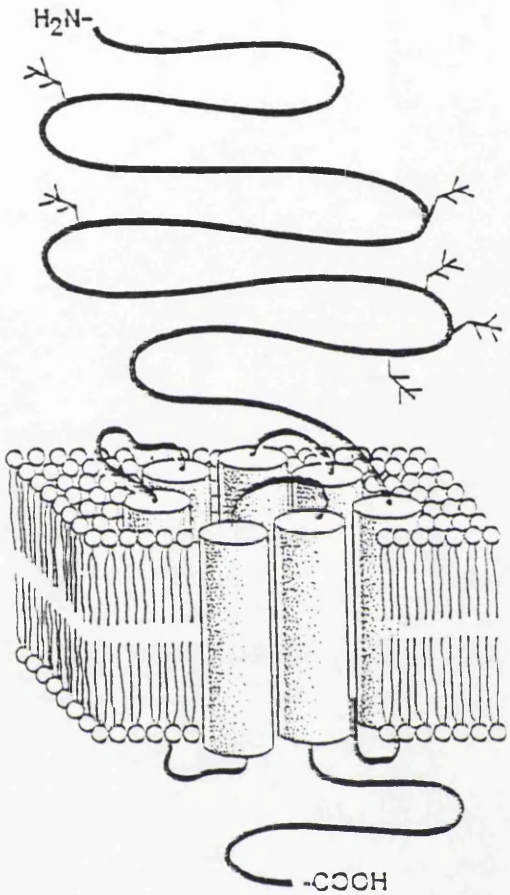
The results of this thesis are divided into three chapters. The first chapter (Chapter 3) describes experiments to develop a granulosa cell culture system to allow study of

luteinization *in vitro*. Changes in steroidogenic enzyme mRNA levels are measured using the *in vitro* cell culture system and compared to changes taking place *in vivo*. In addition expression of SF-1 in luteinising granulosa cells is studied. The second results chapter (Chapter 4) describes expression of the FSH-receptor and alternate transcripts in granulosa cells and determines changes in FSH-receptor expression during luteinization. In the final results chapter (Chapter 5)levels of HBP during luteinization *in vivo* and *in vitro* are shown. During the course of this work it became clear that SR-BI was much more likely to represent the physiological HDL-receptor and the bovine SR-BI (HDL) receptor cDNA was, therefore, isolated and the tissue distribution of expression of the receptor and changes in expression following luteinization were measured.

Figure 1.1

Three dimensional model of the FSH-receptor showing the long extracellular domain, the seven transmembrane domains and the short intracellular domain at the carboxy end. Branched symbols represent known sites of glycosylation.

Below is a drawing of the structure of the gene encoding the FSH-receptor with exons represented as boxes and introns as broken lines. Introns are not to scale.


H_2N-
-COOH

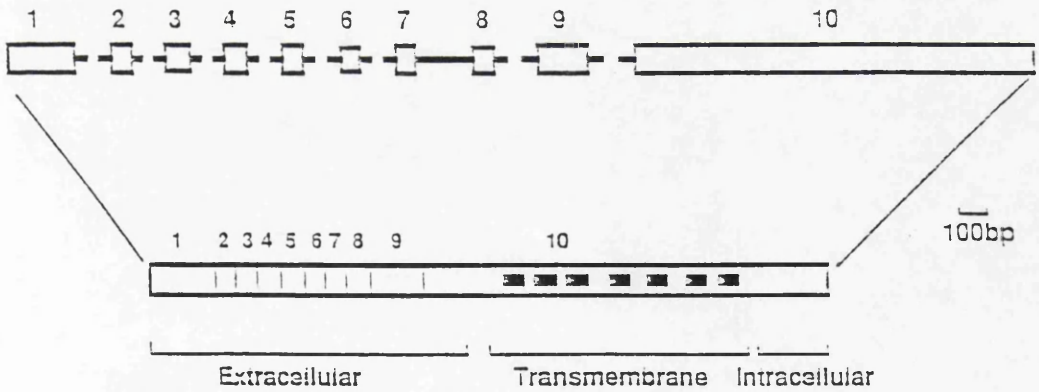

1
2
3
4
5
6
7
8
9
10
1 2 3 4 5 6 7 8 9
10
100bp
Extracellular
Transmembrane
Intracellular

Figure 1.2

FSH-receptor alternate splicing in different species. Exons are numbered 1 to 10 and no attempt has been made to show the introns to scale. Alternate transcripts mostly represent exon removal except in the human where exon 5 may contain intron sequence and in the sheep and rat where splicing may occur to sequences of unknown origin (Gromoll *et al*, 1992; Kelton *et al*, 1992; Khan *et al*, 1993; O'Shaughnessy *et al*, 1994; Tisdall *et al*, 1995; Themmen *et al*, 1995)

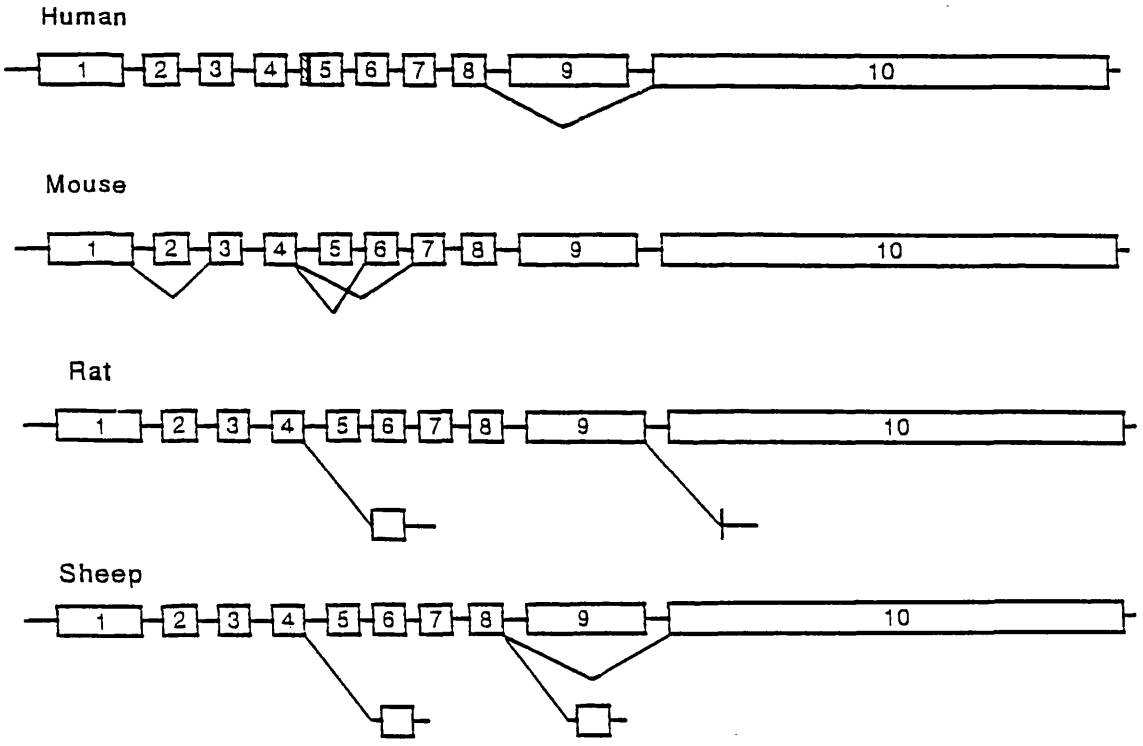
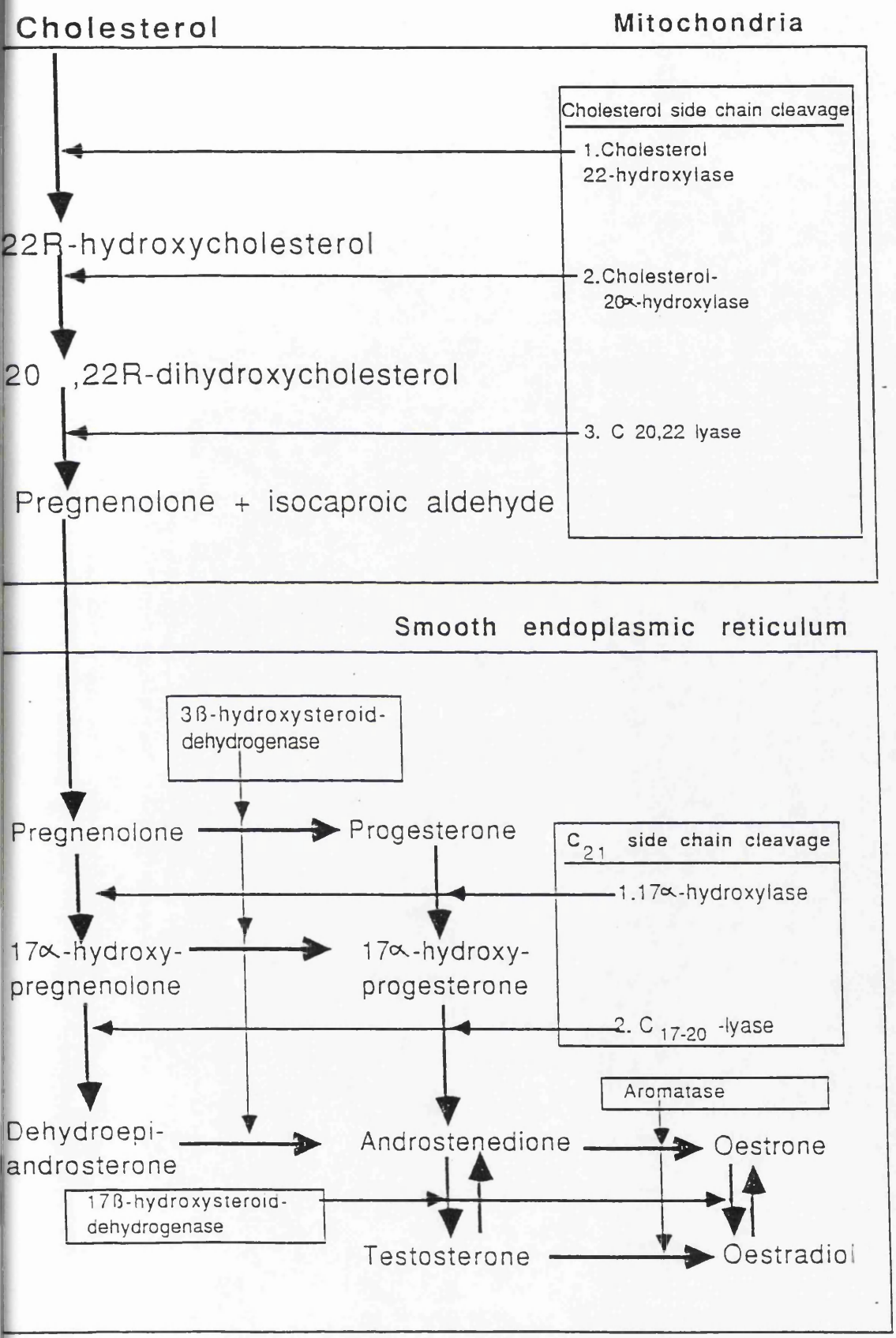

Human
1
2
3
4
5
6
7
8
9
10
Mouse
1
2
3
4
5
6
7
8
9
10
Rat
1
2
3
4
5
6
7
8
9
10
Sheep
1
2
3
4
5
6
7
8
9
10

Figure 1.3

Major steroidogenic pathways which occur in the ovary and enzymes associated with each step of the pathway. The enzymes are shown in boxes with arrows pointing to the steps of the pathway catalysed. The parts of the steroidogenic pathway occurring in the mitochondrion and smooth endoplamic reticulum are also shown.

(Modified from "The Physiology of Reproduction " by Knobil & Neil, Vol 1. pp. 331-386)


Cholesterol
Mitochondria
Cholesterol side chain cleavage
1.Cholesterol 22-hydroxylase
22R-hydroxycholesterol
2.Cholesterol-20α-hydroxylase
20 ,22R-dihydroxycholesterol
3. C 20,22 lyase
Pregnenolone + isocaproic aldehyde
Smooth endoplasmic reticulum
3ß-hydroxysteroid-dehydrogenase
Pregnenolone
Progesterone
C21 side chain cleavage
1.17α-hydroxylase
17α-hydroxy-pregnenolone
17α-hydroxy-progesterone
2. C17-20 -lyase
Aromatase
Dehydroepi-androsterone
Androstenedione
Oestrone
17ß-hydroxysteroid-dehydrogenase
Testosterone
Oestradiol

Chapter 2

Material and Methods

2.1 Collection of Corpus Luteum

Corpora lutea at days 1, 3, 5 and 7 of the luteal phase were collected from slaughtered cows. The stage of oestrous cycle of these animals was established by daily scanning of the ovaries using a real time B-mode scanner equipped with a 7.5 MHz rectal transducer (Dynamic imaging, Livingstone, Scotland). Ovulation was characterised by the collapse of the pre-ovulatory follicle and the day of ovulation was designated as day 0 of the cycle. Animals were killed at a local abattoir and corpora lutea were immediately stored in liquid nitrogen for subsequent RNA extraction. Scanning of animals and establishment of the oestrus stage was performed by Dr L. Robertson (Division of Veterinary Anatomy)

2.2 Granulosa Cell Culture

2.2i Preparation of liquid culture medium using powdered media (DMEM/F12)

Powdered medium (11.82g, pre-weighed; Life Technologies, Paisley, UK) was added to approximately 800-900 ml of water (purified by reverse osmosis) at room temperature and stirred gently to dissolve. Sodium bicarbonate (2.44g) was added and the pH of the medium adjusted to 7.2 using 1N HCl or 1N NaOH. The following additions were made to the medium; 365 mg of glutamine/L, 100 units of penicillin/ml, 100µg of streptomycin/ml, 50 mIU of insulin/ml (dissolved in pH 2 water) (Sigma, Palace) and 5µg of transferrin/ml and the volume was adjusted to 1L with water. The medium was sterilised by passage through a 0.22 micron pore size filter under positive pressure and was stored at 4°C in 50 ml aliquots.

2.2ii Isolation of cell

Ovaries were collected from cows being slaughtered at a local abattoir (the reproductive histories of these animals were not known). These ovaries were transported to the laboratory in ice within one to two hours of the slaughter of the animals and kept in ice until the cells were harvested. Follicles, without sign of obvious atresia (Henderson *et al* 1987) were used to harvest granulosa cells (O'Shaughnessy& Wathes, 1984; Langhout *et al* 1991). Follicles were considered healthy if thecal capillaries were visible and there was a lack of debris in the follicular fluid.

The ovaries were washed and follicles >1cm and <2cm dissected out without causing damage. From this step onward, all processes were carried out in aseptic condition. Follicles were washed with 70% ethanol for about 30 second to remove external contamination. Follicular fluid was aspirated using 18 - 23 G needles and 1 ml sterile plastic syringes and was transferred to culture media to try to prevent clotting (1:20 dilution). A "V" shaped incision was then made in the follicular wall using sterile scissors and the cavity of the follicle gently flushed with culture media using sterile pipettes. This procedure allowed us to collect granulosa cells from the follicular wall without damage to the basement membrane of the follicle. The media with cells were diluted about 20 times to prevent clotting. Then both follicular fluid and flushed medium were centrifuged at 800g for 5 min at 4°C. The supernatant was discarded and the pellet was re- suspended in 1 ml of culture medium and centrifuged again for 5 min at 800g. The supernatant was again discarded and the pellet re-suspended in 1 ml of culture medium.

Viability of the cells was assessed by the trypan blue exclusion method (Langhout *et al* 1991). Briefly, 0.4% trypan blue (Sigma) was added to an equal volume of cell suspension, mixed and the number of unstained cells counted using a haemocytometer.

2.2iii Preparation of culture plates

One ml of donor bovine calf serum (Life Technologies), heat inactivated at 56°C for 30 min, was added to the culture plates and incubated at 37°C overnight with a loose cap in a humidified incubator with 5% CO_2. Next day serum was removed and culture plates were washed with 2-3 ml of culture media before the inoculation with cells.

2.2iv Culturing of cells

Initial experiments (see results) were carried out to determine the best culture dishes to be used for the proposed experiments. Subsequently, plastic cell culture flasks (Nunclon, UK) with a growth area of 25cm^2 were used. One to two million viable granulosa cells were inoculated into culture dishes containing 3 ml of DMEM/F12 culture media. In most experiments the culture medium was supplemented with 1mM dibutyryl cyclic AMP (dbcAMP) (Sigma) to induce full luteinized function of the granulosa cells. Dishes were incubated at 37°C in a 5% CO_2 atmosphere. The caps of the dishes were loose and the incubator was humidified with a water pan. Medium was replaced every 24 hours with new medium preheated to 37°C. The replaced medium was stored at -20°C for subsequent progesterone assay. These cultures were used for the extraction of RNA, cell counting and testing for cholesterol side-chain cleavage (CSCC) activity.

2.2v Cell counting

The medium of culture were removed and 0.5 to 1 ml 0.005% porcine pancreatic trypsin (ICN Biomedical Ltd, Thame, UK) in PBS + 1mM EDTA solution was added to culture plate and flushed several times with a pipette to remove cells from the plate. Then the cells in the solution were counted in haemocytometer (O'Shaughnessy & Wathes 1984).

2.2vi Cholesterol side-chain cleavage activity

Several methods can be used to measure activity of CSCC including measurement of [^{3}H] pregnenolone or isocaproic acid synthesized from added [^{3}H]cholesterol. The problem with these methods is that cholesterol has very low aqueous solubility and is unable to gain access to the inner mitochondrial membrane without disruption of the mitochondrion. These problems may be overcome using hydroxylated cholesterol as substrate. Unfortunately 22R-hydroxycholesterol, which has a high affinity for the CSCC enzyme is not available in the tritiated form. As a consequence non-radioactive 22R-hydroxycholesterol is used in this assay as substrate and the progesterone formed is measured by radioimmunoassay (RIA) as described below.

Culture medium was removed from culture plates, the cells were washed with fresh culture medium, and then 1 ml of prewarmed culture medium, containing 8μg of 22R-hydroxycholesterol, was added and cells were incubated for one hour at 37°C. At the end of one hour the medium was collected for progesterone assay and the number of cells in the plate were counted (O'Shaughnessy, Pearce & Mannan, 1990).

2.2vii Radioimmunoassay for progesterone

2.2viia *Background and theory*

Yallow and Berson first described in 1959 a system for a competitive assay based on the immunological properties of antibodies raised to specific molecules. In general an antibody is raised against a molecule, in the case of a steroid it is usually conjugated to a protein such as BSA. The precise antigen specificity of antibodies makes them versatile and powerful tools that can be used to detect, quantify and localize a large variety of biologically interesting molecules.

Radioimmunoassay is an invaluable technique for measuring minute quantities of substances. In this technique a constant amount of radioactive antigen is added with a constant amount of antibody to a sample containing an unknown quantity of the same antigen in non-radioactive form. Unlabelled antigen will compete with the labelled antigen for antibody binding sites, so that the greater the amount of antigen in the unknown sample the smaller the amount of radioactive antigen bound to the antibody. The free and bound radioactive antigen are separated and radioactivity measured. By reference to standards, in which the concentrations of non-radioactive antigen are known, the quantity of unknown antigen in the sample can be routinely assayed.

2.2viib *Methodology*

Particulars of the RIAs used have been described already (Sheffield & O'Shaughnessy, 1989). Unknowns or standards in a total volume of 0.1 ml were added to small glass tubes (4ml volume). In some cases the unknowns were diluted to ensure that they were within the same range as the standards. To each tube 0.1 ml of [^{3}H]progesterone (Amersham Life Sciences, Little Chalfont, UK) was added (approx 10,000 cpm) followed by 0.1 ml of antibody diluted in buffer. Antibody was diluted so that it would bind approximately 30% of radioactivity added without unlabelled antigen. Assay tubes were incubated overnight at 4°C or for 1 h at 37°C.

At the end of the incubation, dextran coated charcoal was added. Dextran/charcoal binds the steroid that has not already been bound with antibody. The samples were subjected to centrifugation (1500g) for 10 min at 4°C. The supernatant was poured into scintillation vials and radioactivity determined as described below. The counts represent [^{3}H]progesterone bound to the antigen. This can be calibrated against the standard curve that is performed within each assay. The highest and lowest standards were 3200 and 25 fmol/0.lml. The source of the

antiserum was Dr. Foulkes (MAFF, Reading U.K.) and cross reactivity with androstenedione and testosterone was <0.1%. In each assay a quality control sample was included to check each assay and to determine interassay variation.

The buffer used in all assays was phosphate-buffered saline containing azide and gelatin (PAS-G). PAS-G contained 11.2g/l $Na_2PO_4.2H_2O$; 6.08g/l $Na_2HPO_3.2H_2O$; lg/l NaN_3; 9g/l NaCl and lg/l gelatin. Charcoal reagent was made up in PAS-G and consisted of 5g/l activated charcoal and 500mg/l dextran.

2.2viic *Reliability criteria*

The reliability criteria of any RIA are specificity, sensitivity, and precision (after Gupta, 1980). Specificity refers to cross reactivity with other steroids which may be present in the sample and which must be checked and quantified. The theoretical sensitivity depends on activity of antisera used for the steroid to be measured, the concentration of labelled steroid and antibody used and the volume of incubation medium. The biological sensitivity, in contrast, depends on the standard curve sensitivity and blank values which are used to assess background interference in the assay. Problems with precision arise mostly due to worker error and pipette malfunction. Inter-assay variance is evaluated by replicate measurement of the same sample in different assays. Intra-assay variation is estimated by replicate measurement of the same sample in the same assay. The inter- and intra-assay coefficients of variation were 15.3 and 13.5% respectively.

2.3 Molecular Biology Techniques

2.3i RNA extraction

Total RNA from granulosa cells or luteal tissue was extracted using RNAzol B (Biogenesis, Poole, UK) based on the method by Chomcznski & Sacchi (1987). This method relies upon complexing of RNA to guanidinium while proteins are denatured and proteins and DNA are removed from the aqueous phase into the organic (phenol/chloroform) phase and the interphase. The standard protocol provided by the manufacturers was used for RNA extraction with minor modifications. Between 200 and 300 µl of RNAzole B was added to 20 to 30 mg of luteal tissue or freshly isolated granulosa cells and the cells or tissue disrupted by sonication. For cultured cells, the culture media was removed and 500µl RNAzol B was added and the cells were flushed several time with pipettes to ensure lysis. In all cases, a 10% volume of chloroform was added, vortexed and the mixture kept in ice for 5 min. The mixture was centrifuged for 15 min at 10,000g which separated the lower phenol-chloroform phase and upper colourless aqueous phase containing RNA. The upper phase was then transferred to a new microfuge tube, an equal volume of anhydrous isopropanol added and the mixture kept in ice for 30 minutes. The sample was centrifuged for 15 minutes at 14,000g, the supernatant discarded and the pellet washed with 150µl 80% ethanol. The tube was centrifuged for 5 minutes at 10,000g and the ethanol removed using blotting paper (the pellet should not be completely dried). The pellet was resolubilised in RNAse-free water (BDH, Poole, UK) (100µl) and then 10µl of 3M sodium acetate (pH 7) and 220µl of ice cold ethanol (at -20°C) was added and the mixture incubated at -20°C for at least 1h. Again the sample was centrifuged for 15 minutes at 14,000g, the supernatant discarded and the pellet re-dissolved in 15µl of RNAse-free water. The RNA samples were stored in liquid nitrogen until used later.

