THE INSULIN-LIKE GROWTH FACTOR SYSTEM IN THE BOVINE CORPUS LUTEUM

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ABSTRACT

Whilst pituitary-derived luteinising hormone is the primary regulator of corpus luteum (CL) function, it is becoming increasingly apparent that other factors of extra- and intra-ovarian origin have the potential to modulate the luteal response to gonadotrophins. The insulin-like growth factor (IGF) system is thought to play a central role in these interactions, and studies *in vitro* have shown that both IGF-I and –II have wide ranging effects on ovarian function. The aim of this work was to investigate the role of the IGF system in regulating corpus luteum function.

Bovine CL were collected on days 5, 10, and 15 of the oestrous cycle following synchronised oestrus (day of oestrus = day 0). In addition, CL were collected following prostaglandin-induced luteolysis. *In situ* hybridisation detected luteal expression of IGF-I, -II and the type 1 IGF receptor messenger ribonucleic acid (mRNA) throughout the oestrous cycle. The expression of IGF-I mRNA varied significantly during the oestrous cycle. IGF-I mRNA concentrations were significantly higher on day 15 than on day 10, and IGF-I mRNA in the regressing corpus luteum 48 hours after exogenous prostaglandin was significantly greater than in the early or mid-luteal phase (days 5 and 10). In contrast, there was no significant effect of day of the oestrous cycle on IGF-II and the type 1 IGF receptor mRNA concentrations in the corpus luteum. IGF-II mRNA expression was localised to a subset of steroidogenic luteal cells and was also associated with cells of the luteal vasculature. Messenger RNA encoding the type 1 IGF receptor was widely expressed, in a pattern suggestive of steroidogenic luteal cell expression.

The actions of the IGFs are modulated by their association with members of a family of IGF-specific binding proteins (IGFBPs), which regulate the transport of IGFs and their presentation to specific receptors. *In situ* hybridisation detected mRNA encoding IGFBP-2, -3, and –4 in the bovine corpus luteum throughout the luteal phase. IGFBP-2 and –4 mRNA concentrations were low within the corpus luteum, and showed no temporal variation. In addition, a subset of large vessels in the periphery of the CL showed moderate to intense hybridisation for IGFBP-2 mRNA. IGFBP-3 mRNA concentrations were high throughout the luteal phase, and expression was localised predominantly to cells lining microvessels, suggestive of endothelial cell expression.

The administration of gonadotrophin releasing hormone (GnRH) to cattle in the early luteal phase (day 6) induces ovulation and the formation of an additional CL in greater than 80% of animals. Progesterone profiles indicate that GnRH-induced CL are functional, however progesterone levels are reduced and they display a short lifespan when compared to spontaneously formed CL. Based on the ability of the IGFs to modulate the ovarian response to gonadotrophins, we hypothesised that changes in concentrations of mRNA encoding components of the IGF system were responsible. GnRH was administered to cows on day 6 and ovarian dynamics monitored by rectal ultrasound and daily blood sampling. Ovaries were collected on day 10 and 15, and GnRH-induced CL compared to similar aged control CL and spontaneous CL present at GnRH treatment. Induced CL expressed mRNA encoding IGF-I, IGF-II, the type 1 IGF receptor and IGFBP-3. The patterns of expression were not significantly different between control and induced CL, however, concentrations of mRNA expression were

altered. IGFBP-3 mRNA concentrations were increased in induced CL, whilst type 1 IGF receptor mRNA concentrations were decreased when compared to control CL. We suggest that these changes will reduce both the bioavailability and bioactivity of IGF-I and –II, thus compromising the induced CL response to luteotrophic support.

In conclusion, these data demonstrate that the bovine corpus luteum is a site of IGF production, reception and regulation throughout the luteal phase, and further support the hypothesis that the IGF system is important in regulating luteal function in the cow.

DECLARATION

I hereby declare that this thesis has been composed by myself, and has not been submitted for any other degree, in Edinburgh or elsewhere. The work presented herein is my own, and all work of other authors is duly acknowledged. I also acknowledge all assistance given to me during the design and execution of the experiments contained in this thesis and during its preparation.

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Conference Presentations

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Armstrong DG, Baxter G, Gutierrez CG, **Woad** KJ, Sinclair KD, Robinson JJ, McEvoy TG and Webb R (1997) Ovarian IGF-II mRNA expression in cattle fed different energy and protein diets. Journal of Reproduction and Fertility Abstract Series 19: abstract 131

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INTRODUCTION

The corpus luteum has key functions in the control of ovarian cyclicity and embryo survival. The establishment of pregnancy and continued embryo development are dependent on the inhibition of luteal regression and hence maintained secretion of the primary luteal product progesterone. A lack of appropriate progesterone secretion has a detrimental effect on pregnancy success (Mann *et al.*, 1999).