2.3ii Reverse Transcription

In order to use the polymerase chain reaction (PCR) it was necessary to generate cDNA from isolated RNA using the reverse transcription reaction. In most cases the final reaction volume was 10µl and contained buffer (25 mM Tris-HCl pH 8.3 at room temperature plus 37.5 mM KCl), $MgCl_2$ (3 mM), dithiothreitol (1 mM), dNTPs (Advanced Biotechnology, Milton Keynes, UK) (500µM), MMLV Reverse transcriptase (Superscript, Life Technologies)(30 units), RNase inhibitor (Rnasin,Promega, Southampton, UK) (6U), random hexamer (Pharmacia Biotech, St Albans, UK)(5µM) and 1 to 2µl of extracted RNA. The buffer, $MgCl_2$ and water were exposed to UV light ($1J/cm^2$) to destroy any contaminating DNA which may have arisen from previous PCRs.

After mixing of RNA, the reaction mixture was incubated for 5 minutes at room temperature and then incubated at 37°C for one hour in water bath. The product, cDNA was stored at -20°C until used as template for PCR.

2.3iii Polymerase Chain Reaction

2.3iiia *Reactions*

The PCR was used to amplify DNA for visualisation, subsequent sequencing and for semi-quantitation. The reaction mixture for the PCR contained $(NH_4)_2SO_4$ (20mM), Tris-HCl (75mM, pH9.0 at 25°C), $MgCl_2$ (2mM) *Taq* DNA polymerase (Advanced Biotechnologies) (1U/30µl), primers (200nM each), dNTPs (200µM each) and template (0.5-2µl) in a total volume of 30µl. The buffer, $MgCl_2$ and water was exposed to UV light as above to remove amplified DNA contamination. In most experiments mineral oil (about 50µl) was added over the reaction mixture to prevent evaporation although in later experiments this was not necessary as a heated lid thermal cycler was used. In all experiments an automated thermal

cycler was used to cycle temperatures. The PCRs were initiated by heating the reaction mixture to 96°C for 2 min to denature the annealed cDNA/RNA from the reverse transcription reaction. The reaction tubes were then cycled up to 40 times through denaturation temperature (95°C) for 30 secs, annealing temperature (50-65°C) for 30 secs and extension temperature (72°C) for 1 or 2 mins. The annealing temperature was varied with different primers used in PCR. The conditions for different PCR are described, subsequently, where appropriate. At the end of the thermal reaction the oil layer was removed and 6µl of 6 times loading buffer (see appendix 1) added to each tube.

2.3iiib *PCR controls*

Various controls were run to ensure the fidelity of the PCR reactions. Not all controls were run in every reaction but were used where appropriate.

The biggest problem with the PCR technique is the danger of contamination from previously run reactions. In order to avoid this problem buffers were treated with UV light as described above and filter pipette tips were used to prevent pipette contamination. In every PCR reaction a blank tube was run without DNA template to check for contamination in the PCR reactions. In addition, reverse transcription blanks were run with each batch of reverse transcription reactions to ensure that contamination did not arise during this step. As an internal control to ensure that the reverse transcription step had worked the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified using primers based on human sequence (Tso *et al* 1995).

2.3iiic *PCR primers*

The primers used in the PCR reactions (except RACE, see Chapter 5) were:

1) GAPDH (Ercolani *et al* 1988)

5'-GTG AAG GTG GGA GTC AAC G-3' (exon 2)

5'-GGT GAA GAC GCC AGT GGA CTC-3' (exon 5)

Product size 300bp

2) P450scc (Morohashi *et al*, 1984)

5'-GCC CTC TAC AAG ATG TTC CAC AC -3'(exon 4)

5'-GCC ATC TCG TAG TAC AAG TGC CAT TGC-3' (exon 6)

Product size 321bp

3) P450 arom (Hinshelwood *et al* 1993)

5'-GGA TTG GAA GTG CCT GCA ACT ACT-3' (exon 3)

5'-GAG CAT GTT AGA GGT GTC CAG CAT-3' (exon 5)

Product size 399bp

4) FSH-receptor (Houde *et al*, 1994):

5'-CCTTCTGCCAGGAGAGCAAGGTGAC-3' (exon 1)

5'-TGCCTTAAAATAGATTTGTTGCA-3' (Exon 10)

5'-CATTGAATGCACAGTTGTGT-3' (exon 7)

Expected band sizes are described in Chapter 4

5) SF-1 (Honda *et al*, 1993)

5'-TGC TGC AGC TGC ACG CGC T-3' (exon 3)

5'-AGC ATC TCG ATG AGC AGG TTG -3'(exon 4)

Product size 304bp

6) HBP (own sequence)

Primers used to amplify HBP in semi-quantitative assays were

5'-CCA GTT TCC TGA TAA GGA CG-3'

5'-GTC AGG AGC CTT CTC ACT GCT G-3'

Product size 472bp and position of primers is described in Chapter 5. The gene structure of HBP is not known.

7)SR-BI (HDL-receptor) (own sequence)

Primers used to amplify HDL-receptor cDNA in semi-quantitative assays were

5'-GTGTCCTTCCTGGAGTACCG -3' (Bases 437-456)

5'-GAACACGGTGAAGAGGCCAG -3'(Bases 772-753)

The expected band size is 336bp and the position of the primers is described in Chapter 5.

The exon structure of SR-BI is not published.

Primers were synthesised by Oligo Express Ltd, Stanmore, UK.

2.3iiid *Agarose gel electrophoresis*

Agarose gels (1-2%) were prepared in 0.5 times TBE buffer (appendix A). Agarose (Biometra Ltd, Maidstone, UK) was dissolved in buffer by heating in a microwave oven. Ethidium bromide (Sigma) was added at the rate of 0.4μg per ml of gel when it became cool and the gel was allowed to set in a casting tray with combs inserted.

2.3iiie *Electrophoresis*

Products of the PCR reactions were loaded on the gel along with molecular size markers loaded in a separate lane. The molecular size markers were a 100bp ladder (Life Technologies) and 0.5μg was loaded per well. After loading, electrophoresis was carried out in 0.5 times TBE buffer at a constant voltage (100 - 150 V) for an appropriate time. The DNA bands were visualised under UV light with presence of ethidium bromide. Images of gels were recorded using a video camera system and thermal paper or polaroid film.

2.3iv Quantification of mRNA

2.3iva *General principle*

Semi-quantification of mRNA was carried out by RT-PCR using methods based on the study by Murphy *et al* (1990). During the PCR the amount of product formed should increase exponentially, in theory doubling every cycle, until the reaction begins to become less efficient and a plateau phase is reached (e.g. Fig 3.7). This plateau phase may be caused by a number of factors such as one of the reagents becoming limiting or because there is preferential amplification of another product (eg "primer/dimer"). In many cases the cause of the plateau effect is not clear but it is always observed. The fact that the reaction starts to become less

efficient in the latter stages means that any comparison between concentrations of cDNA template by PCR must be done during the exponential phase of the reaction (Fig 3.7).

In the technique used here the amount of a specific cDNA template was measured relative to GAPDH, a housekeeping gene, which is assumed to be expressed in all cells at a relatively constant level. For each template multiple identical tubes are set up to measure the specific cDNA of interest by PCR and a parallel set of tubes is set up to measure GAPDH. Tubes are amplified for different numbers of cycles, usually in the range of 18 to 35 cycles. The amount of product formed is measured by incorporation of [^{32}P]dATP. Products are separated on agarose gels, the gels dried and bands cut out according to ethidium bromide staining and after autoradiography. The incorporation of [^{32}P] is measured in a scintillation counter and the amount of product formed is measured relative to GAPDH over the exponential range of the PCR reactions. An example of such an experiment is shown in Fig 3.7.

2.3ivb *Specific reaction conditions*

Reaction tubes for PCR were set up as above with the exceptions that four or more identical tubes were prepared for each specific cDNA to be measured and, for each template, an equal number of tubes were set up to measure GAPDH. In addition [^{32}P]dATP (specific activity 3000 Ci/mmol, ICN) was added to each tube. PCR reactions were set up as above and tubes were removed from the thermal cycler at different cycle numbers. The PCR products were run of 1-2% agarose gels and the gels then dried on a gel drier at about 70°C under vacuum for about 3 h. The gel was subject to autoradiography and then re-stained with ethidium bromide. Bands visible under ethidium bromide were cut out and other bands were cut out using the autoradiograph as a template. The cut-out bands were put in scintillation vials, 4ml of scintillation fluid added and radioactivity counted in a scintillation counter. Graphs were

drawn of the number of cycles verses the log of the radioactivity (e.g. Fig 3.7) and then, selecting the exponential regions of the curves the relative amount of specific cDNA was calculated relative to GAPDH. The relative amount of cDNA was assumed to reflect the relative amount of mRNA since the efficiency of the reverse transcription step should be the same.

2.3v Southern blotting

For Southern hybridisation of PCR products the DNA was transferred from agarose gels to nylon membranes and hybridised with a [^{32}P]-labelled cDNA probe.

PCRs were run on agarose gels as normal and were documented with a ruler placed alongside the molecular size markers to allow subsequent sizing of products on Southern blots. The agarose gel was then treated with denaturation solution (1.5 M NaCl and 0.5 M NaOH) for 30 minutes with gentle rocking to make single strand DNA. Then gel was then treated with neutralisation solution (1.5 M NaCl plus 1 M Tris, pH 8.0) for 30 minutes with gentle rocking. The transfer of DNA to the nylon membrane was done by capillary blotting.

The capillary blotting required a container with 20xSSC, a raised support for the gel and a wick made from a sheet of filter paper. The gel was placed on the wick and water-resistant film (Nescofilm, Bando Chem, Japan) was placed around the edge of gel to prevent formation of short circuits between the buffer reservoir and the sink that might bypass the DNA-containing part of the gel. A nylon membrane (Boehringer Mannheim, Germany) was cut to size and placed over the gel and then two filter papers were placed over the nylon membrane and over that a 5-10 cm thick layer of paper towel was added as a sink and finally a 500g weight placed over the top of towels. DNA was allowed to transfer overnight and DNA was

then fixed to the membrane by baking 15 min at 80°C. The membrane was then rinsed with 2xSSC and stored at 4°C until hybridised.

2.3vi Preparation of probe for southern hybridisation

2.3via *Isolation of DNA*

Probes for Southern hybridisation were prepared from PCR products. The PCRs were run as above and the appropriate band cut out of the agarose gel under UV. Exposure to UV was kept to a minimum to prevent damage to the PCR product. The band was placed in dialysis bag containing 500µl of 0.5 x TAE buffer. After sealing the two ends of the bag with plastic clips, the dialysis bag was placed in an electrophoresis tank containing TAE and a voltage (150V) applied for 15 to 20 min. This caused the DNA to move out of the agarose gel slice into the TAE buffer in the dialysis bag. The DNA in the TAE was precipitated by addition of 10% (50µl) 3M sodium acetate (pH 5.2) and two volumes of ethanol (at -20°C) and incubation for 1 hour at -20°C . The incubation tube was centrifuged for 15 minutes at 10,000g and the supernatant discarded. The pellet was re-suspended in 10µl of TE buffer and 1µl of this solution was run on a gel to check that the DNA had been recovered. The DNA was stored at -20°C until used.

2.3vib *Labelling of probe*

Probes were labelled using random oligonucleotides and Exo(-) Klenow (Stratagene, Cambridge, UK). The procedure relies on the ability of random nanomers to anneal to multiple sites along the length of the DNA template and to act as priming sites for the polymerase enzyme. The labelling procedure is described here.

Purified DNA probe (10-15ng) was added to water to a volume of 23 µl and 10µl of random nanomers (27 OD units/ml) was added. The reaction tube was put in a boiling water bath for 5 min and then centrifuged briefly. Primer buffer was then added (10µl)(200mM Tris-HCl (pH 7.5), 100mM $MgCl_2$, 250mM NaCl) followed by [^{32}P]dATP (5µl, 50µCi, 3,000Ci/mmole, ICN) and Exo(-) Klenow (1µl, 5 U). The tube was incubated at 37°C for 10 min and then 2µl of stop mixture (0.5 M EDTA, pH 8.0) was added and the probe kept in ice.

2.3vic *Purification of labelled probe*

For the purification of probe, Sephadex-nick columns (Pharmacia Biotech) were used. This allows separation of labelled probe from unincorporated [^{32}P]dATP which is retained by the column. The column was fixed to a stand and washed with 1 ml of TE buffer. TE buffer (50µl) was added to the [^{32}P]-labelled probe reaction mixture and the reaction mixture was added to the Sephadex column followed by 400µl of TE buffer. The probe was eluted from the column with 500µl lots of TE buffer and collected as 500µl aliquots in tubes. The radioactivity of each fraction was measured using a portable monitor and the first 2 to 3 fractions with high radioactivity were used as probes for Southern hybridisation

2.3vid *Hybridisation*

The hybridisation procedure consists of three steps. These are pre-hybridisation, hybridisation and washing.

During pre-hybridisation the nylon membrane with the fixed DNA was placed in a hybridisation bottle and then 12 ml of pre-hybridisation solution (preheated to 63°C) (appendix 1) was added. The membrane was incubated with rotation in the pre-hybridisation solution for 1 h at 63°C. The labelled, purified probe was heated to 98°C for 5 mins and then quickly chilled

on ice and added to pre-hybridisation solution in the hybridisation bottle without allowing direct contact with the membranes. The membrane was then hybridised overnight at 55°C in a rotating bottle. The hybridisation solution was discarded after incubation and the membrane was washed with 50 ml 2 x SSC + 0.1% SDS solution for 30 minutes at 55°C and another 30 minutes with 50 ml of 1 x SSC + 0.1% SDS at 55°C for 30 min. The membrane was then removed from the hybridisation bottle, wrapped in cling film and exposed to autoradiographic for a period depending on the activity of the membrane.

2.3vii DNA Sequencing

Three different methods were used for DNA-sequencing but each was based on the chain termination method. The principle of the chain termination method of sequencing is that a specific oligonucleotide is allowed to anneal to a single-stranded DNA template and to act as a primer for DNA polymerase activity to generate a second strand. Extension of DNA is blocked, however, by random integration of dideoxynucleotide triphospates (ddNTPs). The ddNTPs create base-specific terminations required for sequencing. Radioactivity is incorporated into the growing DNA strand, providing the ability to detect the sequence by autoradiography. The three methods used were direct sequencing of single-stranded DNA, cycle-sequencing and, laterally, a new method using sequenase and [^{33}P]-labelled ddNTP terminators

2.3viia *Direct sequencing*

For direct sequencing single-stranded DNA was prepared and a sequencing kit (Amersham Life Sciences) was used. For preparation of single strand DNA, a kit from Pharmacia Biotech was used. The principle of this preparation is removal of one strand of DNA by using lambda exonuclease, an enzyme which preferentially digests double strand DNA

starting from a phosporylated 5' end. Non-phosphorylated 5' termini are resistant to exonuclease degradation. For this technique to work the DNA must contain one phosphorylated 5' terminus which was generated during PCR amplification with use of a phosphorylated primer. Primers were phosphorylated using T4 polynucleotide kinase. This enzyme acts to transfer the γ-phosphate of ATP to a 5' terminus of DNA.

Primer phophorylation Primer (250 pmol) was mixed with 3µl of 10×buffer (100mM Tris acetate, 100mM Mg acetate, 500mM K acetate, pH 7.5), 3µl of 10 mM ATP and 20U of T4 polynucleotide kinase. The final volume was adjusted to 30 µl by adding water. The above reaction mixture was incubated for 30 minutes at 37°C. The reaction was stopped by heating the mixture to 65°C for 10 minutes the primer was stored at -20°C until used.

Generation of single-stranded DNA DNA (minimum of 0.5µg of double strand DNA) was mixed with 2.5µl of 10×buffer (0.67M glycine-KOH (pH 9.3), 25mM $MgCl_2$) and 8-10U of lamda exonuclease. The volume was adjusted to 25µl with water. The reaction was carried out at 37°C for 20 minutes. After 20 minutes an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1) was added to the sample and vortexed for 30 seconds followed by centrifugation at 10,000g to separate two phases. The upper aqueous layer of the phenol-extracted sample was removed and the single stranded DNA precipitated using 3M sodium acetate (pH 5.2) and ethanol.

Sequencing reaction Single stranded DNA (approx 0.5pmol) and sequencing primer (5-10pmol) were mixed in a total volume of 10µl, heated to 100°C and then rapidly cooled. At the same time four termination tubes were prepared containing one ddNTP (8µM) and the other three dNTPs (80µM) in a total volume of 2.5µl. To the DNA mixture 2µl reaction buffer (200mM Tris-HCl (pH 7.5), 100mM $MgCl_2$, 250mM NaCl), 1µl dithiothreitol (DTT) (100mM), 5µCi [^{35}S]dATP (0.5µl) and 2µl sequenase DNA polymerase in a total volume of

17.5µl. From this reaction mix 3.5µl was added to each termination tube and incubated for 10 min at 37°C. The reactions were stopped by addition of 4µl stop solution (95%formamide , 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) and heated to 72°C for 2 min before loading on a sequencing gel.

2.3viib *Cycle sequencing*

Cycle sequencing combines the principle of PCR with that of sequencing. Tubes are set-up largely as they would be for PCR except that ddNTP is included. For each template four tubes are set-up with one ddNTP in each tube. The tubes are subject to a normal PCR cycling protocol and, according to the principle above, ddNTP terminates extensions on the growing DNA strand. The tubes are then heated to denature the double-stranded DNA and the cycle is repeated. Using this method sequence can be obtained from relatively low amounts of template DNA.

Sequencing reaction For cycle sequencing of DNA, the Exo-*Pfu* DNA sequencing kit (Stratagene) was used. Template for sequencing was prepared by RT-PCR and the bands were electroeluted and precipitated as described above (probe preparation). For each template four termination tubes were prepared and 3µl of the appropriate ddNTP (1.5 mM) was added to each tube. The following reaction components were then prepared on ice and mixed thoroughly: about 200 pmol of template, 1 pmol of primer, 4 µl of sequencing buffer (200mM Tris-HCl, 100mM KCl, 20mM $MgSO_4$, 1% Triton, 1mg/ml BSA, 20µM dATP, 50µM dCTP, 50µM dGTP and 50µM dTTP), 10µCi of [^{35}S]dATP, 2.5 U of Exo-*Pfu* DNA polymerase, 4µl of DMSO and water up to 30µl. An aliquot (7µl) of this reaction mixture was then added to each of the termination tubes and the reaction mix overlayed with oil. The tubes were placed in a thermal reactor and heated to 95°C for 5 min followed by 30 cycles of 95°C for 30 secs, 55-60°C for 30 secs and 72°C for 1 min. At the end of reaction, 5µl of stop solution (80%

formamide, 50mM Tris-HCl. 1mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) was added to each tube. The samples were heated to 80°C for 2-5 minutes before loading onto a sequencing gel.

2.3viic *Sequencing using [^{33}P]-labelled ddNTP terminators*

Towards the end of this project a new variation of the direct sequencing method became available which was used on the later sequencing of the HDL-receptor (SR-BI) cDNA. The principle is the same as that for direct sequencing except that instead of incorporating [^{35}S]-labelled dNTP into the growing DNA strand in order to visualise the product the radioactivity ([^{33}P]) is incorporated within the dideoxyterminators. Otherwise the protocol is very similar to that described above.

Sequencing reaction A reaction mix was set up containing 2µl buffer (260mM Tris-HCl, pH 9.5, 65mM $MgCl_2$), 25-250fmol DNA, 0.5-2.5pmol primer, 2µl thermo sequenase polymerase and water to a total volume of 20µl. Termination tubes were prepared by the addition of [α-^{33}P]ddNTP (one for each tube) and a mix of 7.5µM dNTP. To each termination tube 4.3µl of the reaction mix was added and tubes were placed in a thermal cycler and cycled at conditions described above (2.3viib). Stop solution (4µl) (95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol)was added, samples were heated to 70^{0}C for 2 min before loading on sequencing gel.

2.3viid *Discussion of different sequencing methods*

All three methods provided good sequencing infomation although it was clear that the labelled terminator method gave the most consistent data and provided the longest runs. It is likely that this is because only those reactions that terminate correctly with addition of a ddNTP will be seen on the sequencing gel. It is unfortunate that this technique was not

available until the later parts of the project. Direct sequencing with single stranded DNA also gave good results at times although it was more variable. This could be caused by a number of reasons including inefficient phosphorylation of primers or unwanted digestion of both strands of the DNA by the lamda exonuclease which can digest from non-phosphorylated ends although less efficiently than from phosphorylated ends. Cycle sequencing was usually successful although the quality of the sequence was variable and run lengths were not as good as those with labelled dideoxyterminators.

2.3viie *Preparation of polyacrylamide sequencing gel*

A 40% acrylamide stock solution was first prepared by mixing 38 g of acrylamide, 2 g of NN bis acrylamide and 60 ml of water. The mixture was heated to 37°C, mixed with a stir bar until dissolved and then stored at 4°C. To prepare the polyacrylamide gel solution15 ml of 5× TBE, 18 ml of water and 36 g of urea were mixed with heating until dissolved and 15 ml of acrylamide stock solution was added. Two glass plates from the gel apparatus were cleaned thoroughly with ethanol and fixed with gum tape to prevent leaks when pouring the gel. The gap between the plates was about 0.3 mm. One of the glass plate was siliconised with "Sigmacote" to prevent adhesion of the gel on subsequent dismantling. Once the glass plates were ready the gel solution was mixed with 750μg of ammonium persulphate (Sigma) (dissolved in 750 μl of water) and 22.5μl of TEMED (Sigma). The solution was immediately poured into the space between the two glass plates without allowing bubbles to form. The gel was then allowed to polymerise for at least 1 hour with a comb inserted in the upper boarder. Once polymerisation was completed the gel was mounted in the gel apparatus.

The gel was pre-run for at least 1 hour to warm the gel before loading samples. Voltage and current were adjusted to achieve a power output of 70W. Reaction mixture (2-5μl) was loaded and the gel was run for up to 6 h. The gel apparatus was then dismantled and the

siliconised glass plate was removed. The gel was then fixed in 10% acetic acid and 10% methanol solution for about 10-15 minutes, dried at 80°C for 3 hours in a vacuum gel drier and then exposed to autoradiographic film.

Chapter 3

Changes in granulosa cell steroidogenesis during luteinization *in vitro* and *in vivo*

3.1 Introduction

The purpose of this thesis was to look at changes in expression of specific receptor mRNAs during luteinization *in vivo* and *in vitro*. To examine changes *in vitro* a granulosa cell culture system was utilised in which cells luteinize in culture. In this Chapter the granulosa cell culture system used is described and changes in granulosa cell steroidogenesis are reported.

3.1i Granulosa cell culture

There have been numerous reports on granulosa cells in culture. This is partly because the cells are very easy to isolate from ovaries of most species, not requiring collagenase or other treatment to separate the cells from the basement membrane or extracellular matrix. In addition, granulosa cells are popular for cell culture because they will remain active *in vitro* and in many cases will undergo luteinization in culture. This latter phenomenon can be a problem for the study of granulosa cells *per se* but it is very useful for the study of corpus luteum development.

Early studies by Channing and co-workers described morphological and functional changes in granulosa cells during luteinization in culture (Channing, 1966; 1969a,b,c; Channing & Grieves, 1969; Channing, 1970a,b; Van Thiel *et al*, 1971). In this early work cells from mare, rhesus monkey, human and pig were described while others examined luteinization in bovine granulosa cells (Crillo *et al*; 1969). These studies showed that granulosa cells from large follicles would luteinize spontaneously in culture while cells from small or medium sized follicles could be induced to luteinize using LH and FSH. Luteinization in this case was measured by an increased progesterone synthesis accompanied by an enlargement of cell size and appearance of lipid droplets in the cell cytoplasm. It was later shown that, in the bovine, luteinization is also associated with a loss of P450arom enzyme activity both *in vivo* and

in vitro (Henderson & Swanston, 1978; Henderson & Moon, 1979; Kuran 1995; Luck *et al*, 1990; Skinner & Osteen, 1988; Meidan *et al*, 1990; Saumande, 1991) and this is a useful marker of luteinization in this species.

All of the early studies on granulosa cell function and morphology in culture used serum (5-20%) in the culture medium to aid cell attachment, growth and function. The potentially deleterious effects of serum on granulosa cell function in culture became an issue in the late 1970s and early 1980s. It was shown at this time that serum would act to inhibit the action of FSH on rat granulosa cell function and would inhibit granulosa cell steroidogenesis (Orly *et al*, 1980; Erickson *et al*, 1983; Lowsky & Farooki, 1989). More recently it has also been shown that serum would inhibit oxytocin secretion by luteinized granulosa cells in culture (Luck 1989; Luck *et al*, 1990). The effects of serum on steroid production by bovine granulosa cells in culture is more complex. Serum appears to increase progesterone synthesis by granulosa cells from large follicles (Roberts & Echternkamp, 1994) but it is likely that this is, at least partly, related to the lipoproteins present in serum which will act to stimulate progesterone production through an increase in cholesterol substrate (O'Shaughnessy *et al*, 1990). Another group, in contrast, has shown that serum will inhibit granulosa cell steroidogenesis in culture, particularly after several days in culture (Luck *et al*, 1990). This illustrates an additional problem of serum that the contents are undefined which may lead to inconsistent results and casts doubts on observed effects in culture which may be due to interactions with unknown serum factors.

The problems with serum led to the development of serum-free culture systems, first in rats and more recently in cattle. In rat granulosa cells use of serum-free conditions allowed demonstration that FSH acts on cultured granulosa cells to increase LH-receptor expression (Erickson *et al*, 1983). In bovine granulosa cell cultures most studies which have attempted to use serum-free conditions have used serum to coat the culture dishes or have used serum in the

medium for the first few hours of culture (Luck *et al*, 1990; Langhout *et al*, 1991; Wrathall & Knight, 1993; Spicer *et al*, 1993; Gong *et al*, 1994; Roberts & Echternkamp, 1994). This is to increase the attachment of the cells to the culture dishes. This approach has been criticised (Gutierrez *et al*, 1997) because cells will be exposed to serum components and the technique may introduce unspecified contaminants into the culture. Recently, and after most work on this thesis was completed, a completely serum-free culture system was reported (Gutierrez *et al*, 1997) in which responsiveness to FSH, aromatase activity and morphological characteristics of the cells were maintained. From this work it appears that one of the more important factors in determining subsequent granulosa cell activity in culture is the size of the follicle used with medium (4-8mm) and small follicles (<4mm) preferred.

3.1ii Changes in granulosa cell function in vivo and in vitro during luteinization

The most noticeable functional change induced by luteinization is the marked increase in progesterone production which occurs both *in vivo* and *in vitro*. Studies *in vivo* have shown that this increase in steroidogenesis is associated with an increase in activity and mRNA expression of the steroidogenic enzymes P450scc, 3βHSD (Rodgers *et al*, 1987; Couet *et al*, 1990) with a decrease in activity and expression of the enzymes P450c17 and P450arom (Henderson & Moon, 1979; Rodgers *et al*, 1987). Studies *in vitro* have also seen a loss of P450arom (Henderson & Swanston, 1978; Luck *et al*, 1990; Kuran *et al*, 1995) and an increase in P450scc (O'Shaughnessy *et al*, 1990) in bovine granulosa after luteinization in culture. As discussed in Chapter 1 the other marked changes which occur in granulosa function during luteinization are an increase in oxytocin production and a decrease in inhibin production. These changes are also seen in granulosa cells which luteinize *in vitro* (Henderson & Franchimont, 1981; 1983; Luck *et al*, 1990).

Thus, a number of previous studies have suggested that granulosa cells luteinize in culture and that the characteristics of this (increased progesterone and oxytocin production and decreased oestradiol production) are very similar to changes which occur *in vivo*. It was our intention to use granulosa cells luteinizing in culture to examine changes in expression of cell-surface receptors but as a preliminary we wished to establish further the similarity between luteinization *in vivo* and *in vitro*. In this study changes in expression of mRNA encoding the key steroidogenic enzymes P450scc and P450arom have been measured during granulosa cell luteinization in culture and corpus luteum formation *in vivo*. In addition, since SF-1 has been established as a key regulator of steroidogenic enzyme expression we have examined SF-1 expression in cultured granulosa cells and in corpora lutea.

3.2 Materials and Methods

3.2i Cells and tissues

Granulosa cells were obtained from antral follicles of ovaries collected at a local abattoir as described in Chapter 2. Corpora lutea from animals on days 1, 3, 5 and 7 of the luteal phase were collected as described in Chapter 2. In addition, mid-cycle corpora lutea, as determined by the method of Ireland *et al* (1980) were obtained from a local abattoir.

3.2ii Granulosa cell culture

Cells were cultured as described in Chapter 2. The culture vessels used were initially round culture plates (45 mm diameter) (Sterilin, Staffs, UK) and later plastic culture flasks (Nunclon, Life Technologies Ltd, UK) with a growth area of 25cm^2.

3.2iii Measurement of cell function

Cells were initially cultured under basal conditions (ie without dbcAMP addition) in the presence or absence of serum. After 18h of culture dbcAMP (1mM) was added to the stimulated group. Cell numbers, progesterone production, and mRNA levels were measured as described in Chapter 2

3.2iv Statistics

Results were analysed by analysis of variance using a computer running GraphPad Prism software. Further differences between means was assessed using the Neuman Keul test. Statistical analysis was carried out with the assistance of Prof P.J. O'Shaughnessy

3.3 Results

3.3i Development of cell culture system

3.3ia *Cell attachment*

In initial experiments the effect of culturing cells on serum-coated or non-coated culture dishes was tested. Cells in DMEM/F12 culture medium were added to control culture dishes or dishes which had been coated in serum as described in Chapter 2. In the absence of serum coating we were unable to detect any cells in culture after 24 h. In the presence of serum-coating granulosa cells flattened out to form a monolayer of cells with a typical rounded morphology containing discreet granules within the cytoplasm and apparent lipid inclusions.

3.3ib *Effect of serum on cell proliferation and function*

Since serum appeared to be required for cell attachment the effect of serum in the culture medium on cell proliferation and cell function was tested. All cells were cultured on serum-coated plates and one group was cultured in DMEM/F12 culture medium alone while the other group had 10% bovine calf serum (BCS) added to the culture medium. After 24 h in culture dbcAMP was added to half the plates in each group. The effect of serum and dbcAMP on cell number is shown in Fig. 3.1A while progesterone production is shown in Fig. 3.1B and cholesterol side-chain cleavage activity in Fig. 3.1C. Results in Fig. 3.2 show similar data from a second experiment.

Cell numbers It is clear from both experiments that in the absence of serum in the culture medium there was little or no change in cell number over the 6 day course of the experiment. In the presence of 10% serum there was a 3 fold increase in cell number between days 2 and 4 with little change at day 6 (Fig 3.1A). Addition of dbcAMP had no affect on cell number in the absence of serum but appeared to cause a slight, but not significant, inhibition of cell growth in the presence of serum. A similar pattern of cell proliferation was seen in the second experiment although the effects of dbcAMP were more marked and were significant (Fig. 3.2A).

Progesterone production Basal progesterone production was low over the first 24h in culture then showed a marked increase between 2 and 4 days in culture (Fig 3.1B). Addition of dbcAMP caused a marked increase in basal progesterone production at 2 days and less dramatic increases at 4 and 6 days. In the presence of 10% serum progesterone production was similar to that seen in basal cultures while the addition of dbcAMP caused a very marked increase in progesterone production after 4 and 6 days in culture (Fig. 3.1B). In the second experiment

there was a higher overall level of progesterone production although the pattern of effects of dbcAMP and serum were similar (Fig. 3.2B).

Cholesterol side-chain cleavage activity The activity of the rate-limiting enzyme cholesterol side-chain cleavage was measured using 22R-hydroxycholesterol and measuring progesterone production. In the absence of serum and dbcAMP there was a steady rise in cholesterol side-chain cleavage activity in culture (Fig 3.1C). The presence of dbcAMP in the culture medium accentuated the increase in enzyme activity, particularly during the first 2 days of culture. In contrast, serum inhibited cholesterol side-chain cleavage activity both in the presence and in the absence of dbcAMP. Again results in the second experiment were similar although enzyme activity was higher (Fig 3.2C).

3.3ii Culture conditions for harvesting granulosa cell RNA

From these preliminary experiments conditions were chosen to culture granulosa cells for the harvest of RNA before and after luteinization. Cells were cultured in the absence of serum and in the presence of dbcAMP to accelerate luteinization. The cells were grown in culture plates and cells were harvested at 2 hours, 1 day, 2 days, 3 days and 5 days. Figure 3.3 shows combined progesterone production data from three experiments in which cells were cultured for RNA extraction. Cell numbers were not measured in these experiments and data have been normalised by expressing progesterone production as a percentage of the highest level of production. It is clear that there was a marked increase in progesterone production between 24 and 48 hours which ties in with data described above.

3.3iii Detection of P450scc and P450arom by RT-PCR

Using primers described in Chapter 2 and PCR conditions described there both P450scc and P450arom were detectable by RT-PCR in freshly isolated granulosa cells (Figs. 3.4 and 3.5). In granulosa cells in culture P450scc continued to be detectable by RT-PCR (Fig. 3.4) while P450arom became undetectable after day 1 (Fig 3.5) A similar pattern of expression was observed in developing corpora lutea (see below).

3.3iv Detection of SF-1 by RT-PCR

Using primers described in Chapter 2 SF-1 could be detected barely in agarose gels by ethidium bromide staining. To increase sensitivity Southern blots were used (Fig 3.6). Results show bands of the correct size for SF-1 (305bp) in granulosa cells in culture (Fig 3.6A) and in luteal tissue (Fig 3.6B). In culture there appeared to be a slight increase in SF-1 mRNA levels after 2h and this was maintained up to 2 days. There was no apparent difference, however, in intensity of bands in granulosa cells compared to luteal tissue (Fig 3.6B). In addition to bands of the correct size for SF-1 additional, a higher molecular weight band was also seen, particularly in cultured cells. Because of the apparent low levels of SF-1 mRNA expression no quantitative studies were done.

3.3v Semiquantitative measurement of mRNA by RT-PCR

To measure changes in levels of mRNA encoding P450scc and P450arom a semi-quantitative RT-PCR method was developed based on previous work in this laboratory and others (Murphy *et al*, 1990; O'Shaughnessy & Murphy, 1993; O'Shaughnessy & Mannan, 1994; O'Shaughnessy & Gray, 1995). Figure 3.7A shows changes in incorporation of [^{32}P] into P450scc PCR product with increasing cycle number. It is clear that over cycles 21 to 24 there

was an exponential increase in levels of P450scc and GAPDH cDNA being generated by the PCR. After 24 cycles the rate of increase in GAPDH PCR product started to slow and by 27 cycles a clear plateau phase had been reached. Levels of P450scc continued to increase with cycle number but started to slow after 27 cycles. To show that this method can be used to determine relative amounts of PCR product PCRs were set up with differing amounts of substrate. As can be seen from Fig 3.7B the relationship between the levels of starting substrate was maintained after 21 to 27 cycles. It is clear, however, from Fig. 3.7 that levels of P450scc could only be reliably measured during the exponential phase of amplification as the relationship between P450scc PCR product and GAPDH PCR product changes after the plateau phase started. In this study levels of P450scc and P450arom (and, subsequently, HBP and SR-BI (HDL-receptor)) were measured relative to GAPDH during the exponential phase. To ensure that measurements were made during the exponential phase variations of the experiment shown in Fig. 3.7A were carried out for every substrate measured.

3.3vi Changes in P450scc and P450arom during luteinization in vivo and in vitro

In cultured granulosa cells levels of P450scc mRNA showed a marked decline initially in culture and then began to rise again after day 1 and showed a fairly marked increase between days 3 and 5 (Fig 3.8A). *In vivo* there was a rapid increase in P450scc levels on day 1 of the luteal phase followed by an apparent decline between day 1 and day 5 (Fig 3.8B).

In contrast to changes in P450scc, levels of P450arom mRNA decreased very rapidly in culture to become undetectable by day 1 (Fig 3.9A). There was a similar rapid decline *in vivo* although P450arom remained detectable at low levels in the developing corpora lutea (Fig 3.9B).

3.4 Discussion

Results described in this chapter describe work designed to set up a method for the study of granulosa cell luteinization *in vitro*. In addition the basic changes in steroidogenic enzyme mRNA levels taking place during granulosa cell luteinization *in vitro* have been compared with changes taking place *in vivo*.

In the first series of experiments to look at granulosa cells in culture it was clear that the cells would not attach to tissue culture plastic in significant numbers unless the culture plates were serum-coated. Others have reported that granulosa cells can be cultured without serum coating (Gutierrez *et al*, 1997). The reason for the discrepancy with the current results is not clear but in the report by Gutierrez *et al* (1997) cells were recovered from smaller antral follicles (<8mm) which may be related to their ability to attach.

On serum-coated dishes granulosa cells did not show any proliferative behaviour over the period of the culture. This contrasts with the situation when serum (10%) is present in the medium. Under these conditions marked proliferation occurs. This phenomenon has been described before (Gospodarowicz *et al*, 1977; Roberts & Echternkamp, 1994) and is probably due to the presence of growth factors in the serum such as IGF-1 and insulin (Gong *et al*, 1994) and factors such as HDL which have been shown to stimulate granulosa cell proliferation (O'Shaughnessy *et al*, 1990). The high proliferative behaviour in the presence of serum was accompanied by a large output of progesterone by the cultured cells in the presence of dbcAMP. The response was considerably greater than that seen in the absence of serum. Nevertheless, when CSCC was measured using 22ROHC it was clear that in the presence of serum there was a marked decline in enzyme activity. The likely explanation is that the serum contains significant levels of lipoprotein which increase progesterone output even in the presence of reduced CSCC enzyme activity. Previous studies have shown that bovine luteal

cells require an external source of cholesterol for most progesterone production when this production is at a high rate (Pate & Condon, 1982; Savion *et al*, 1982; O'Shaughnessy & Wathes, 1985; Carroll *et al*, 1992; Bao *et al*, 1995). This is discussed further in Chapter 5. So far as CSCC is concerned, however, it shows that there is a reduction in enzyme activity in the presence of serum.

Results from these experiments suggested that luteinization, in the absence of serum in the medium, was occurring between 24 and 48h in culture. To examine changes in granulosa cell function during this period more fully molecular biological techniques were used to measure changes in steroidogenic enzyme mRNA levels. It was also intended to measure oxytocin mRNA levels during this part of the experiment but preliminary studies by Prof O'Shaughnessy indicated that we were unable to amplify bovine oxytocin/neurophysin by RT-PCR. The reason for this is not clear although other workers have indicated a similar problem. It may be related to the sequence of the oxytocin/neurophysin cDNA which has a high G/C content which may inhibit efficient amplification by PCR (Ivell & Richter, 1984)

Results in this chapter show that a semi-quantitative RT-PCR method can be used to measure levels of P450scc and P450arom mRNA relative to a house-keeping gene such as GAPDH. The method is based on previous published methods (Murphy *et al*, 1990; O'Shaughnessy & Murphy, 1993; O'Shaughnessy & Mannan, 1994; O'Shaughnessy & Gray,1995) and is dependent upon measuring levels of an unknown and GAPDH during the exponential phase of amplification during PCR. In these studies GAPDH was used as the housekeeping gene rather than the more commonly used β-actin. This is because it has been indicated, though not published, that levels of β-actin mRNA may change during granulosa cell luteinization (Voss & Fortune, 1993a). A number of other methods of quantitative or semi-quantitative RT-PCR have been described but the one described here has the benefit of being

simple and easily adaptable. The more rigorous approach is to construct an internal standard for each unknown you wish to measure and use this to quantify the unknown (e.g. Feldman *et al*, 1991). This would be extremely time-consuming if you wanted to measure more than one unknown. The approach used here gives reliable information on the relative levels of mRNA species and suffers the only disadvantage that it is relatively time-consuming doing each sample since several tubes, run over different cycles, must be used.

There was a dramatic increase in levels of P450scc mRNA *in vivo* after ovulation. This supports previous data showing an increase in P450scc mRNA and protein after ovulation. (Rodgers *et al*, 1987b; Rodgers et al, 1986). One difference between this study and that of Rodgers *et al* 1987 is that those authors were unable to detect P450scc in granulosa cells from bovine follicles whereas in the current studies P450scc mRNA was readily detectable. This may be due to the methods used. The previous study used Northern blots which are considerably less sensitive than the RT-PCR method used here. Nevertheless it is clear that there is a 3-fold increase in P450scc mRNA (relative to GAPDH) very rapidly after formation of the corpus luteum. This shows that the developing corpus luteum will have a considerably greater potential for steroidogenesis than the antral follicle. There was an apparent decline in P450scc levels after day 1 but this is likely to be due to the increased mass of the developing corpus luteum which will increase levels of GAPDH without necessarily increasing P450scc. During the first ten days of the luteal phase there is a 20 to 30-fold increase in luteal mass in the cow (Zheng *et al*, 1994). If the levels of P450scc mRNA were to be measured per corpus luteum there would then be a further marked increase after day 1. During this period of corpus luteum development tissue levels of progesterone increase by ten fold (Wathes *et al*, 1984 ;Zheng *et al*, 1994).

In contrast to the situation *in vivo* levels of P450scc mRNA decreased in culture during the first 24h before increasing again up to day 5. This is unlikely to be due to a general decline

in cellular mRNA levels as levels of P450scc mRNA were measured relative to GAPDH mRNA. The phenomenon observed, however, is similar to that seen by Voss & Fortune (1993b) who showed that P450scc levels in bovine granulosa cells decreased *in vivo* after the ovulatory LH surge and also decreased in culture over the first 24 h before increasing again around (3 days). This indicates that there is an initial decrease in P450scc mRNA during luteinization followed by a subsequent increase. No decrease was observed *in vivo* because the corpora lutea used were taken after ovulation not after the LH surge and so any decrease in P450scc would have happened previously. The decrease in P450scc mRNA was accompanied by an increase in progesterone production. This is similar to the results described by Voss & Fortune (1993b). This may indicate that there was an increase in other aspects of the luteinized cell function such as cholesterol synthesis. There would also be continued synthesis of P450scc protein and there may be changes in rate of P450mRNA degradation so that levels of P450scc protein may increase. This would have to be measured by Western blotting.

Levels of P450arom mRNA declined rapidly after luteinization *in vivo* and *in vitro*. This has been documented before (Henderson & Swanston, 1978; Henderson & Moon, 1979; Luck *et al*, 1990; O'Shaughnessy, *et al*, 1990; Kuran *et al*, 1995) and loss of P450arom appears to be a reliable indicator of luteinization in bovine granulosa cells. Interestingly, low levels of P450arom mRNA were detectable by RT-PCR in bovine corpora lutea. Since granulosa cells appear to lose enzyme activity completely after luteinization this may be an indication that P450arom is expressed at low levels in another cell type, possibly luteal cells derived from the thecal layer.

Expression of SF1 was very low in all tissues measured. This was surprising because of the known role of SF-1 in regulation of P450scc levels (Ikeda *et al*, 1993; Honda *et al*, 1993).

It would appear, therefore, that SF-1 is not involved in development of expression of P450scc after luteinization and another, as yet unknown, transcription factor must regulate the process.

As described in the introduction there have been a large number of studies into granulosa cell behaviour in culture. In most of the early studies it was clear that the cells in culture were undergoing luteinization as judged by changes in progesterone production. More recently, studies of granulosa cells in culture have been aimed more at maintenance of granulosa cell phenotype and prevention of luteinization (Gutierrez *et al*, 1997). In this study we wanted to induce luteinization and it is clear that under the conditions used luteinization was taking place. This was in the presence or absence of dbcAMP although dbcAMP was present in the medium in subsequent cultures to ensure luteinization. Characteristics of changes in progesterone production and, to a lesser extent, enzyme mRNA levels were similar enough to give confidence that luteinization *in vitro* was a similar phenomenon to luteinization *in vivo.*

Figure 3.1

Cell number (A), progesterone production (B) and cholesterol side-chain cleavage activity (C) in granulosa cells cultured in the presence or absence of serum. Granulosa cells were plated on serum coated dishes in culture medium alone or culture medium plus 10% bovine serum. After 24h culture dbcAMP was added to half the plates and culture continued. Cell number, progesterone production and cholesterol side-chain cleavage activity were measured after 1, 2, 4 and 6 days of culture.

Statistical analysis of data

Data were analysed as a series of single factor or two factor analyses. For each parameter measured changes in basal values and values with serum were measured by single factor analysis followed by the Neuman-Keul test. In the summary below groups with different letter superscripts were significantly different. Within each time point effects of dbcAMP and serum were analysed by two factor analysis. $P<0.05$ was considered significant.

A) Cell number:

Single factor analysis

Changes in basal not significant, there was a significant increase in cell numbers in serum ($1d^a$ vs $2d^a$ vs $4d^b$ vs $6d^b$).

Two factor analysis

At 2 days; no significant difference overall

At 4 days; The effect of serum was significant but both the interaction and effect of dbcAMP were not significant

At 6 days: The effect of serum was significant but both the interaction and effect of dbcAMP were not significant.

B) Progesterone production

Single factor analysis

There was a significant increase in basal production with time ($1d^a$ vs $2d^a$ vs $4d^b$ vs $6d^b$) and in the presence of serum ($1d^a$ vs $2d^a$ vs $4d^b$ vs $6d^b$).

Two factor analysis

At 2 days; the effect of dbcAMP was significant but the effect of serum and the interaction were not significant.

At 4 days; effects of dbcAMP and serum were significant but there was a significant interaction.

At 6 days; effects of dbcAMP and serum were significant but there was a significant interaction

C) Side chain cleavage activity

Single factor analysis

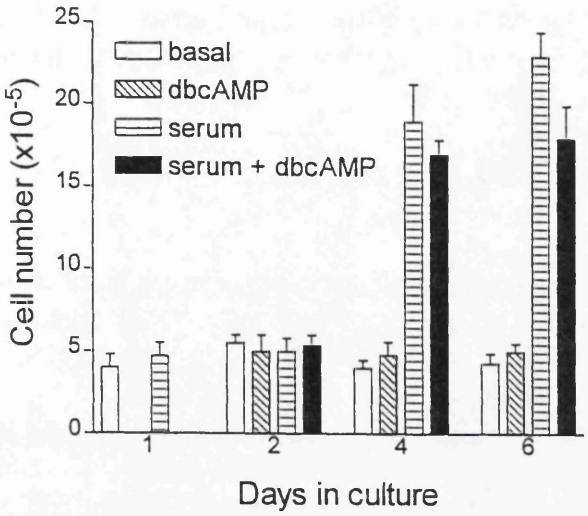
There was a significant increase in basal production with time ($1d^a$ vs $2d^a$ vs $4d^b$ vs $6d^c$) but no change in the presence of serum.

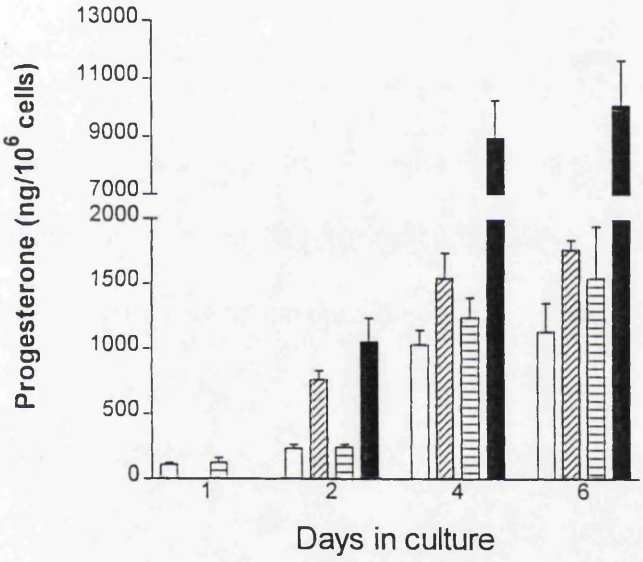
Two factor analysis

At 2 days; the effects of dbcAMP and serum were significant but there was a significant interaction.

At 4 days; the effects of dbcAMP and serum were significant and there was no significant interaction.

At 6 days; the effects of dbcAMP and serum were significant and there was no significant interaction


basal
dbcAMP
serum
serum + dbcAMP
Cell number (x10-5)
25
20
15
10
5
0
1
2
4
6
Days in culture


Progesterone (ng/10^6 cells)
13000
11000
9000
7000
2000
1500
1000
500
0
1
2
4
6
Days in culture

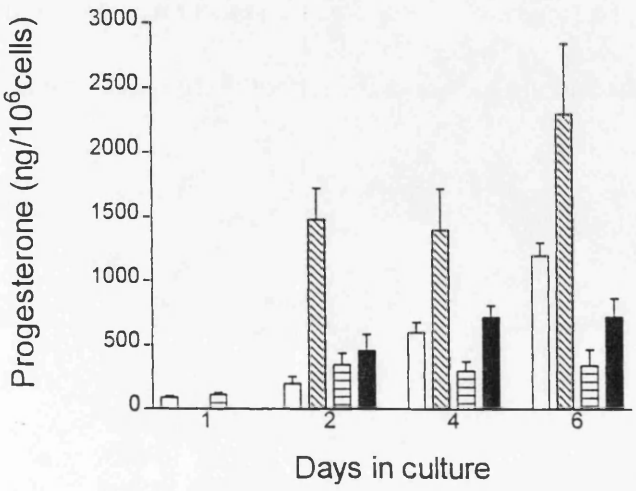

Progesterone (ng/10^6cells)
3000
2500
2000
1500
1000
500
0
1
2
4
6
Days in culture

Figure 3.2

Repeat of experiment described in Fig. 3.1. Cell number (A), progesterone production (B) and cholesterol side-chain cleavage activity (C) in granulosa cells cultured in the presence or absence of serum.

Statistical analysis of data

Data were analysed as described in Fig. 3.1

A) Cell number:

Single factor analysis

Changes in basal not significant, there was a significant increase in cell numbers in serum ($1d^a$ vs $2d^a$ vs $4d^b$ vs $6d^c$).

Two factor analysis

At 2 days; no significant difference overall

At 4 days; The effect of serum was significant but both the interaction and effect of dbcAMP were not significant

At 6 days: The effect of serum was significant but both the interaction and effect of dbcAMP were not significant.

B) Progesterone production

Single factor analysis

There was a significant increase in basal production with time ($1d^a$ vs $2d^b$ vs $4d^b$ vs $6d^b$) and in the presence of serum ($1d^a$ vs $2d^{ab}$ vs $4d^{bc}$ vs $6d^c$).

Two factor analysis

At 2 days; the effect of dbcAMP was significant but the effect of serum and the interaction were not significant.

At 4 days; effects of dbcAMP and serum were significant but there was a significant interaction.

At 6 days; effects of dbcAMP and serum were significant but there was a significant interaction

C) Side chain cleavage activity

Single factor analysis

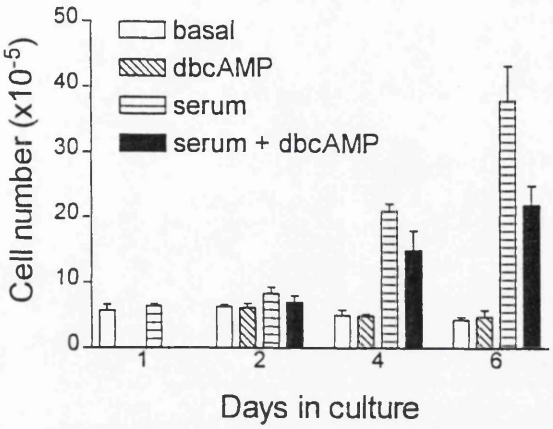
There was a significant increase in basal production with time ($1d^a$ vs $2d^{ab}$ vs $4d^{bc}$ vs $6d^c$) but no change in the presence of serum.

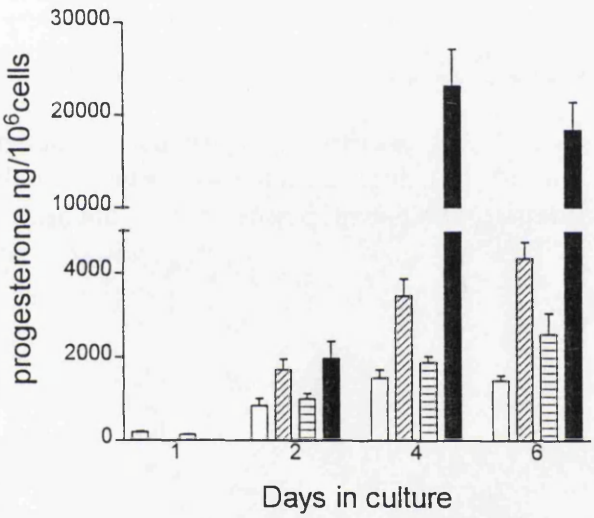
Two factor analysis

At 2 days; the effects of dbcAMP and serum were significant but there was a significant interaction.

At 4 days; the effects of dbcAMP and serum were significant and there was no significant interaction.

At 6 days; the effects of dbcAMP and serum were significant but there was a significant interaction


basal
dbcAMP
serum
serum + dbcAMP
Cell number (x10-5)
50
40
30
20
10
0
1
2
4
6
Days in culture


progesterone ng/10^6cells
30000
20000
10000
4000
2000
0
1
2
4
6
Days in culture

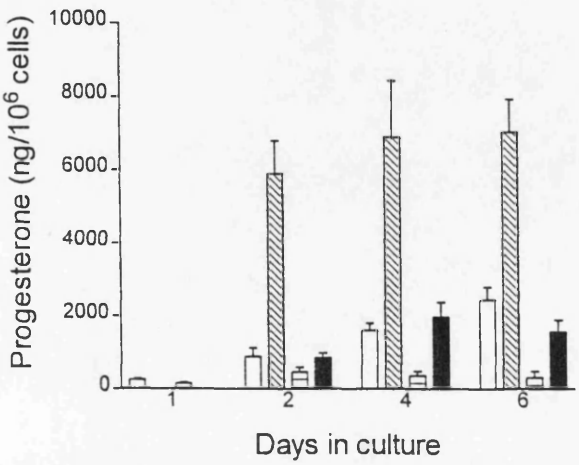

Progesterone (ng/10^6 cells)
10000
8000
6000
4000
2000
0
1
2
4
6
Days in culture

Figure 3.3

Pooled data from three experiments showing progesterone production (per hour) by granulosa cells cultured to recover mRNA. Cell numbers were not measured in this series of experiments and so to normalise data results between experiments data are expressed as a percentage of the maximum production. Analysis of variance showed a significant difference between groups ($1d^a$ vs $2d^b$ vs $3d^c$ vs $5d^c$)

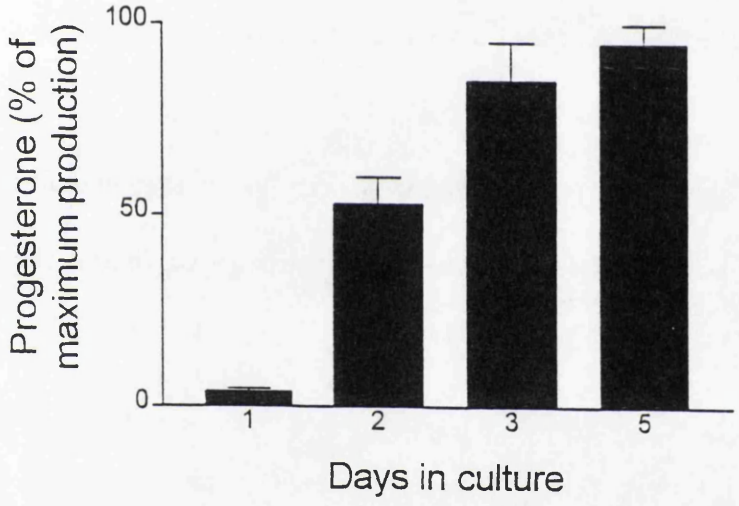
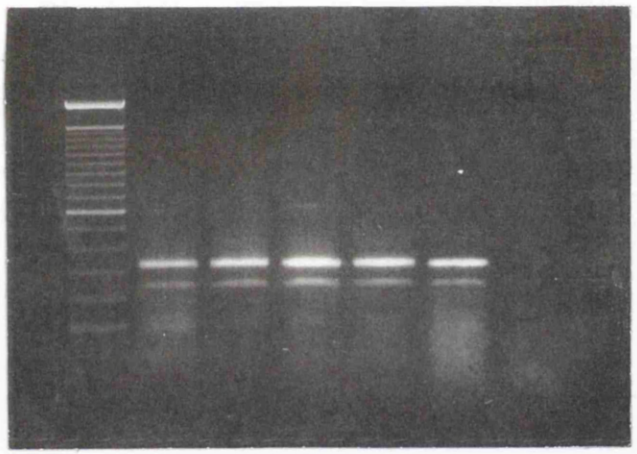

Progesterone (% of maximum production)
100
50
0
1
2
3
5
Days in culture

Figure 3.4

Agarose gel showing P450scc cDNA following PCR.

Expression of P450scc in newly isolated granulosa cells (g.c.) and in granulosa cells in culture.

The left lane shows a 100bp ladder and the last lane (-) is a negative control. The expected size of the P450scc PCR product is 321bp.



g.c. 1 2 3 5 -

Days in culture

Figure 3.5

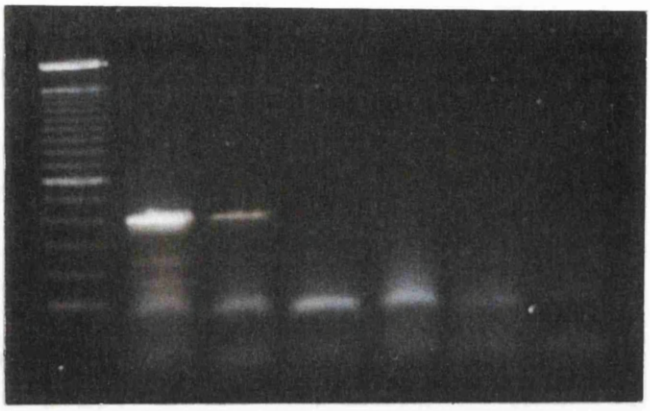
Agarose gel showing P450arom and GAPDH cDNA following PCR.

A) Expression of P450arom in newly isolated granulosa cells (g.c.) and in granulosa cells in culture.

The left lane shows a 100bp ladder and the last lane (-) is a negative control. The expected size of the P450arom PCR product is 399bp.

B) Expression of GAPDH in freshly isolated granulosa (g.c.) cells and in granulosa cells in culture. The last lane (-) is a negative control. The expected size of the GAPDH PCR product is 300bp.

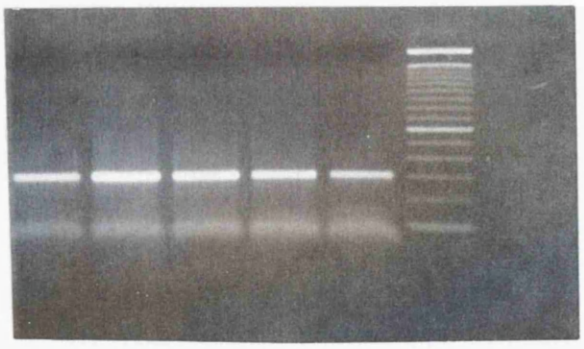
A)



g.c. 1 2 3 5 -

Days in culture

B)



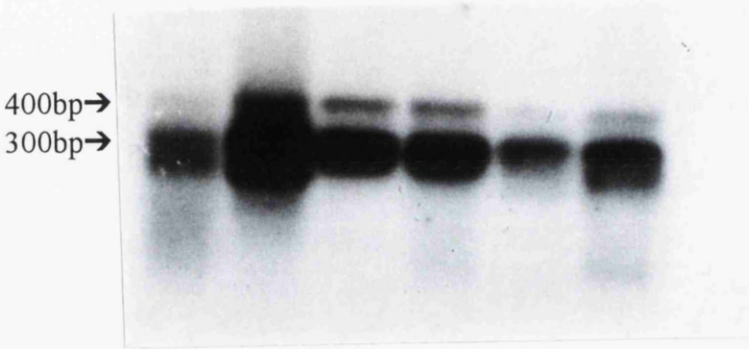
g.c. 1 2 3 5 -

Days in culture

Figure 3.6
Southern blot of RT-PCR products showing SF-1. The expected size of band was 304 bp
A) Expression of SF-1 in freshly isolated granulosa cells (gc) and in granulosa cells i
culture up to 5 days. The identity of the extra band at 400 bp is unknown. The lane marke
-ve is a negative control without cDNA.
B) Expression of SF-1 in granulosa cells and developing corpus luteum.

The expected size of the SF-1 PCR product was 304bp.

A)



B)

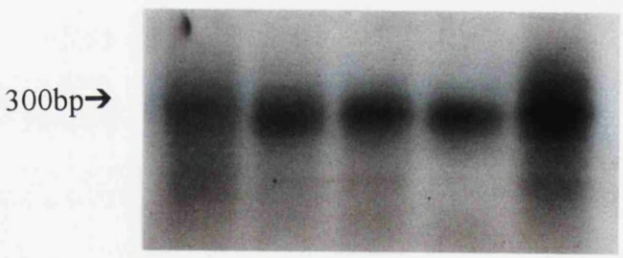
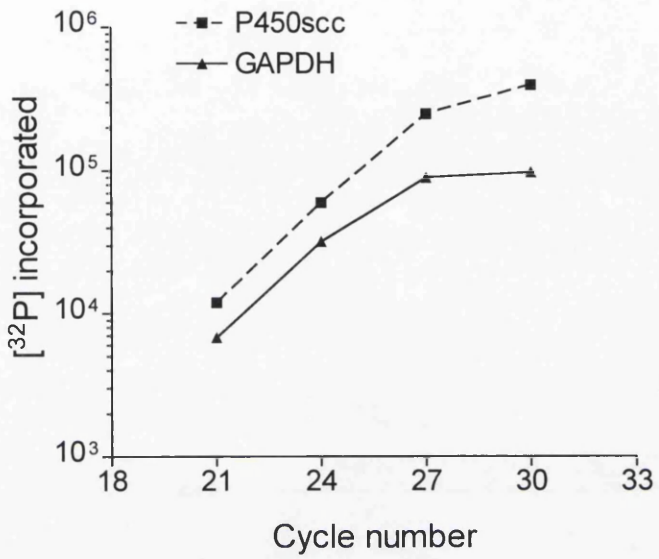


Figure 3.7

Characteristics of PCR quantitative assay. RNA was extracted from a corpus luteum on day 2 and cDNA was amplified as described in Chapter 2 in the presence of [^{32}P]dATP. Radioactivity incorporated was measured by radioactive counting as described.

A) Incorporation of [^{32}P] into PCR products is shown over different cycle numbers for P450scc and GAPDH.

B) Incorporation of [^{32}P] into P450scc PCR products with differing concentrations of starting template used. PCR tubes were set up with x, 2x and 4x amounts of template and amplified over different cycle numbers as shown.


P450scc
GAPDH
[32P] incorporated
10^6
10^5
10^4
10^3
18
21
24
27
30
33
Cycle number

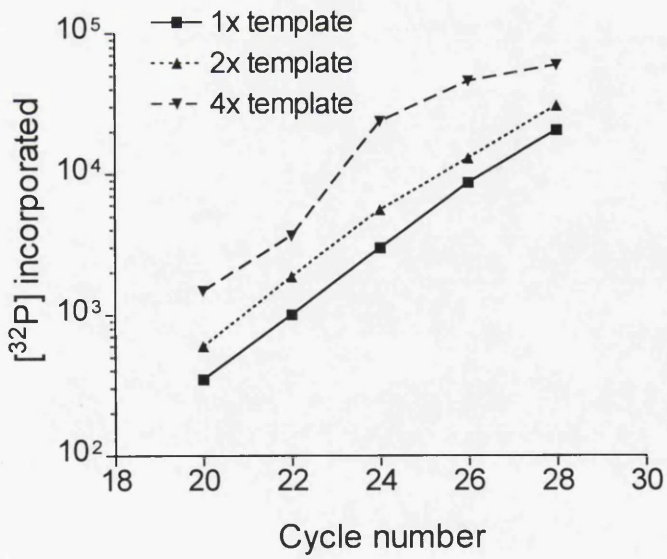
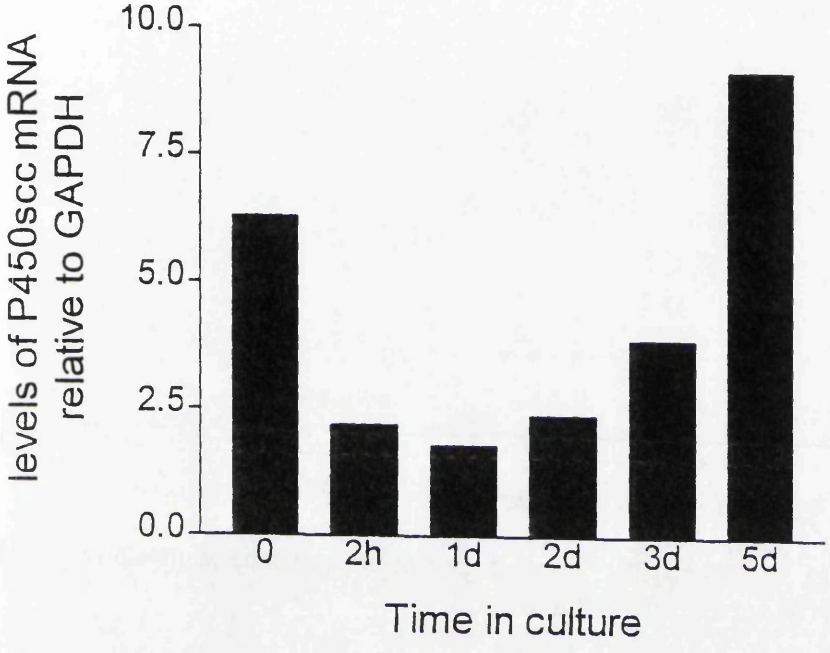

1x template
2x template
4x template
[32P] incorporated
10^5
10^4
10^3
10^2
18
20
22
24
26
28
30
Cycle number

Figure 3.8
Expression of P450scc (relative to GAPDH) by bovine granulosa cells in culture and by bovine luteal tissue at different times after ovulation.

A) Levels of P450scc mRNA in cultured granulosa cells as measured by PCR. Time 0 represents the uncultured granulosa cells.

B) Levels of P450scc in isolated granulosa cells and in luteal tissue up to 5d after ovulation.

A)


levels of P450scc mRNA relative to GAPDH
10.0
7.5
5.0
2.5
0.0
0
2h
1d
2d
3d
5d
Time in culture

B)

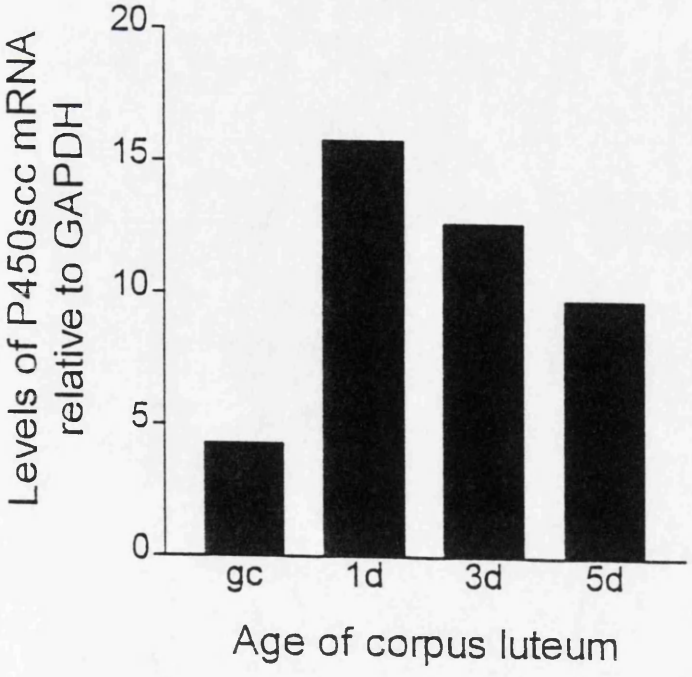
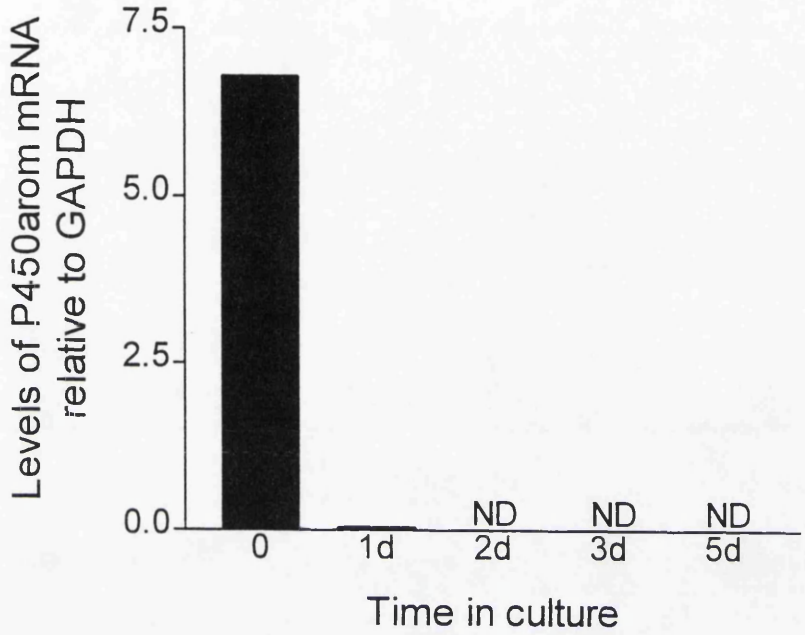

Levels of P450scc mRNA relative to GAPDH
20
15
10
5
0
gc
1d
3d
5d
Age of corpus luteum

Figure 3.9
Expression of P450arom (relative to GAPDH) by bovine granulosa cells in culture and by bovine luteal tissue at different times after ovulation.

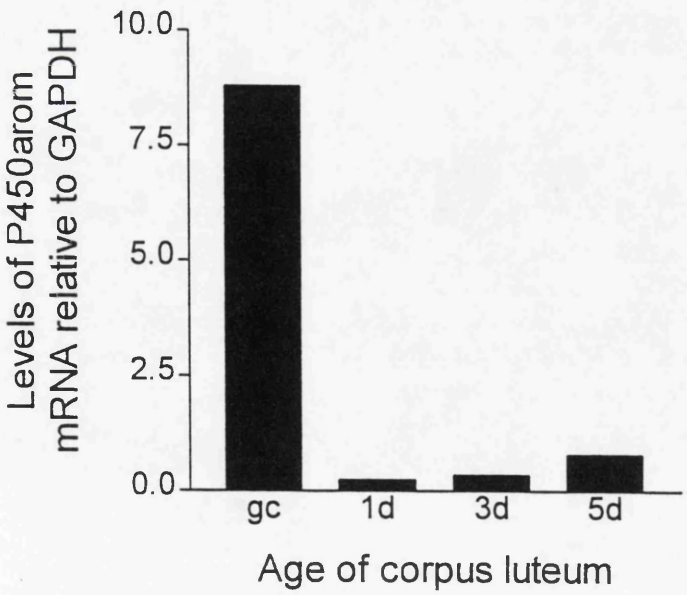
A) Levels of P450arom mRNA in cultured granulosa cells as measured by PCR. Time 0 represents the uncultured granulosa cells.

B) Levels if P450arom in isolated granulosa cells and in luteal tissue up to 5d after ovulation.

A)


Levels of P450arom mRNA relative to GAPDH
7.5
5.0
2.5
0.0
ND
ND
ND
0
1d
2d
3d
5d
Time in culture

B)


Levels of P450arom mRNA relative to GAPDH
10.0
7.5
5.0
2.5
0.0
gc
1d
3d
5d
Age of corpus luteum

Chapter 4

Expression of follicle-stimulating hormone-receptor mRNA during luteinization

4.1 Introduction

Ovarian granulosa cell function is completely dependent upon the action of follicle-stimulating hormone (FSH) during growth from the primary stage until the late antral stage (Goldenberg *et al*, 1972; Richards, 1980; Richards *et al*, 1987). As the mature antral follicle develops under the influence of FSH and oestrogen, granulosa cells express receptors for luteinizing hormone (LH) (Zeleznick *et al*, 1974; Segaloff et al, 1990) and LH released during the LH-surge acts to cause ovulation and follicle luteinization. During this stage of LH-dependence it is uncertain whether the granulosa cells retain FSH-receptor mRNA transcripts and whether there are changes in alternate splicing of the primary transcript as seen during early development of FSH-receptor mRNA expression (O'Shaughnessy *et al*, 1994; Rannikki *et al*, 1995). A bovine FSH-receptor cDNA has been cloned and sequenced (Houde *et al*, 1994) and this has allowed us to examine (i) alternate splicing of the receptor primary transcript in this species and (ii) changes in FSH-receptor expression following luteinization using corpora lutea obtained from cattle at known times of the early luteal phase. In addition, changes in FSH-receptor expression have been measured in bovine granulosa cells undergoing luteinization in culture as described in Chapter 3.

4.2 Materials & Methods

4.2i Animals and Tissues

Luteal tissue was recovered from cows slaughtered at a known stage of the oestrus cycle as described in Chapter 2. Granulosa cells were isolated from bovine ovaries collected from a local abattoir and transported to the laboratory in ice. Medium (4-8mm) and large

(>8mm) follicles with no obvious signs of atresia (Henderson *et al*, 1987) were dissected out and granulosa cells isolated as described in Chapter 2. Harvested cells were either stored in liquid N_2 for subsequent RNA extraction or were used for cell culture.

4.2ii Granulosa cell culture

Isolated granulosa cells were cultured as described in Chapter 3 using serum-free medium with serum-coated dishes and in the presence of dbcAMP (1mM) to ensure luteinization.

4.2iii Reverse transcription and polymerase chain reaction

Total RNA was isolated from cultured cells or luteal tissue, reverse transcribed and the cDNA used for PCR as described in Chapter 2. The position of primers used for PCR amplification of the FSH-receptor are shown in Fig.4.1A.

Blotting and sequencing were as described in Chapter 2. In this part of the study cycle sequencing was used.

4.3 Results

4.3i Alternate splicing of FSH-receptor in bovine ovary

Amplification of bovine granulosa cell cDNA by PCR using FSH-R primers on exons 1 and 10 revealed a number of products which hybridised to an FSH-receptor cDNA probe following Southern blotting (Fig 4.1B). The largest of these products was of the molecular size expected (805bp) from the position of the PCR primers and subsequent sequencing confirmed that it contained the full expected sequence. The smaller bands in Fig. 2 had molecular sizes of about 725, 600, 500, 400 and 300bp. Of these smaller products only those at 500 and 600

bp were consistently observed in all granulosa cell RNA preparations tested. Sequencing of the 500 and 600bp products showed that the 600bp species lacked exon 9 of the full-length FSH-receptor cDNA while the 500bp species lacked exons 4, 5 and 9 (Fig 4.1A).

Using primers on exons 1 and 7 three major amplification products were observed following Southern blotting (Fig 4.2B). The larger product was of the molecular size expected (482bp) from the position of the primers while the other products had molecular sizes of approximately 400 and 325bp.

4.3ii FSH-receptor mRNA expression during luteinization in vivo

Expression of FSH-receptor mRNA in granulosa cells from large antral follicles and in corpora lutea 1, 3, 5, 7 and 10 days after formation is shown in Fig 4.2. Using PCR primers on exons 1 and 10 FSH-receptor mRNA transcripts were present immediately after luteinization but there was a marked decrease in the overall level of hybridisation (Fig 4.2A). After day 1 there was little or no amplification of FSH-receptor transcripts using primers on exons 1 and 10. In contrast, using primers on exons 1 and 7 of the FSH-receptor two major products (325 and 480bp) were clearly present in corpora lutea up to at least day 10 (Fig. 4.2B). In all groups there was clear expression of GAPDH (Fig 4.2C).

4.3iii FSH-receptor mRNA expression during luteinization in vitro

In granulosa cells FSH-receptor transcript expression, measured using primers on exons 1 and 10, became undetectable after 2h in culture (Fig 4.3A). Using primers on exons 1 and 7 to amplify FSH-receptor transcripts in cultured granulosa cells it was clear that the major products at 325 and 500bp continued to be expressed throughout luteinization *in vitro* (Fig 4.3B).

4.4 Discussion

Studies using a number of different species have shown that the FSH-receptor primary transcript undergoes alternate splicing. The structure of the different spliced transcripts varies between species as shown in Figs. 1.1 and 4.1A although transcripts lacking exon 9 are present in 4 out of 6 species studied so far (including dog, McBride unpublished). It is possible that other alternate transcripts may exist in the bovine since any lacking exon 10 (as in the sheep) would not have been amplified in PCR experiments described here. The function of the alternate transcripts in the bovine ovary remains uncertain but they all contain exon 10 and, therefore, the transmembrane domain. The alternate transcripts also contain amino acids 9-30 and 300-310 which have been implicated in binding between FSH and its receptor (Dattatreyamurty & Reichert, 1992; Leng *et al*, 1995; Sharma & Catterall 1995). This means that the translated proteins may function as receptors, possibly with altered affinity for the hormone or with altered response to hormone binding (Braun *et al*, 1995). Recent work in the sheep ovary has identified two alternate transcripts of the FSH-receptor which may have physiological significance (Sairam *et al*, 1996; Sairam *et al*, 1997). One transcript differs at the carboxy terminus and is shorted by 25 bases (Sairam *et al*, 1996) and fails to activate adenylate cyclase. In addition the receptor acts to inhibit the action of FSH and agents such as forskolin and cholera toxin which bypass the receptor. It is proposed, therefore that this is a dominant negative form of the receptor (Sairam *et al*, 1996). The other alternate transcript of the receptor encodes only the first eight exons of the receptor along with a carboxy terminus which contributes a possible single transmembrane domain (Sairam *et al*, 1997). This transcript could act as a possible growth factor receptor in the ovary. It is not known if these transcripts are expressed in the bovine ovary but data in Figs 4.2 and 4.3 show that shortened transcripts are formed lacking exon 10.

In a previous study alternate transcripts of the FSH-receptor were not found in bovine granulosa cells following RT-PCR amplification of the extracellular domain (Houde *et al*, 1994). The reason for this earlier failure to find alternate transcripts is uncertain but may be related to the position of the primers which was not reported in the earlier study.

While LH has been accepted as the main luteotrophin in cattle the possible role of FSH in regulating corpus luteum function in cattle has been unclear. Direct studies have failed to show an effect of FSH on luteal steroidogenesis *in vivo* or *in vitro* (Hansel *et al*, 1973) although Manns *et al* (1984) have reported that the bovine corpus luteum contains specific FSH binding sites throughout the cycle. Results from this study show, however, that full-length FSH-receptor transcripts become negligible in the corpus luteum within two days of its formation. Results from a previous study have shown that the mid-cycle corpus luteum lacks full-length FSH-receptor mRNA indicating that the early loss is maintained throughout the cycle (Houde *et al*, 1994). Studies using granulosa cells in culture show that expression of full-length FSH-receptor mRNA is lost as cells undergo luteinization. This suggests that the loss of expression may be an integral part of luteinzation rather than purely as a result of exposure to surge levels of gonadotrophins around ovulation.

Developmental studies have shown that prior to expression of full-length, FSH-receptor transcripts shortened transcripts appear which would only be expected to code for the extracellular domain of the receptor (O'Shaughnessy *et al*, 1994; Rannikki *et al*, 1995). Studies in this thesis show that as FSH-receptor transcripts encoding the full-length receptor disappear after granulosa cell luteinization transcripts encoding the extracellular domain persist. This raises the possibility that expression of the FSH-receptor gene is constitutive in follicular (pre-granulosa) cells, granulosa cells and granulosa-derived luteal cells but that splicing control mechanisms regulate the presence or absence of the full-length transcript. It may be unlikely

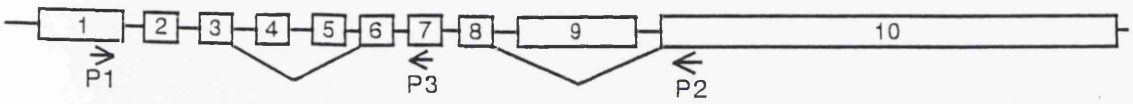
that the shortened transcripts have any function in modulating FSH action since, if translated, the proteins would lack the transmembrane domain. The studies described above in sheep show, however, that these shortened transcripts could have a function which is different from that of transducing effects of FSH (Sairam *et al*, 1997). Studies on the LH-receptor have shown that alternate transcripts lacking exon 11 which encodes the transmembrane and intracellular domains are translated and can bind hormone but do not appear to be released from the cell (Koo *et al*, 1994). It remains to be determined, therefore, whether the shortened transcripts have a role to play in the function of the bovine corpus luteum. The ability of shortened transcripts to bind hormone may, however, account for the presence of FSH-binding sites detected earlier in the corpus luteum (Manns *et al*, 1984).

Figure 4.1

A) FSH-receptor gene structure and primer position in the cow. The positions of primers used in this study are indicated by arrows. The deduced structure of alternate transcripts is indicated by lines joining non-adjacent exons.

B) Southern blot hybridisation of PCR products obtained using FSH-receptor primers on exons 1 and 10. Template for the PCR reactions was obtained by reverse transcription of RNA from granulosa cells of large, medium or small-sized antral follicles. The position of molecular size standards (in bp) is shown. The expected full-length PCR product was 805bp.

A)



B)

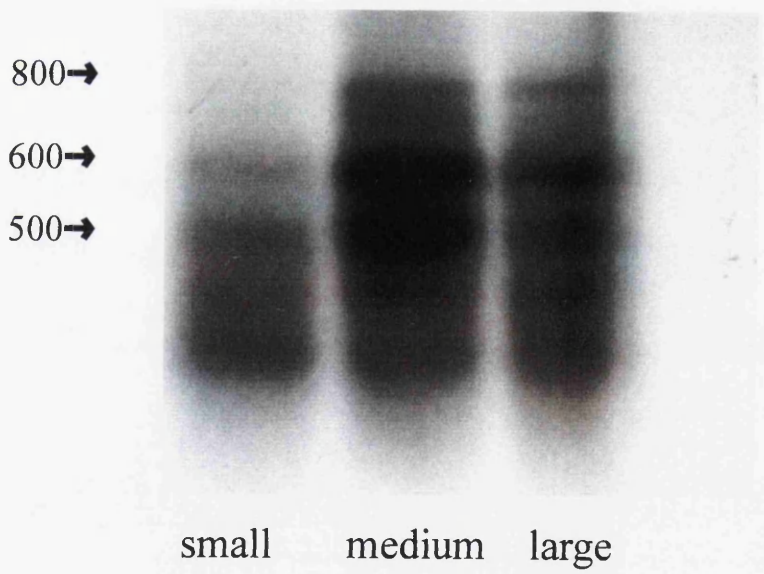


Figure 4.2

PCR amplification of cDNA derived from granulosa cells (g.c.) or corpora lutea of different ages.

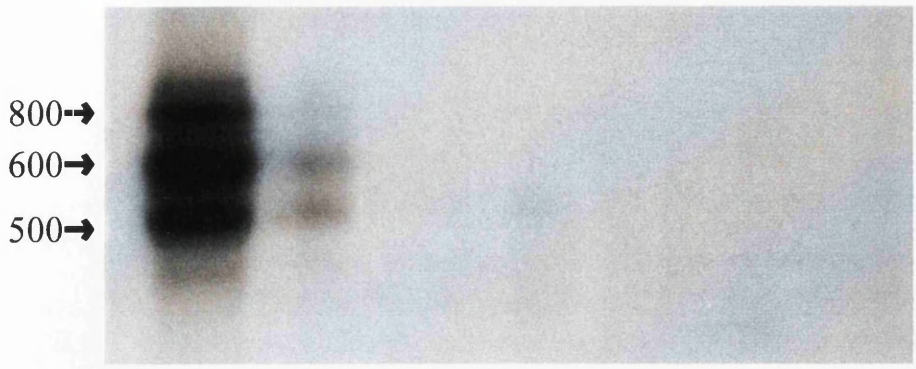
A) Southern blot hybridisation following PCR amplification using FSH-receptor primers on exons 1 and 10. The expected full-length PCR product was 805bp.

B) Southern blot hybridisation following PCR using FSH-receptor primers on exons 1 and 7. The expected full-length PCR product was 482bp.

C) Ethidium bromide staining of PCR products using GAPDH primers. The expected PCR product was 300bp.

The positions of molecular size markers are indicated and numbers under each figure indicate the age (in days) of the corpora lutea used in the experiments.

A)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g.c.
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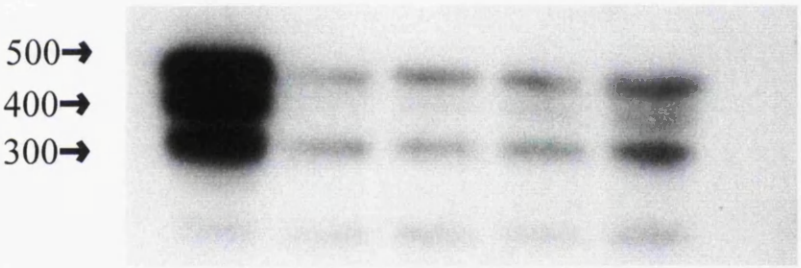
blank

B)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300
g.c.
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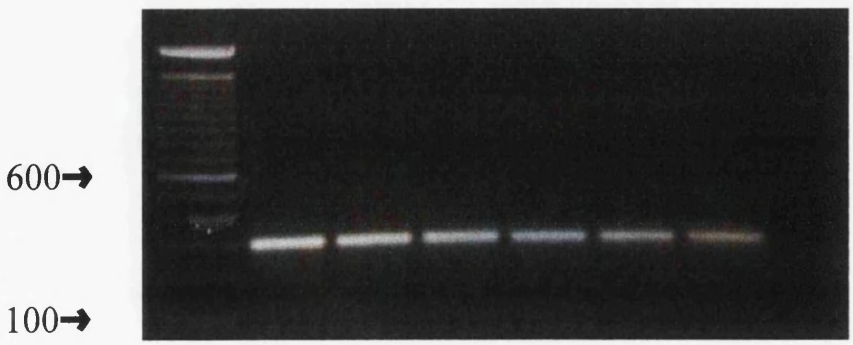
5
7
10
blank

C)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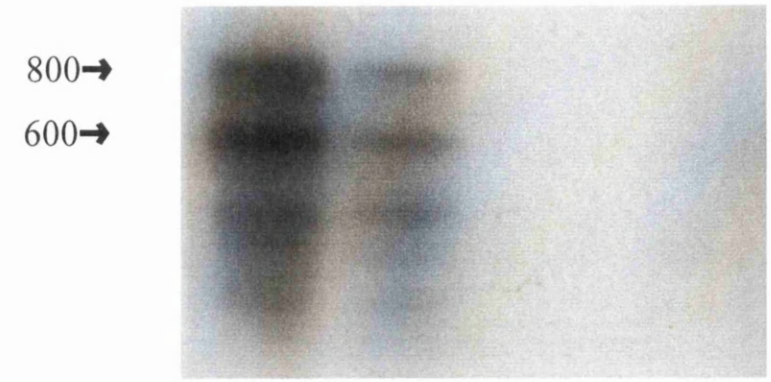
Mw
g.c.
1
3
5
7
10
blank

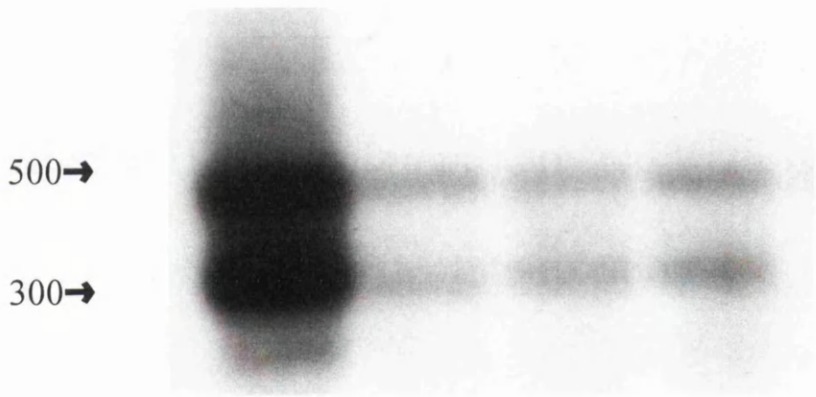
Figure 4.3

PCR amplification of cDNA derived from granulosa cells after different times in culture.
A) Southern bolt hybridisation following PCR amplification using FSH-receptor primers on exons 1 and 10. The expected full-length PCR product was 805bp.
B) Southern bolt hybridisation following PCR amplification using FSH-receptor primers on exons 1 and 7. The expected full-length PCR product was 482bp.
C) Ethidium bromide staining of PCR products using GAPDH primers. The expected PCR product was 300bp.
Numbers below each figure represent days in culture while g.c. indicates non-cultured granulosa cells and 2h represents 2 hours in culture.

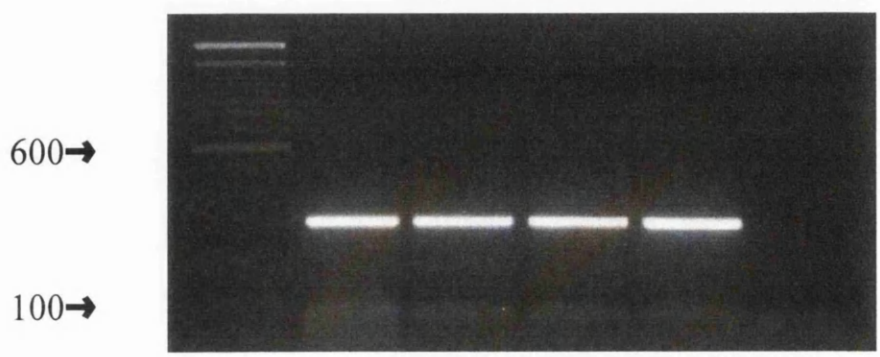
A)



B)



C)



Chapter 5

Expression of HDL-binding protein and HDL-receptor (SR-BI) during luteinization

5.1 Introduction

In most mammalian species progesterone production by the ovary increases markedly after ovulation as the corpus luteum develops. This change in steroidogenic activity is due, largely, to a huge increase in progesterone production by the luteinizing granulosa cells (Carr *et al.*, 1982; O'Shaughnessy *et al.*, 1990; Smith *et al.*, 1994). It has been proposed that the increased progesterone production following ovulation in humans is due to vascularization of the granulosa cell layer making cholesterol substrate available from circulating low-density lipoprotein (LDL) (Carr *et al.*, 1982). Bovine luteal cells, in contrast to the human, can utilise cholesterol from both LDL and high-density lipoprotein (HDL) for progesterone synthesis with HDL more likely to be of importance in maintaining luteal steroidogenesis (Pate & Condon, 1982; Savion *et al.*, 1982; O'Shaughnessy & Wathes, 1985; Carroll *et al.*, 1992; Bao *et al.*, 1995). Concentrations of HDL are relatively high in bovine follicular fluid (Savion *et al.*, 1982; Brantmeier *et al.*, 1987) and granulosa cells are, therefore, exposed to lipoprotein before ovulation. There is, however, little or no response of bovine granulosa cells to HDL before luteinization suggesting that HDL-receptors are at low levels or are absent in the pre-ovulatory follicle (O'Shaughnessy *et al.*, 1990; Bao *et al.*, 1995).

At the start of this work the HDL-binding protein (HBP) described by McKnight *et al* (1992) in the human and by Chen & Menon (1994) in the rat was the only candidate HDL-receptor available. It was decided to isolate and sequence a partial bovine HBP cDNA and to use this to design primers for RT-PCR to measure changes in HBP levels during granulosa cell luteinization *in vivo* and *in vitro*. Towards the end of the work on HBP the class B scavenger receptor SR-BI was identified as an HDL-receptor fulfilling all the physiologically relevant criteria (Acton *et al.*, 1996; Rigotti *et al.*,1996; Wang *et al.*, 1996, see Chapter 1). This was a considerably stronger candidate for the physiological HDL-receptor and it was decided

to measure levels of SR-BI mRNA in granulosa cells and following luteinization *in vivo* and *in vitro*. In order to study SR-BI the bovine receptor mRNA was isolated and sequenced. Measurement of expression of SR-BI mRNA, and to a lesser extent HBP, provided the opportunity to test the hypothesis that granulosa cells are limited in steroidogenesis by lack of HDL-receptors.

5.2 Materials and Methods

5.2i Animals and tissues

Luteal tissue and granulosa cells were collected as described in Chapters 2 and 4. Other bovine tissues were collected from a local abattoir.

5.2ii Granulosa cell culture

Granulosa cells were cultured and allowed to luteinize in culture as described in Chapters 3 and 4.

5.2iii Isolation of bovine HDL-receptor (SR-BI) cDNA by RACE

5.2iiia *General description*

A variation of the rapid amplification of complementary ends (RACE) technique (Frohman *et al.*, 1988)(Clontech Marathon cDNA Amplification Kit, Cambridge Bioscience, UK) was used to isolate bovine HDL-receptor cDNA ends. The RACE technique is a method for isolating 5' and 3' ends of a cDNA and thus allowing sequencing of full-length cDNA (Fig 5.1). It is an alternative to constructing and screening a cDNA library. The technique depends upon knowing a short nucleotide sequence (>30 bp) of the cDNA you wish to amplify in order to design gene-specific primers. In studies described here this nucleotide sequence was

determined by PCR using primers designed on nucleotide sequences conserved between species (described in more detail in Results).

In the adaption of the RACE technique used here mRNA was reversed transcribed using an oligo dT primer with 2 degenerate nucleotide positions at the 3' end (Table. 5.1 reverse transcription primer). These nucleotides position the primer at the start of the poly A^+ tail and eliminate 3' heterogeneity which can occur if only oligo dT is used. Following second strand synthesis adaptors are ligated at the 5' and 3' ends (Fig 5.1 and Table 5.1) . Primers which recognise sequence in the adaptors are then used along with gene-specific primers in PCR reactions which will amplify either 5' or 3' ends. These PCR products can then be sequenced. As a refinement of the technique used here the adaptors were partially single stranded (Fig5.1 and Table 5.1). This means that adaptor ligated cDNA does not contain a binding site for the adaptor primer AP1 (Fig 5.1) which can only be generated during PCR with the gene-specific primer. Non-specific products are reduced because the AP1 binding site will not be created in the general population of cDNA molecules.

5.2iiib *Specific RACE conditions*

Total RNA was isolated from bovine corpora lutea on day 5 of the luteal phase using RNAzol (Biogenesis Ltd, UK) as described in Chapter 2. The poly A^+ fraction was separated using an oligo(dT) cellulose column (Life Technologies Ltd, UK). It is considered important to use poly A^+ for the RACE reaction to increase the yield and reduce background. The Poly A^+ column was washed with loading buffer (50mM Na citrate (pH 7.5), 0.5 LiCl, 1mM EDTA and 0.1% SDS) and the total RNA applied in water. The column was washed again in loading buffer and the effluent added again to the column. Elution buffer (10mM Na citrate (pH 7.5), 1mM EDTA and 0.05%SDS) was added and the RNA collected. This process was repeated

to further enrich the poly A^+ fraction. After this procedure the RNA fraction will contain 70-90% poly A^+ RNA.

In initial studies cDNA was generated by reverse transcription using M-MLV reverse transcriptase (Superscript, Life Technologies Ltd, Paisley, U.K.) and random hexamers as previously described (O'Shaughnessy & Murphy 1993; O'Shaughnessy *et al.*, 1994). A 434bp fragment of the bovine HDL-receptor was amplified by PCR using primers based on the sequences of mouse, human and hamster HDL-receptor (Calvo & Vega, 1993; Acton *et al.*, 1994; Acton *et al.*, 1996). This fragment was sequenced and used to design bovine gene-specific primers for subsequent isolation and sequencing of the full-length receptor cDNA 5' and 3' ends (See in Results).

First-strand cDNA synthesis was carried out using MMLV reverse transcriptase and the modified oligo$(dT)_{30}$ primer (Table 5.1). Approximately 1μg of RNA was added to 1μl primer (10μM), 2μl buffer (250mM Tris-HCl, pH 8.3, 30mM $MgCl_2$, 375 mM KCl), 1μl dNTP, 1μl enzyme (100 units/μl) and water to a total of 10μl. Tubes were incubated at 42^0C for 1 h. Second strand synthesis used all of the product of the reverse transcription reaction and mixed them with 16μl buffer (500mM KCl, 50mM NH_4 sulphate, 25mM $MgCl_2$, 0.75mM β-NAD, 100mM Tris HCl, pH 7.5, 0.25 mg/ml BSA), 1.6 μl dNTPs (10mM), 4μl enzyme mix (DNA polymerase I (6 units/μl), DNA ligase (1.2 units/μl, RNase H (0.25units/μl)) and water to 80μl total volume. The tubes were incubated at 16^0C for 1.5h then 2μl (10 units) of T4 DNA polymerase was added and the tubes incubated for a further 45 min at 16^0C. The DNA was isolated by precipitation following phenol/chloroform extraction. After second-strand synthesis

a partially double-stranded adaptor (Table 5.1) was blunt-end ligated to both ends of the double stranded cDNA. The reaction mix contained 5µl ds cDNA, 2µl adaptor (10µM), 2µl buffer (250mM Tris-HCl, pH 7.8, 50mM $MgCl_2$, 5mM DTT, 5mM ATP and 25% polyethylene glycol) and 1µl T4 DNA ligase in a total volume of 10µl. Reactions were overnight at 16^0C and the product was ready for the RACE reaction.

5.2iv Reverse transcription and polymerase chain reaction

Total RNA was isolated from cultured cells or luteal tissue using RNAzol and reverse transcribed using random hexamers. This cDNA was used as a template for PCR. For semi-quantitative PCR HBP and SR-BI mRNA levels were measured relative to GAPDH mRNA as described in Chapters 2 and 3.

5.2v Southern blotting and sequencing

Southern hybridisation was as described in Chapter 2. All three methods of sequencing described in Chapter 2 were used in this project to sequence the partial HBP and the full-length SR-BI. The use of [^{33}P]-ddNTP towards the end of the sequencing project speeded up considerably the rate at which useful sequence was obtained.

5.2vi Statistics

Results were analysed by analysis of variance followed by the Neuman-Keul test.

5.3 Results

5.3i HDL-binding protein (HBP)

5.3ia *Isolation of HBP cDNA sequence*

A line up of the known sequence of human and rat HDP cDNAs was made (Fig 5.2) and from this sequence two primers were designed based on two regions of homology between the two sequences (boxed in Fig 5.2). These two primers amplified a product of the correct size. A partial sequence of this product was obtained and shown to be very similar to the human and rat sequences apart from a region corresponding to nucleotides 3557 to 3620 of the human sequence. Ignoring this region overall identity with the human sequence was 91% and with the rat sequence it was 90% at the nucleotide level. Including this sequence identity fell to 75% for the human sequence and 73% for the rat sequence. There was a number of "missing" nucleotides which may be due to sequencing error but at this stage we were more interested in confirming the identity of the product and measuring expression levels so that further confirmatory sequencing was not carried out.

5.3ib *Expression of HBP mRNA during luteinization in vivo and in vitro*

Using the underlined upstream primer in Fig 5.2 and the boxed downstream primer (based on human and rat sequences) a single band of the correct size 472bp was obtained (Fig 5.3A). Preliminary studies on granulosa cells and corpora lutea indicated that HBP was expressed in granulosa cells and in all developing corpora lutea (Fig 5.3B).

Semi-quantitative RT-PCR was carried out to measure levels of HBP relative to GAPDH in granulosa cells luteinizing in culture and in developing corpora lutea. As before, incorporation of [^{32}P]dATP into PCR products was measured over a number of cycles (e.g. Fig 5.4A) and it was shown that amplification of GAPDH parallelled that of HBP cDNA.

Levels of HBP increased rapidly in culture and then declined to below starting levels by day 5 (Fig 5.4B). In contrast, in vivo there was a delay of 3 days before levels of HBP increased and this increase was sustained for up to 7 days (Fig 5.4C).

5.3ii HDL-receptor (SR-BI)

5.3iia *Isolation of HDL-receptor cDNA*

In initial studies primers for PCR were designed from the mouse and hamster sequences of SR-BI which were the ones known at the time (Acton *et al*, 1994; Acton *et al*, 1996). The two primers were from an area conserved between the two species and were designed to give a fragment of about 463bp. The primer sequences were:

1) 5'-ACC TTC AAT GAC AAT GAT ACT (bases 466-486 of hamster sequence)

2) 5'-ATT GAT CAT GTT GCA CTG CTC (bases 920-900 of hamster sequence)

These primers were designated as P1 and P2. Choice of the primers was arbitrary within the conserved regions except that the 3'-end of the primers was chosen to coincide with an area coding for an amino acid with no degeneracy in the genetic code. The primers were chosen to amplify an area approximately midway through the known open reading frame (Fig 5.5). A schematic diagram showing the position of the main primers used in the RACE reactions is shown in Fig 5.5

The first pair of primers chosen (P1/P2) amplified a band of the correct size (Fig 5.6A) and sequencing of this product confirmed that it was from the bovine HDL-receptor (Fig 5.6B). This known sequence of the bovine HDL-receptor was then used for the RACE reactions as described in Materials and Methods. Within the determined sequence two primers were

designed for further RACE and these were designated GSP1 (gene specific primer 1) and GSP2. PCR using GSP1 and GSP2 produced a band of the correct size (Fig 5.6A) and these primers were subsequently used for semi-quantitative RT-PCR (see below)

Initial 5' RACE studies using primers AP1 and GSP1 failed to produce a PCR product of the correct size using ethidium bromide staining. A nested PCR reaction was then used with the product of the AP1/GSP1 PCR subjected to a second PCR using AP2 and a primer nested to GSP1. Using this technique a strong band close to the expected size was obtained. This was sequenced and found, unfortunately, to be bovine satellite DNA (Fig 5.7). From the sequence it is clear that this product was amplified because it contains an upstream region with homology to AP2 (Fig 5.7). To avoid this problem arising again a number of measures were taken. Firstly a number of new gene specific primers were designed based on the sequence obtained before and also on extended sequence which was obtained as above with primers designed on regions of homology between human, mouse and hamster sequences. To increase the specificity of the second, nested PCR the product of the first reaction was run on an agarose gel and the area of the gel corresponding to the size of the expected product was cut out and the DNA eluted. Before sequencing any bands obtained by these methods a number of tests were carried out to check that the band was of interest. These included running a PCR with only the AP2 primer present to check for possible contamination with bovine satellite DNA and running a nested PCR using only gene specific primers. If these primers amplified it was a good indication that the isolated band was derived from SR-BI.

Using these techniques a product was finally obtained which contained 106 bp of 5' untranslated region and allowed the sequence of the open reading frame to be determined up to GSP1 (Fig 5.8)..

The 3' end of the SR-BI cDNA was obtained using similar techniques although it was found necessary to extend the known sequence towards the 3' end of the open reading frame using primers designed to be homologous to mouse and hamster sequence (Fig 5.5). The cDNA turned out to have a long 3' untranslated region and this is the likely reason for failure to pick up full-length RACE products using GSP2.

The sequence of the HDL-receptor cDNA, as determined by the RACE reaction products, is shown in Fig. 5.8. The isolated sequence contains a 5' untranslated region of 106 nucleotides, an open reading frame of 1527 nucleotides and a 3' untranslated region of 945 nucleotides before the poly A^+ tail. The translated protein contains 509 amino acids with 76.7, 78.6, 82.9 and 76.4% identity and 83.7, 84.5, 87.3 and 82.9% similarity to hamster, mouse, human and rat sequences (Fig. 5.9) (Calvo *et al.*,1993; Acton *et al.*, 1994; Acton *et al.*, 1996; Mizutani *et al*, 1997). In common with sequences from other species, the bovine HDL-receptor protein contains two hydrophobic regions (Fig. 5.9).

5.3iib *Tissue distribution of HDL-receptor mRNA*

Tissue distribution of HDL-receptor mRNA was determined using RT-PCR and Southern blotting (Fig. 5.10). Highest expression was seen in the adrenal cortex and in the corpus luteum with lower expression in the liver and spleen and no detectable expression in skeletal muscle or cardiac muscle.

5.3iic *HDL-receptor expression in the ovary*

Using RT-PCR and primers GSP1 and GSPP2 HDL-receptor cDNA was detectable by PCR in bovine granulosa cells and corpus luteum (Fig. 5.11A). To determine relative changes in HDL-receptor mRNA levels during luteinization the semi-quantitative RT-PCR method was

used as described above. Results in Fig. 5.11B show one experiment in which HDL-receptor mRNA from a day 5 corpus luteum was measured relative to GAPDH. Amplification was exponential until the later cycles with a similar efficiency for each set of primers. In Fig 5.12A, the accumulated results of several experiments show the changes in HDL-receptor mRNA levels following luteinization *in vivo*. From the data it is clear that there is a marked, 7-fold, increase in HDL-receptor mRNA levels after luteinization. Cultured granulosa cells showed a similar 5-fold increase in HDL-receptor mRNA levels after luteinization *in vitro* (Fig 5.12B).

5.4 Discussion

It is likely that HDL can deliver cholesterol esters to most tissues through a mechanism of lipoprotein internalization and degradation (Steinberg, 1996). In some cells, however, a more selective mechanism occurs whereby the HDL binds to a specific cell-surface receptor allowing cholesterol esters to be delivered to the cell before dissociation of the HDL particle (Pittman & Steinberg, 1984; Steinberg, 1996). This occurs primarily in the liver and the non-placental steroidogenic tissues (testis, ovary, adrenal) in which the cholesterol ester acts as a substrate for steroid synthesis (Gwynne & Strauss, 1982; Murphy *et al*, 1985; Steinberg, 1996).

The first possible specific HDL-receptor to be isolated was HBP (McKnight *et al*, 1992; Chen & Menon, 1994) which has some expected characteristics of an HDL-receptor. For example, over expression of the protein leads to an increase in HDL binding in mammalian cells (McKnight *et al*, 1992) while hCG causes an increase in HBP expression in rat luteal cells (Chen & Menon, 1994). Nevertheless, HBP is expressed in a wide variety of tissues including heart, lung and kidney (Chen & Menon, 1994) which would argue against it being the mediator of a selective mechanism for binding HDL seen in the steroidogenic tissues. At the time of these

studies HBP was the only candidate HDL-receptor available for study and we isolated a partial sequence which was used to study expression in the developing corpus luteum. The isolated sequence showed high homology with rat and human sequences apart from a small area in which there was significant diversity. The significance of this is not known at this time since functional domains of the protein have not yet been established. Overall expression of HBP mRNA was not high but appeared to show an increase after luteinization *in vivo* which would be consistent with the known role of HDL in cattle. In culture there was a rapid increase in HBP mRNA levels, before luteinization would be expected *in vitro*. This may be a response of the cells to culture and the subsequent decline in HBP expression would suggest that luteinization *in vitro* is not associated with an increase in HBP mRNA levels. It is possible that the increase in HBP seen in the developing corpus luteum is due to expression in luteinizing thecal cells and that luteinization of granulosa cells is associated with a decrease in HBP expression. This would agree with studies by Chen & Menon (1994) who observed higher levels of HBP in rat granulosa cells than corpora lutea using *in situ* hybridisation.

More recently, the class B scavenger receptor SR-BI was identified as an HDL-receptor which binds HDL with a high affinity and mediates selective cholesterol uptake by the cell (Acton *et al*, 1996). These and subsequent studies which showed that in SR-BI knockout mice levels of cholesterol in the adrenal fell by 72% indicated that SR-BI was the physiological HDL-receptor which mediated HDL-uptake in steroidogenic tissues. To determine, therefore, whether the increase in progesterone production seen in cattle after luteinization is due to an increase in HDL-receptors we have isolated and sequenced the bovine SR-BI HDL-receptor and have examined changes in receptor expression during luteinization.

The sequence of bovine SR-BI HDL-receptor mRNA codes for a protein of 509 amino acids showing high homology with the HDL-receptor in mouse, rat, hamster and human (Calvo

& Vega, 1993; Acton *et al*, 1994; Acton *et al*, 1996; Mizutani *et al*, 1997). The receptor also shows homology with the CD36 family of cell surface glycoproteins including the presence of two internal hydrophobic domains which may serve as membrane-spanning domains in the receptor (Vega *et al*, 1991; Acton *et al*, 1994). Distribution of the SR-BI HDL-receptor in the cow was similar to that described for the rat and mouse with high expression in the adrenal and corpus luteum which is consistent with a role in steroidogenesis (Acton *et al*, 1996; Landschulz *et al*, 1996).

The increased steroidogenic capacity of granulosa cells following luteinization is caused by a number of changes in the cells including increased activity of the steroidogenic enzymes P450 cholesterol side-chain cleavage (P450scc) and 3β-hydroxysteroid dehydrogenase (3βHSD) and increased 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in *de novo* cholesterol biosynthesis (Rodgers *et al*, 1987a; Rodgers *et al*, 1987b; Couet *et al*, 1990; O'Shaughnessy *et al*, 1990). Despite these changes, luteal cells, of most species, show a marked decrease in steroidogenesis when deprived of lipoproteins while inhibition of HMG-CoA reductase has little effect on steroidogenesis in the presence of lipoproteins (McNamara *et al*, 1981; Carr *et al*, 1982; Strauss *et al*, 1982; O'Shaughnessy & Wathes, 1985; McLean & Miller, 1986; Carroll *et al*, 1992; Bao *et al*, 1995). Thus, luteal cells of most species are unable to produce sufficient cholesterol for steroidogenesis through *de novo* synthesis and require cholesterol esters provided by circulating lipoproteins.

In humans, luteal cells utilise LDL, rather than HDL, and increased steroidogenesis after luteinization appears to be related to availability of LDL through the proliferating capillary network as much as to an increase in LDL receptor number (Carr *et al*, 1982; Rodgers *et al*, 1987b). In contrast, in most species so far studied, including cattle, sheep and rodents, HDL appears to be the major lipoprotein involved in maintenance of luteal steroidogenesis

(McNamara *et al*, 1981; Pate & Condon, 1982; Strauss *et al*, 1982; McLean & Miller, 1986; Carroll *et al*, 1992; Bao *et al*, 1995), while rabbits probably utilise both HDL and LDL (Wiltbank *et al*, 1990). Unlike LDL, HDL can enter the follicular fluid and granulosa cells of these species are exposed to lipoprotein prior to luteinization (Savion *et al*, 1982; Brantmeier *et al*, 1987). Bovine granulosa cells, however, show little or no response to HDL prior to luteinization (O'Shaughnessy *et al*, 1990; Bao *et al*, 1995) which is consistent with the low levels HDL-receptor mRNA seen in these cells. After luteinization, there is a clear increase in HDL-receptor mRNA expression which is seen both *in vivo* and *in vitro*. This is supported by *in situ* and immunolocalization studies in the rat ovary which show that the corpus luteum and, to a lesser extent, the theca are the main sites of HDL-receptor expression in this tissue (Landschulz *et al*, 1996; Mizutani *et al*, 1997). Changes in HDL-receptor expression after ovulation may be an integral part of granulosa cell luteinization or may be induced in response to the increased utilization of cholesterol by the luteinized cells (Rigotti *et al*, 1996). Overall, it is likely that the increase in HDL-receptor expression, linked to changes in activity of P450scc and 3βHSD, will explain the significant increase in progesterone production seen in the developing corpus luteum.

Table 5.1

Primers and adaptors used in Marathon RACE

Name	Sequence
reverse transcription primer	5'- TTCTAGAATTCAGCGGCCGA$(T)_{30}N_1N$-3' (N_1 is G or A: N is G, A, C or T)
cDNA Adaptor	5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3' 3'-CCCGTCCA-5'
Adaptor primer (AP1)	5'-CCATCCTAATACGACTCACTATAGGGC-3'
Nested adaptor primer (AP2)	5'-ACTCACTATAGGGCTCGAGCGGC-3'

Figure 5.1

Outline of the RACE technique for isolation of full-length cDNAs.
A) Annealing of the cDNA synthesis primer (Table 5.1)
B) First strand cDNA synthesis
C) Second strand cDNA synthesis
D) Adaptor ligation using adaptors described in Table 5.1

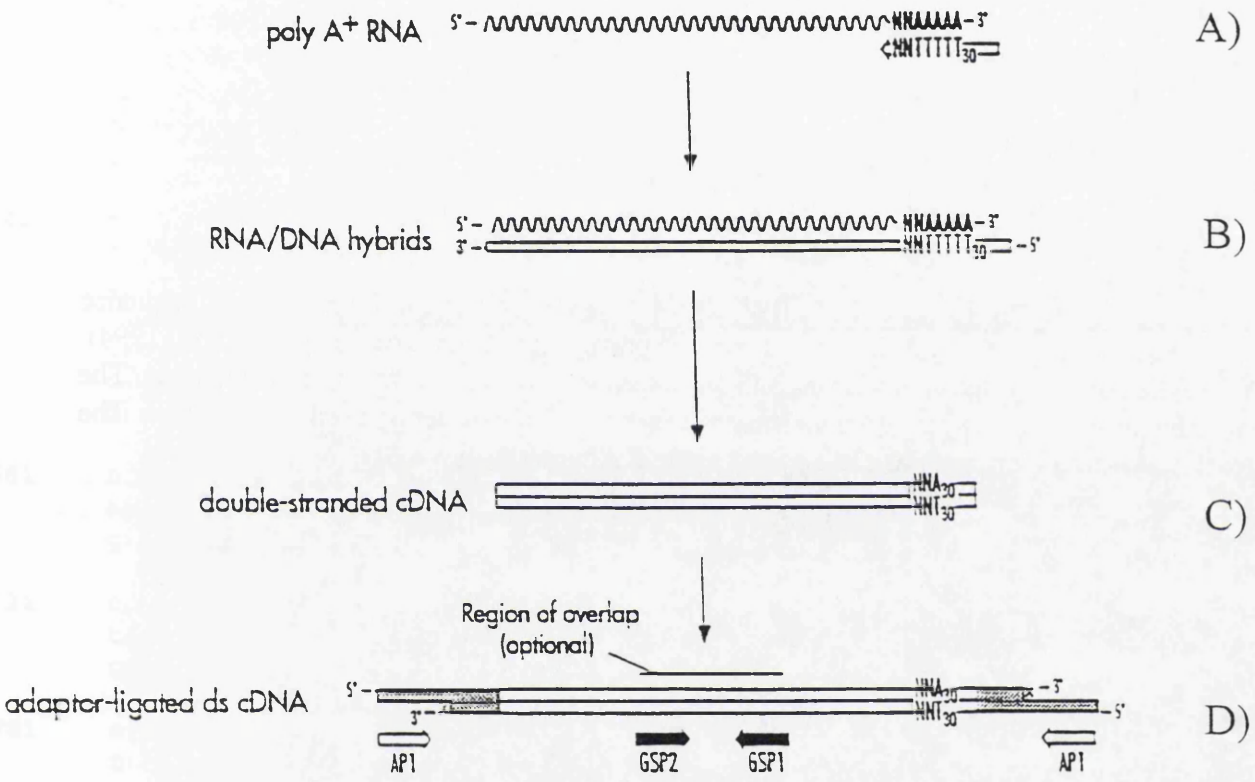

poly A+ RNA
A)
RNA/DNA hybrids
B)
double-stranded cDNA
C)
Region of overlap
(optional)
adaptor-ligated ds cDNA
AP1
GSP2
GSP1
AP1
D)

Figure 5.2

Human and rat sequences of HBP cDNA taken from the full-length human sequence described by McKnight *et al* (1992) and the partial rat sequence of Chen & Menon (1994). The boxed regions are the area from which primers were taken for initial sequencing. The underlined bovine sequence is the upstream primer subsequently used for RT-PCR. The downstream primer was the original sequencing primer (boxed).

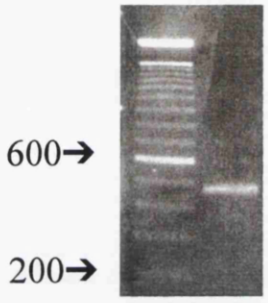
```
                    3318↘
human   tttaaggagt tttaagctga gtgtcactgt agaccccaaa taccatccca agattatcgg
rat                    tga gtgtcactgt agaccccaaa taccatccca aaattatcgg
bovine

3361    gagaaagggg gcagtaatta cccaaatccg gttggagcat gacgtgaaca tccagtttcc
        gagaaaaggg gcagtgatca cacagatccg gttggagcat gacgtgaaca tccaatttcc
                  ggtgatca cccagatccg cctggagcat gacgtgaaca tccagtttcc

3421    tgataaggac gatgggaacc agccccagga ccaaattacc atcacagggt acgaaaagaa
        tgataaggac gatgggaatc agccccagga ccaaatcacc atcacagggt atgagaagaa
        tgagaaggac gatgggagcc agccc.agga ccaaatcacc atcacaggct acgagaagaa

3481    cacagaagct gccagggatg ctatactgag aattgtgggt gaacttgagc agatggtttc
        cactgaagct gcccgtgatg ctatcctgaa aattgtgggt gagcttgagc agatggtttc
        cacagaggct gcccggg... ccatcctgaa gatcgtgggt gagcttgaac agatggtttc

3541    tgaggacgtc ccgctggacc accgcgttca cgcccgcatc attggtgccc gcggcaaagc
        tgaggatgtt cccttggact accgtgtgca tgcccgaatc attggtgcca gaggcaaagc
        tgaggacgtc cgctagacac gcgcctgaga ggatacctgg ccgacgtggt ggacagcgag

3601    cattcgcaaa atcatggacg aattcaaggt ggacattcgc ttcccacaga gcggagcccc
        catcaggaaa atcatggatg agttcaaggt ggacattcgc ttcccacaga gcggagctcc
        gcactgcagc tacctgaagc aattgaaggt cgacattccc agcccac... gaggagctcc

3661    agaccccaac tgcgtcactg tgacggggct cccagagaat gtggaggaag ccatcgacca
        agaccctaac tgcgtcactg tgacaggact cccggagaat gtggaggaag ctatcgacca
        gccacccaac tgcgtgac.g tgacggggct cccagaga.c gtgga.gaag ccatcgacca

3721    catcctcaat ctggaggagg aatacctagc tgacgtggtg gacagtgagg cgctgcaggt
        tatcctcaac ctagaagagg aatatctggc tgatgtggtg gacagtgagg cgctgcaagt
        catcctcaac ctggaggagg .atacctggc cgacggg

3781    atacatgaaa cccccagcac acgaagaggc caaggcacct tccagaggct ttgtggtgcg
        ttacatgaga cccccagcac atgaagagtc caaggcacca tccaaaggct ttgtggtgcg


3841    ggacgcaccc tggaccgcca gcagcagtga gaaggctcct gacatgagca gctctgagga
        agatgctccc tggacctcca acagcagtga gaaggctcct gacatgagca gct
```

Figure 5.3

Amplification of HBP by PCR. A) Amplification of day 5 corpus luteum cDNA by PCR using primers designed to amplify HBP. A 100bp ladder is shown on the left-hand side. The single product was of the expected size 472bp.

B) Amplification of cDNA from different ages of corpus luteum using primers designed to amplify HBP. The age of the corpora lutea is shown in days while lane 2 contained cDNA from granulosa cells (gc). Lane 1 contained a 100bp ladder. The mid-cycle corpus luteum is from an abattoir specimen. The last lane is a negative control.

A)



B)

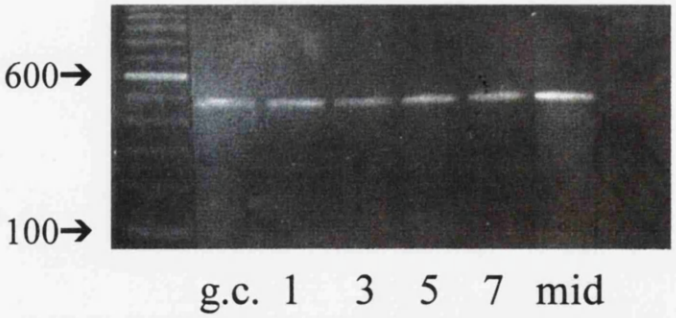
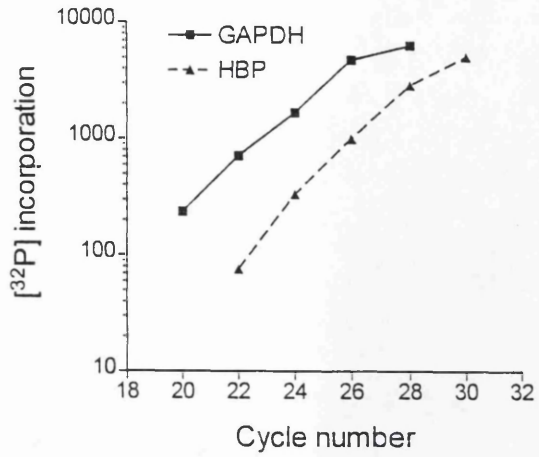


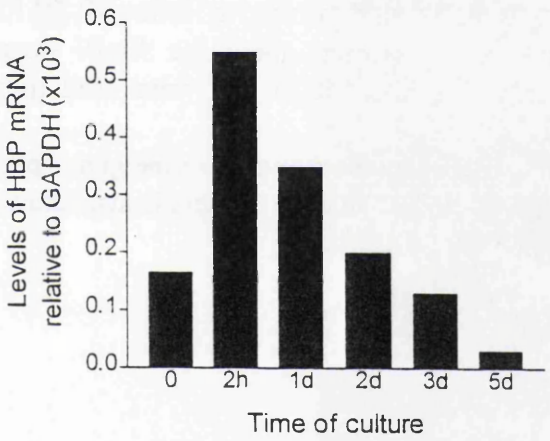
Figure 5.4

Measurement of HBP mRNA expression in developing corpus luteum and in granulosa cells luteinizing in culture.
A) Semi-quantitative measurement of bovine HBP mRNA in fresh granulosa cells using RT-PCR. Tubes were amplified for different numbers of cycles as indicated and then bands cut out and incorporated [^{32}P] measured as described in Material & Methods. Primers used were designed to amplify HBP or GAPDH.
B) Results of semi-quantitative RT-PCR showing levels of HBP mRNA in fresh granulosa cells and in granulosa cells cultured for up to 5 days. Levels of HBP are expressed relative to GAPDH.
C) Results of semi-quantitative RT-PCR showing levels of HBP mRNA in granulosa cells and in developing corpora lutea.

A)


10000
1000
100
10
GAPDH
HBP
[32P] incorporation
18
20
22
24
26
28
30
32
Cycle number

B)


0.6
0.5
0.4
0.3
0.2
0.1
0.0
Levels of HBP mRNA relative to GAPDH (x10³)
0
2h
1d
2d
3d
5d
Time of culture

C)

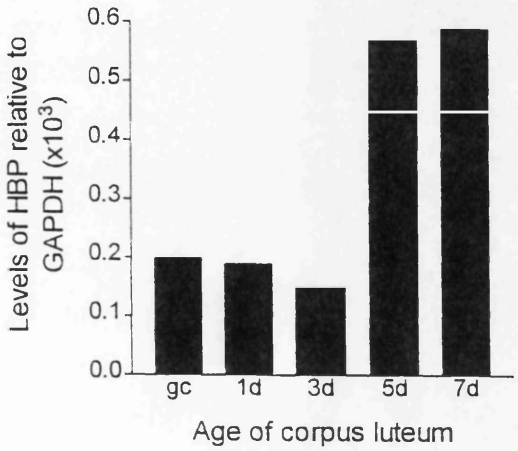

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Levels of HBP relative to GAPDH (x10³)
gc
1d
3d
5d
7d
Age of corpus luteum

Figure 5.5

Schematic diagram of RACE technique used to isolate SR-BI full-length cDNA. Primers are indicated by arrows. The primers above the SR-BI sequence were based on the sequence of hamster and mouse SR-BI and were used to generate bovine-specific sequence for RACE.

The primers below the SR-BI sequence were bovine gene-specific and were used in the RACE reactions. The position of adaptor primers is also shown.

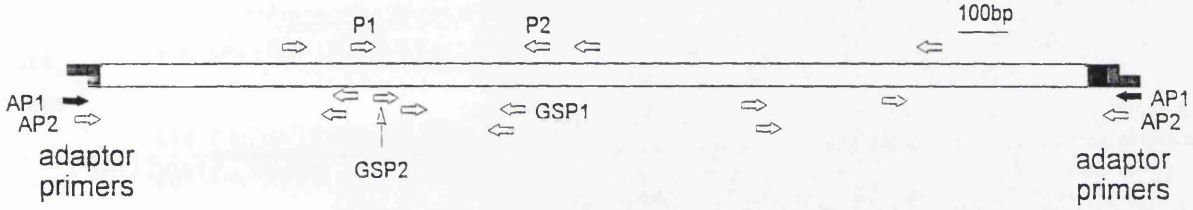
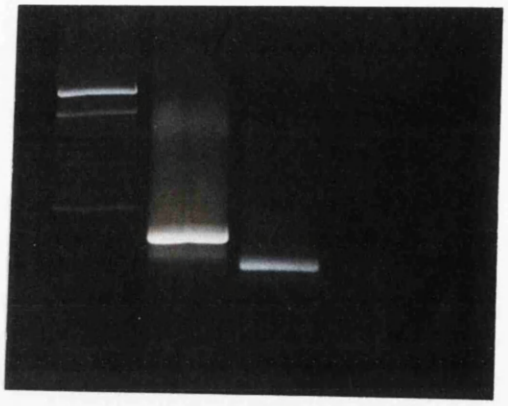

P1
P2
100bp
AP1
AP2
adaptor
primers
GSP1
GSP2
AP1
AP2
adaptor
primers

Figure 5.6

A) Amplification of bovine corpus luteum cDNA using primers P1 and P2 (A) and GSP1 and GSP2 (B). Lane one contained a 100bp ladder.

B) Sequence obtained after sequencing PCR product using P1 and P2 and comparison to mouse and hamster sequences. Primers P1 and P2 are underlined in the hamster sequence. Bovine nucleotides not determined from this sequence are marked with dots.

A)



A B

B)

```
Hamster     460 ACATCACCTTCAATGACAATGATACTGTGTCCTTTGTGGAGCACCGCAGC 509
                ||||| |||||||||||||||| || ||||||||| ||||||| |||||||||
Mouse       355 ACATCACCTTCAATGACAACGACACCGTGTCCTTCGTGGAGAACCGCAGC 404
Bovine                                  ......................

            510 CTCCATTTCCAGCCGGACAGGTCCCACGGCTCTGAGAGTGACTACATTAT 559
                |||||||||||||| |||| ||| || ||||| ||||||||||||||| |
            405 CTCCATTTCCAGCCTGACAAGTCGCATGGCTCAGAGAGTGACTACATTGT 454
                 ||||||| || ||||| |  |||   ||||| |||||||| ||
                .......TCCAGCCGGATAAGTCCCGCGGCCAGGAGAGCGACTACATCGT

            560 ACTGCCTAACATTCTGGTCTTGGGGGGCGCAGTAATGATGGAGAGCAAGT 609
                |||||||||||  ||||| |||||||| |  || ||||||||||||||
            455 ACTGCCTAACATCTTGGTCCTGGGGGGCTCGATATTGATGGAGAGCAAGC 504
                 |||| |||||| ||||| || | |  || ||  ||||||||| || ||
                CATGCCCAACATCCTGGTCTTGAGTGCGTCAATGATGATGGAGAACAGGC

            610 CTGCAGGCCTGAAGCTGATGATGACCTTGGGGCTGGCCACCTTGGGCCAG 659
                |||   |||||||||||||||||||||||| ||||| |||| ||||||||
            505 CTGTGAGCCTGAAGCTGATGATGACCTTGGCGCTGGTCACCATGGGCCAG 554
                | | |  | |||||||||||||||||||||||| |   |||  | |||||||
                CCGGGCTCTTGAAGCTGATGATGACCTTGGCGTTCAGCACGCTTGGCCAG

            660 CGTGCCTTTATGAACCGAACAGTTGGTGAGATCCTGTGGGGCTATGAGGA 709
                ||||| |||||||||||| |||||||||||||||||||||||||||||| ||
            555 CGTGCTTTTATGAACCGCACAGTTGGTGAGATCCTGTGGGGCTATGACGA 604
                ||||| || |||||||||||| ||||| |||||| |||||||||||||| ||
                CGTGCCTTCATGAACCGCACTGTTGGCGAGATCATGTGGGGCTATGATGA

            710 TCCCTTCGTGAATTTTATCAACAAATACTTACCAGACATGTTCCCCATCA 759
                |||||||||| ||||| |||||| ||| | ||||||||| | ||||| |
            605 TCCCTTCGTGCATTTTCTCAACACGTACCTCCCAGACATGCTTCCCATAA 654
                |||||  |  ||  |  ||||  |||| ||||| ||  ||| ||| | |
                CCCCTTAATCCACCTCATCAATCAGTACTTCCCAAACTCGCTCCCCTTCA

            760 AGGGCAAGTTCGGCCTGTTTGTTGAGATGAACAACTCAGACTCTGGGCTC 809
                ||||||| || |||||||||||||| |||||||||||  | |||||| ||
            655 AGGGCAAATTTGGCCTGTTTGTTGGGATGAACAACTCGAATTCTGGGGTC 704
                ||||||| || ||  | |||| || | | || |||||  |||||||| ||
                AGGGCAAGTTCGGCTTATTTGCTGAGCTCAACAACTCCGATTCTGGCCTC

            810 TTCACTGTGTTCACGGGCGTCCAGAACTTCAGCAAGATCCACCTGGTGGA 859
                 |||||||| |||||||||||||||||| ||||||| |||||| ||||||||
            705 TTCACTGTCTTCACGGGCGTCCAGAATTTCAGCAGGATCCATCTGGTGGA 754
                 |||||  || ||||| || ||| | || |||||||||||||||| |||||
                TTCACCGTGTTCACAGGGGTCAAAAACTTCAGCAGGATCCATCTCGTGGA

            860 CAGATGGAATGGGCTCAGCAAGGTCAACTACTGGCATTCAGAGCAGTGCA 909
                || |||||| || |||||||| || | || |||||||||||||||| |
            755 CAAATGGAACGGACTCAGCAAGATCGATTATTGGCATTCAGAGCAGTGTA 804
                |||  |||| |||
                CAAGTGGAATGGA.....................................

            910 ACATGATCAATGGCACTTCCGGGCAGATGTG
                |||||||||||| ||||||||||||||||||
            805 ACATGATCAATGGGACTTCCGGGCAGATGTG

                ..............................
```

Figure 5.7
Sequence of bovine testis satellite DNA and a potential binding site for primer AP2. It is likely that the primer annealed to this site and, possibly, to an adaptor at the 3' end of the sequence.

ovine testis satellite DNA

```
      1 ccctcatctc ccgatgacgg gggagtctag gggttgttct cgagcggcgg ccccagtgtg
                                        ||      | |  || ||||||||
AP2 primer                              actca ctatagggct cgagcggc

     61 cggtttctca cgaggtacga cggcgaggtc agtgagcctc tcgtggggcg ccagggaagt

    121 cgggtctcca tgcgagtaac gaggggggagc gcgccattgc tcccgagcca tgggaggaga

    181 atctggcctc gagacgtgtt gaagagggtc tctcgaggtc ttccccgggt gcaggcaggc

    241 agccctgggt ccctcgactt gtgcaggtga cctcaggggg cttctcgtgg tggctctgag

    301 aagccaggga aactggaggt gggaggggcc tctcgggact ccactgggct tggtgcattg

    361 gaagagggcc tcatctccag ttgaggcagg aaccgcaggg tacctctgat ttcagactcc

    421 gatcgcaggg tccctgcaga ctggggacag gagagtcagg cctcgtcttg ggttgaggca

    481 tggaactccg cttgcctctc gagatgtccc cggggagaga ggccgcttgt cgagctgtat

    541 ttggaacctg gggttttttc cgaacggtgc acggagaagc tgccccttcg tgttgactgc

    601 attcacaggg gtggagttcg gagaggtgtc cgggcatcgg gttcttatca agaggggacc

    661 gggaaatcgg ggtcccacgg aacgtggaac cacccacgag gccacgtctg gaatgtcttc

    721 gtgagaccgg cctcatcctg aggtgcgacc ggaaggcaac cccttccaga caaagcaggg

    781 g
```

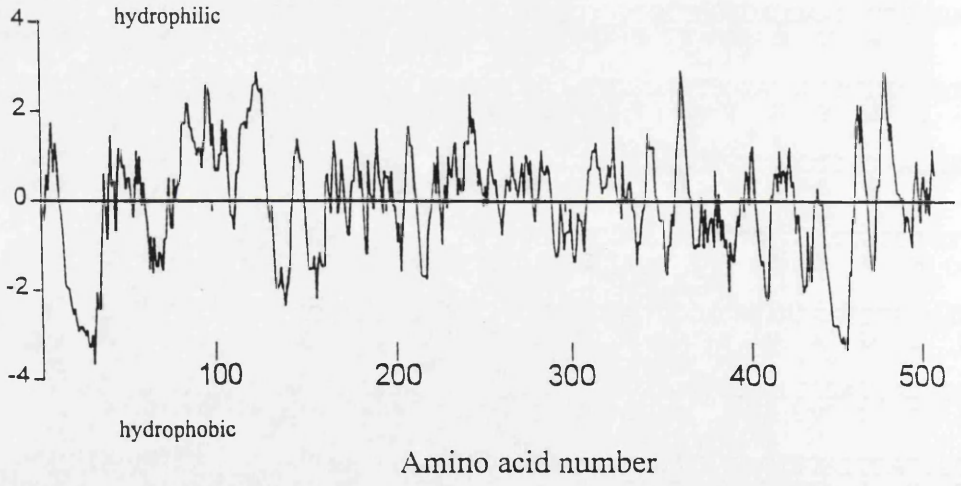
Figure 5.8

Nucleotide and predicted amino acid sequence of bovine HDL-receptor (SR-BI). Untranslated regions are in lower case while the open reading frame is in upper case. The start and stop codons are in bold and the putative poly $(A)^+$ signal is underlined. The primers GSP1 and GSP2 are underlined. This sequence has GenBank accession number AF019384.

```
                                                                gcgggcctcgtcctcggagtcgctgcct 28
ctatcacctccgtccccgtctcggtccctccacaggcgcggaaccccgcgagcaccgcggcgcgcggggcgcgcagac 106

ATGGGCAACCTCTCTAGAGCGCGCCGGGTGACCGCGGCGCTGGGTTTCATCGGGCTCCTCTTCGCCGTGCTGGGCATT 184
M  G  N  L  S  R  A  R  R  V  T  A  A  L  G  F  I  G  L  L  F  A  V  L  G  I (26)

ATCATGATCGTGATGGTGCCCTCAATCATCAAGCAGCAGGTCCTCAAGAATGTGCGCATCGACCCCAACAGCCTGTCT 262
I  M  I  V  M  V  P  S  I  I  K  Q  Q  V  L  K  N  V  R  I  D  P  N  S  L  S (52)

TTCAACATGTGGAAGGAAATCCCTGTCCCCTTCTACCTCTCTGTCTACTTTTTCAACATCGTCAACCCTGAGGGGATC 340
F  N  M  W  K  E  I  P  V  P  F  Y  L  S  V  Y  F  F  N  I  V  N  P  E  G  I (78)

ATCCAAGGCCAGAAGCCACAGGTGCAGGAGCACGGGCCTTATGTGTACAGGGAGTTCAGGCACAAGAGCAACATCACC 418
I  Q  G  Q  K  P  Q  V  Q  E  H  G  P  Y  V  Y  R  E  F  R  H  K  S  N  I  T (104)

TTCAACAACAATGACACGGTGTCCTTCCTGGAGTACCGCAGCTACCAGTTCCAGCCGGATAAGTCCCGCGGCCAGGAG 496
F  N  N  N  D  T  V  S  F  L  E  Y  R  S  Y  Q  F  Q  P  D  K  S  R  G  Q  E (130)

AGCGACTACATCGTCATGCCCAACATCCTGGTCTTGAGTGCGTCAATGATGATGGAGAACAGGCCCGGGCTCTTGAAG 574
S  D  Y  I  V  M  P  N  I  L  V  L  S  A  S  M  M  M  E  N  R  P  G  L  L  K (156)

CTGATGATGACCTTGGCGTTCAGCACGCTTGGCCAGCGTGCCTTCATGAACCGCACTGTTGGCGAGATCATGTGGGGC 652
L  M  M  T  L  A  F  S  T  L  G  Q  R  A  F  M  N  R  T  V  G  E  I  M  W  G (182)

TATGATGACCCCTTAATCCACCTCATCAATCAGTACTTCCCAAACTCGCTCCCCTTCAAGGGCAAGTTCGGCTTATTT 730
Y  D  D  P  L  I  H  L  I  N  Q  Y  F  P  N  S  L  P  F  K  G  K  F  G  L  F (208)

GCTGAGCTCAACAACTCCGATTCTGGCCTCTTCACCGTGTTCACAGGGGTCAAAAACTTCAGCAGGATCCATCTCGTG 808
A  E  L  N  N  S  D  S  G  L  F  T  V  F  T  G  V  K  N  F  S  R  I  H  L  V (234)

GACAAGTGGAATGGAGTCAGCAAGGTCAACTATTGGCATTCCGATCAGTGCAACATGATTAACGGAACTTCCGGGCAG 886
D  K  W  N  G  V  S  K  V  N  Y  W  H  S  D  Q  C  N  M  I  N  G  T  S  G  Q (260)

ATGTGGGCACCATTTATGACCCCTGAATCCTCGCTGGAATTTTACAGCCCTGAGGCCTGCCGGTCCATGAAGCTCGTC 964
M  W  A  P  F  M  T  P  E  S  S  L  E  F  Y  S  P  E  A  C  R  S  M  K  L  V (286)

TACAAGGAGCAGGGGGTGTTTGGAGGCATCCCCACCTTTCGCTTCGTGGCTCCCAGCACCTTATTTGCCAACGGGTCC 1042
Y  K  E  Q  G  V  F  G  G  I  P  T  F  R  F  V  A  P  S  T  L  F  A  N  G  S (312)

GTCTACCCGCCCAACGAGGGCTTCTGCCCCTGCCGGGAGTCCGGAATCCAAAACGTCAGCACCTGCAGGTTCAATGCG 1120
V  Y  P  P  N  E  G  F  C  P  C  R  E  S  G  I  Q  N  V  S  T  C  R  F  N  A (338)

CCCTTGTTTCTCTCCCATCCTCACTTCTACAACGCTGACCCAGTCCTGGCAGAGGCAGTGTCTGGCCTCCATCCTAAC 1198
P  L  F  L  S  H  P  H  F  Y  N  A  D  P  V  L  A  E  A  V  S  G  L  H  P  N (364)

CCAAAAGAACATTCCTTGTTCCTGGACATCCACCCGGTCACCGGAATCCCCATGAACTGCTCTGTGAAACTGCAGCTG 1276
P  K  E  H  S  L  F  L  D  I  H  P  V  T  G  I  P  M  N  C  S  V  K  L  Q  L (390)

AGCCTCTTCGTTAAGTCTGTGAAAGGCATCGGACAAACTGGGAACATCCAGCCAGTGGTCCTGCCGCTAATGTGGTTC 1354
S  L  F  V  K  S  V  K  G  I  G  Q  T  G  N  I  Q  P  V  V  L  P  L  M  W  F (416)

GAAGAGAGTGGAGCAATGGAGGGTGAGACCCTTGAGACGTTCTATATCCAGCTGGTGTTGATGCCCAAAGTGCTACAC 1432
E  E  S  G  A  M  E  G  E  T  L  E  T  F  Y  I  Q  L  V  L  M  P  K  V  L  H (442)

TATGCCCAGTATGTGCTCCTTGCCCTGGGCTGCGTCCTACTGCTCATCCCCATCATCTACCAAATCCGGAGCCAAGAG 1510
Y  A  Q  Y  V  L  L  A  L  G  C  V  L  L  L  I  P  I  I  Y  Q  I  R  S  Q  E (468)

AAATGCTATTTATTTTGGATTAGTTTTAAAAAGGGCTCAAAGGATAAGGAGGCCGTTCAGGCCTATTCTGAATTCCTG 1588
K  C  Y  L  F  W  I  S  F  K  K  G  S  K  D  K  E  A  V  Q  A  Y  S  E  F  L (494)

ATGACATCAGCTCCCAAGGGAACTGTACTGCAAGAAGCAAGACTGTAGggttccaaagatgccatcagccagccctgt 1666
M  T  S  A  P  K  G  T  V  L  Q  E  A  R  L  *                                 (509)

ctggccactcagccagaccaacttcccagcccctacaccccgcttcttcaggactcgctccgtggacagcccaccagt 1744
cccaaagtctgagccccccacctcctgcacattgcacgcacgcacgcgcacacacacacacacacacacatgcccctg 1822
gcacgtgtccacacgtgtgcacaggtgtgtgcagatactcaggatggagccgctgctgagagacttgggggaaagact 1900
ggtcaacaagggctgttctggaaacttctctccaagtaatccacaggcctgaccgtaggtgctatgggcacggggtcc 1978
cctagcccaggctcttatgactcacgtcacctctccccagcggcccgctccatctgtgaaacactgcagccccttcct 2056
ggtggctcatccatgcaggacaggcatttgccctgagaggccgccactgtccccaaatccagcctaggagtgctgact 2134
tcttgtgcctgaacctggtgggcaaggttttgtggttcagcacccagcccggaccaaagctgtagccactggacagcc 2212
atctgggcctcagaaacggagatgcatcctaaacccccctgcaggatcatgggtttgggtgtgcgggtcccttccctcc 2290
agcctaaactgacatcatcctgtatactgagctggtcacttcctgactggggtggcaggaagctgtgccctgagctgc 2368
cccccaccaaggtctgcagctaccaggtgccagggcagaggtagagaggctccgtctgggacgtcagcctgggtgtct 2446
ggtcaggagaccatctgtctgggactcctggtctctgggacaagcctctgtctcttttctactggaagaaaaactgaa 2524
tatcatcttttaaaaataattcacaattgaagtaataaaccttttttaaaaagtgaagaaaaaaaaaaaaaaaaa..   2598
```

Figure 5.9

Amino acid alignment of bovine, hamster, mouse , human and rat HDL-receptor (SR-BI). Identical amino acids are indicated with a dash. Two hydrophobic regions common to all species are boxed and shaded. The hydrophobic regions were determined by generation of hydropathy profiles according to Kyte and Doolittle (1982) as shown below.



```
bovine    MGNLSRARRV TAALGFIGLL FAVLGIIMIV MVPSIIKQQV LKNVRIDPNS 50
hamster   --GSA---W- AVG--VV--- C----VV--L VM--L----- --------S-
mouse     --GS----W- ALG--AL--- --A--VV--L ----L----- --------S-
human     --CSAK--WA AG---VA--- C----AV--- ----L----- --------S-

bovine    LSFNMWKEIP VPFYLSVYFF NIVNPEGIIQ GQKPQVQEHG PYVYREFRHK 100
hamster   ---A------ ---------- EV---SE-LK -E--V-R-R- ----------
mouse     ---G------ ---------- EV---NEVLN ----V-R-R- --------Q-
human     ---------- I--------- DVM--SE-LK -E----R-R- ------S---

bovine    SNITFNNNDT VSFLEYRSYQ FQPDKSRGQE SDYIVMPNIL VLSASMMMEN 150
hamster   A-----D--- ---V-H--LH ----R-H-S- ----IL---- --GGAV---S
mouse     V-----D--- ---V-N--LH ------H-S- -----L---- --GG-IL--S
human     ---------- -------TF- ---S--H-S- ---------- --G-AV----

bovine    RPGLLKLMMT LAFSTLGQRA FMNRTVGEIM WGYDDPLIHL INQYFPNSLP 200
hamster   KSAG------ -GLA------ ---------L ---E--FVNF --K-L-DMF-
mouse     K-VS------ --LV-M---- ---------L ------FV-F L-T-L-DM--
human     K-MT---I-- ---T---E-- ---------- ---K---VN- --K---GMF-

bovine    FKGKFGLFAE LNNSDSGLFT VFTGVKNFSR IHLVDKWNGV SKVNYWHSDQ 250
hamster   I-------V- M--------- -----Q---K -----R---L --------E-
mouse     I-------VG M---N--V-- -----Q---- ---------L --ID----E-
human     --D------- ---------- -----Q-I-- ---------L ---DF-----

bovine    CNMINGTSGQ MWAPFMTPES SLEFYSPEAC RSMKLVYKEQ GVFGGIPTFR 300
hamster   ---------- --------Q- ----F----- -----T-HDS ---E----Y-
mouse     ---------- ---------- ----F----- -----T-N-S R--E----Y-
human     ---------- --P------- ---------- -----M-K-S ---E----Y-

bovine    FVAPSTLFAN GSVYPPNEGF CPCRESGIQN VSTCRFNAPL FLSHPHFYNA 350
hamster   -T--K----- ---------- ---L------ ------G--- ----------
mouse     -T--D----- ---------- ---------- ------G--- ----------
human     ----K----- --I------- ---L------ ------S--- -------L--

bovine    DPVLAEAVSG LHPNPKEHSL FLDIHPVTGI PMNCSVKLQL SLFVKSVKGI 400
hamster   ----S---L- -N-D-R---- ---------- ---------- --YI-A----
mouse     ----S---L- -N-------- ---------- -------M-- --YI------
humans    --------T- ----QEA--- ---------- ---------- --YM---A--

bovine    GQTGNIQPVV LPLMWFEESG AMEGETLETF YIQLVLMPKV LHYAQYVLLA 450
hamster   ----K-E--- ---L---Q-- --G--P-N-- -T------Q- -Q-V------
mouse     ----K-E--- ---L---Q-- --G-KP-S-- -T------Q- ---------G
human     ----K-E--- ---L--A--- -------H-- -T-------- M---------

bovine    LGCVLLLIPI IYQIRSQEKC YLFWISFKKG SKDKEAVQAY SEFLMTSAPK 500
hamster   --GL---V-V ---L------ F---SGS--- -Q----I--- --S--SP-A-
mouse     --GL---V-- -C-L------ F---SGS--- -Q----I--- --S--SP-A-
human     -------V-V -C-------- ----S-S--- ------I--- --S-------

bovine    GTVLQEARL* 509        %similarity %identity
hamster   -------K-*                 83.7       76.7
mouse     -------K-*                 84.5       78.6
human     -S-----K-*                 87.3       82.9
```

Figure 5.10

Distribution of SR-BI HDL-receptor in bovine tissues. Total RNA from each tissue was reverse transcribed, amplified by PCR and visualized by Southern blot hybridization. Lanes 1 to 6 represent cDNA amplified from heart (1), adrenal (2), liver (3), skeletal muscle (4), spleen (5) and corpus luteum (6) while lane 7 is a negative control. Position of molecular size markers is indicated on the left. The expected PCR product size was 336bp.

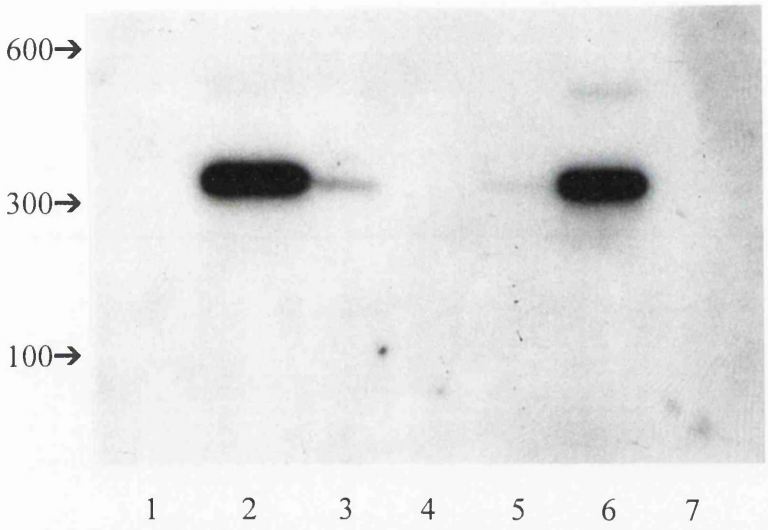

600→
300→
100→
1
2
3
4
5
6
7

Figure 5.11

A) PCR amplification of bovine HDL-receptor cDNA from granulosa cells and corpora lutea using primers GSP1 and GSPP2. The left lane contained a 100bp ladder, lane 1 contained cDNA amplified from granulosa cells (g.c.), lanes 2 and 3 contain cDNA amplified from corpora lutea taken 1 day or 5 days after ovulation. Lane 4 (-) was a negative control. The expected PCR product size was 336bp.
B) Semi-quantitative measurement of bovine SR-BI HDL-receptor mRNA in a day 5 corpus luteum using RT-PCR. Tubes were amplified for different numbers of cycles as indicated and then bands cut out and incorporated [^{32}P] measured as described in Chapter 2. Primers used were designed to amplify HDL-receptor or GAPDH.

A)

g.c. 1d 5d -

corpus luteum

B)

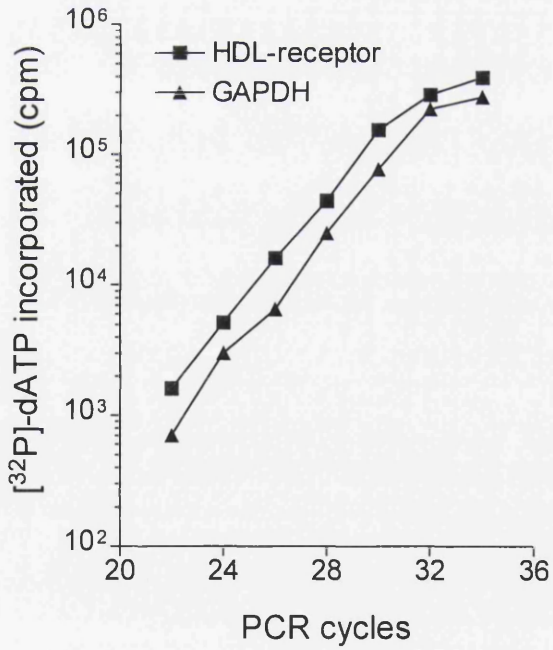
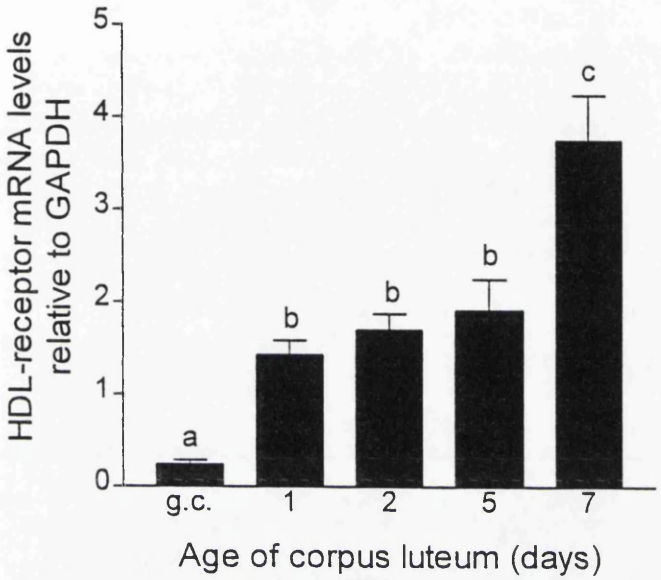


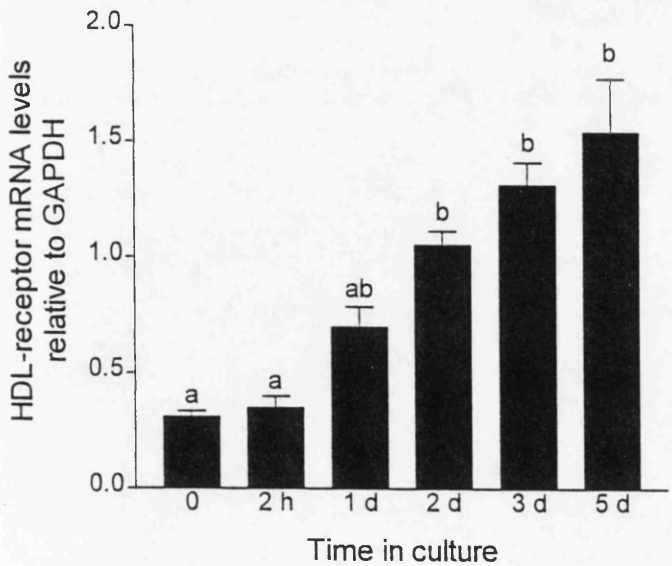
Figure 5.12

A) Accumulated results showing changes in SR-BI HDL-receptor mRNA (relevant to GAPDH) measured in corpora lutea at different stages after ovulation or in granulosa cells obtained from antral follicles. Each group represents the mean ± SEM of 3 or 4 corpora lutea or granulosa cell preparations. Groups with different letter superscripts are significantly different.
B) Changes in SR-BI HDL-receptor mRNA content (relevant to GAPDH) measured in granulosa cells luteinized in culture. Results show the mean ± range of two separate experiments with the mean of each experiment determined from triplicate culture dishes. Groups with different letter superscripts are significantly different.

A)


HDL-receptor mRNA levels relative to GAPDH
5
4
3
2
1
0
a
b
b
b
c
g.c.
1
2
5
7
Age of corpus luteum (days)

B)


HDL-receptor mRNA levels relative to GAPDH
2.0
1.5
1.0
0.5
0.0
a
a
ab
b
b
b
0
2 h
1 d
2 d
3 d
5 d
Time in culture

Chapter 6

General Discussion

In this thesis I have described studies in which I examined changes in granulosa cell function during luteinization *in vitro* and *in vivo*. In particular, I examined changes in two cell surface receptors, the FSH-receptor and the SR-BI HDL-receptor. In addition, changes in steroidogenic enzyme mRNA levels were examined. The results show the dynamic changes that take place in granulosa cells during the process of luteinization with some genes showing a marked increase in expression (eg P450scc and SR-BI HDL-receptor) while others are apparently down regulated (eg P450arom and the FSH-receptor). It is noticeable that neither P450arom nor the FSH-receptor genes are completely "switched off." A residual level of P450arom is detectable in the corpus luteum by PCR although it needs to be determined whether this is granulosa cell-derived or whether it is from the luteinised thecal cells. In addition, the FSH-receptor continues to be expressed although splicing prevents expression of the full-length receptor. It is noticeable, also, that the genes which show a marked increase in expression after luteinization (eg SR-BI HDL-receptor and P450scc) are also expressed in granulosa cell, although at a lower level. Thus luteinization appears to be a change in levels of gene expression rather than a complete differentiation of the cell with new genes "switched on" and others "switched off."

This idea that the corpus luteum is just a modified follicle requires testing of course. Changes in granulosa cell function during luteinization in culture are very similar to those seen *in vivo* which means that this cell culture system could be used for a systematic search for "new" genes expressed in the developing corpus luteum. Changes in granulosa cells in culture are not identical to changes which take place *in vivo* (eg loss of P450arom is complete in culture) but the similarities are sufficient to give confidence that the resuls would be meaningful. The advantage of using a cell culture system for looking for changes in gene expression is that results are usually much more clear cut, mainly because only one cell type is present. A number

of different methods have been described for selecting genes expressed in one cell type and not another including subtractive hybridisation and differential display (Hedrick *et al*,1984; Liang & Pardee, 1992). Each of these methods can work very well but there is a certain amount of chance involved in selecting genes of interest and it is easy to pick out genes that are expressed at high levels but which are probably not of great interest. More recently, a method has been described called serial analysis of gene expression (SAGE) which allows a systematic search for differences in gene expression between different cells or tissues (Velculescu *et al*, 1995). This method relies on generation of expressed sequence tags (ESTs) and gives a quantitative measure of expression of different genes in different tissues. Using this method it would be possible to identify genes expressed in luteinized granulosa cells which were not expressed in non-luteinized cells.

From results reported here and from the work of others it is possible to describe a working model of how changes in steroidogenesis are brought about following luteinization in cattle. Prior to luteinzation levels of progesterone production by granulosa cells are limited because levels of P450scc are low and, also, because cholesterol uptake through HDL-receptors is also low. Oestrogen production by the same cells is high because of high levels of P450arom although this relies on androgen substrate coming from the thecal cell compartment (Falck, 1959; Moor, 1977). After luteinization there is an increase in expression of P450scc, 3βHSD (Couet *et al*, 1990) and the HDL-receptor which leads to an increase in cholesterol uptake and an increase in progesterone production. There is a decrease in P450c17 mRNA levels in the luteinizing thecal cells (Rodgers *et al*, 1987b) which prevents androgen production and this is reinforced by loss of P450arom activity which leads to complete loss of oestrogen production. The questions that remain to be determined are what factors control these changes in steroidogenic enzyme acitivity and HDL-receptor expression. One likely candidate was SF-1

which plays a key role in many aspects of development of reproductive function (Ingraham *et al*, 1994). Levels of this factor were very low, however, in the developing corpus luteum and in granulosa cells luteinizing in culture so it is unlikely to be involved in regulating the luteinization process. To identify factors which regulate changes in steroidogenic enzyme gene expression will require gene identification studies of the kind described above. It is clear from studies reported here that expression of FSH-receptor mRNA levels decreases markedly after luteinization. Residual expression continues although alternate splicing of the primary transcript means that only the extracellular domain of the receptor would be expressed. This change in FSH-receptor expression appears to be related to luteinization since FSH-receptors are clearly at high levels in large antral follicles (this study and Tisdall *et al*,1995; Liu *et al*, 1998). It is not clear why the expression of the FSH-receptor should be reduced so markedly after luteinization. The bovine corpus luteum is dependent, to an extent, upon LH (Donaldson & Hansel, 1965; Snook *et al*, 1969; Carlson *et al*, 1971) and, possibly, prolactin (Bartosik *et al*, 1967; Bevers *et al*, 1988) for maintenance of steroidogenesis. If FSH-receptors continued to be expressed it is likely that they would function synergistically with the LH receptors to support luteal function. It may be that because FSH-receptor function is not necessary there is a mechanism for stopping expression although functional redundancy is more normal in these situations where more than one mechanism exists for any one function.

It is possible, alternatively, that the truncated FSH-receptor continues to have a function in the corpus luteum. There is considerable evidence that the corpus luteum has a large component of independence from pituitary control in ruminants. Work, particularly in sheep, has shown that hypophysectomy or treatment with a GnRH antagonist does not stop the corpus luteum remaining functional although steroid production may be reduced by up to half (Kaltenbach *et al*, 1968; Denamur *et al*, 1973; McNeilly *et al*, 1992). Thus, there appears to

be an LH-independent component to luteal function. This function may be taken up by local growth factors such as prostaglandins (Milvae & Hansel, 1985) or TGFβ or, it is possible, that a truncated isoform of one of the gonadotrophin receptors may act to stimulate luteal cell function. As discussed in Chapter 4 a shortened version of the FSH-receptor has been described which contains only an extracellular domain and a carboxy terminus which may act as a single-pass transmembrane domain (Sairam *et al*, 1997). This receptor has similarities to growth hormone receptors and it is possible that such a receptor, or an isoform of the LH-receptor, may function to stimulate luteal cell activity. This work would require isolation of the various forms of the FSH- and LH-receptors and expression in cultured cells to determine which, if any, of the forms was able to increase cyclic AMP production without the presence of gonadotrophins.

As described in Chapter 1 the bovine corpus luteum, in common with many other species, has two different cell types - the large and small cells. Available evidence suggests that the large cells are derived from the granulosa cell layer while the small cells are thecal-derived (O'Shea *et al*, 1980; Alila & Hansel, 1984). The large cells appear to have a high basal progesterone production but show little response to LH while the small cells have a low basal progesterone production but respond to LH (Lemon & Loir, 1977; Urseley & Leymarie, 1979; O'Shea *et al*, 1980; Hoyer *et al*, 1984; Weber *et al*, 1987). Future studies of the SR-BI HDL-receptor may be aimed at determining in which cells the receptor is expressed. It might be predicted that HDL-receptors will be in the large cells if they are derived from granulosa cells although that would leave open the question of how the smaller cells are able to obtain enough cholesterol for steroidogenesis. Distribution of LDL-receptors is also not known in the mature corpus luteum and it may be that different cells have different lipoprotein receptor types.

Overall these studies have shown that there are marked changes in gene expression in granulosa cells as they undergo luteinization. Each of these changes appears to be related to increasing the potential of the cell to produce progesterone although the loss of FSH-receptor expression is less easily explained from a functional view. Future research might look at trying to identify factors which act to induce the described changes in granulosa cell function.

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Appendix

Denhart's (50x)
5g Ficoll
5g polyvinylpyrrolidone
5g bovine serum albumin
water to 500ml

Loading buffer
0.25% bromophenol blue
0.25% xylene cyanol FF
40% sucrose in water

Prehybridisation solution
100 µg of herrin sperm DNA /ml
0.1% SDS
5 x SSC
5 x Denharts

SSC (20x)
3M Na Cl
0.3M Na citrate

TAE (1x)
40mM Tris-acetate
1mM EDTA

TBE (0.5x)
45mM Tris borate
1mM EDTA

TE
10mM Tris HCl (pH 7.6)
1mM EDTA (pH 8.0)