During the past twenty years there has been a marked decline in the reproductive performance of the UK dairy herd. The pregnancy rate to first service has fallen annually by approximately 1%, and is now only 39.7%. This declining fertility is associated with an increase in the proportion of animals displaying atypical ovarian hormone patterns, and abnormal luteal function is thought to be a primary contributory factor (Royal *et al.*, 2000). Sub-fertility represents a significant loss of revenue for the dairy farmer through reduced milk output and calf production, and increased insemination and replacement costs (Esslemont and Peeler, 1993; Stott *et al.*, 1999). In addition, there are considerable animal welfare implications associated with increased veterinary intervention and a high rate of culling. An increased understanding of the mechanisms regulating luteal function should lead to the development of procedures aimed at improving reproductive efficiency, and help to reverse the current trend of decreasing fertility in the national dairy herd.

This study is concerned with the role of the IGF system in the regulation of luteal function. IGF-I and –II have multiple effects on the regulation of follicular development

and steroidogenic activity. IGF-I stimulates follicular steroidogenesis via enhanced gonadotrophin receptor numbers, sterol delivery, and steroidogenic enzyme activity (Adashi *et al.*, 1985c; Veldhuis, 1989; Magoffin *et al.*, 1990; Magoffin and Weitsman, 1993; Balasubramanian *et al.*, 1997). Evidence also suggests that IGF-II augments the steroidogenic response to gonadotrophin stimulation (Garmey *et al.*, 1993). Comparatively little is known about the effects of the IGFs on luteal function. However, an important role for the IGFs in the CL is supported by the regulatory actions of the IGFs on steroidogenesis, as demonstrated in the follicle, and by the stimulation of progesterone production by luteal cells *in vitro* in response to both IGF-I and IGF-II (McArdle and Holtorf, 1989; Talavera and Menon, 1991; Sauerwein *et al.*, 1992; Khan-Dawood *et al* 1994; Devoto *et al.*, 1995; Yuan and Lucy, 1996a). The IGFs may also have important functions in addition to the regulation of steroidogenesis, including the stimulation of angiogenesis (Hansson *et al.*, 1989; Grant *et al.*, 1993; Vialettes *et al.*, 1994) and control of apoptosis (Chun *et al.*, 1994), and therefore have great potential to regulate luteal events. This study aims to characterise the IGF system during the growth and development of the bovine CL.

Chapter 1. Literature Review

1.1 The Bovine Corpus Luteum; Formation, function and regression.

1.1.1 Historical background

The first detailed description of the corpus luteum (CL) is credited to Regnier de Graaf (cited by Short, 1977) whose publication on female reproduction in 1672 describes the formation of "globular bodies" that form to replace "ova". In addition, de Graaf observed that the "testicles of the female" did not always contain these "globules" rather that they could be seen to diminish and disappear.

The term "corpus luteum" was first ascribed to these globules by the anatomist Malpighi (1628-1694) following his observation of the "yellow body" of the cow (cited by Short, 1977). However, it was not until the late 1800s that specific functions were suggested for the corpus luteum. In 1897, John Beard proposed that the CL prevents ovulation during pregnancy and that the degeneration of the structure towards the end of gestation, or in the absence of fertilisation removes inhibition and allows for a new ovulation (cited by Amoroso, 1968). Prenant, in 1898, was the first to suggest that the corpus luteum acts as an endocrine gland. Based on his histological observations, he proposed that the corpus luteum was "a gland of internal secretion, releasing one or more products into the bloodstream"(cited by Short, 1977).

Confirmation of the role of the corpus luteum in the maintenance of pregnancy was first provided by Fraenkel (1903) and Magnus (1901) who showed that removal of the ovaries or luteal ablation induced abortion or embryo resorption (cited by Amoroso, 1968). A further role for the corpus luteum in pregnancy preparation and

survival was demonstrated by Bouin and Ancel (1910) who showed in the rabbit that endometrial gland proliferation was CL-dependent, thus providing a bioassay that enabled luteal extracts to be tested for their active principle (cited by Amoroso, 1968). Corner and Allen (1929) extended the results of the CL ablation studies by successfully inducing endometrial proliferation and maintaining early pregnancy in ovariectomised animals following the administration of a porcine luteal extract. Subsequently progesterone was isolated and purified as the primary endocrine secretion of the corpus luteum (Allen and Wintersteiner, 1934).

1.1.2 Follicular and luteal dynamics

A pool of approximately 2-3 million primordial follicles are established in the ovary of the cow during fetal development (Gosden and Telfer, 1987), each consisting of primary oocytes arrested in prophase I of meiosis, surrounded by a single layer of pre-granulosa cells and enclosed by a basal lamina. These arrested follicles enter the growth phase during the lifetime of the animal in response to an as yet undetermined stimulus (Hirshfield, 1991; Webb *et al.*, 1999). The initiation of primordial follicle growth is characterised by a change in granulosa cell shape from flattened to cuboidal, granulosa cell proliferation and oocyte enlargement. With continued growth the granulosa cells of the primary follicle become multi-layered and a zona pelucida is secreted around the enlarging oocyte.

Outside the basement membrane, the theca interna and externa are formed by differentiation of stromal cells, and a blood capillary network is increasingly apparent. Once the follicle has reached 100-300μm in diameter, spaces between granulosa cells join together to form a fluid-filled antral cavity (Lussier *et al.*, 1987).

Having left the resting pool a follicle faces one of two fates, continued growth and ovulation, or degeneration and atresia, and only a small percentage of follicles proceed to the final stages of follicular development (Figure 1.1).

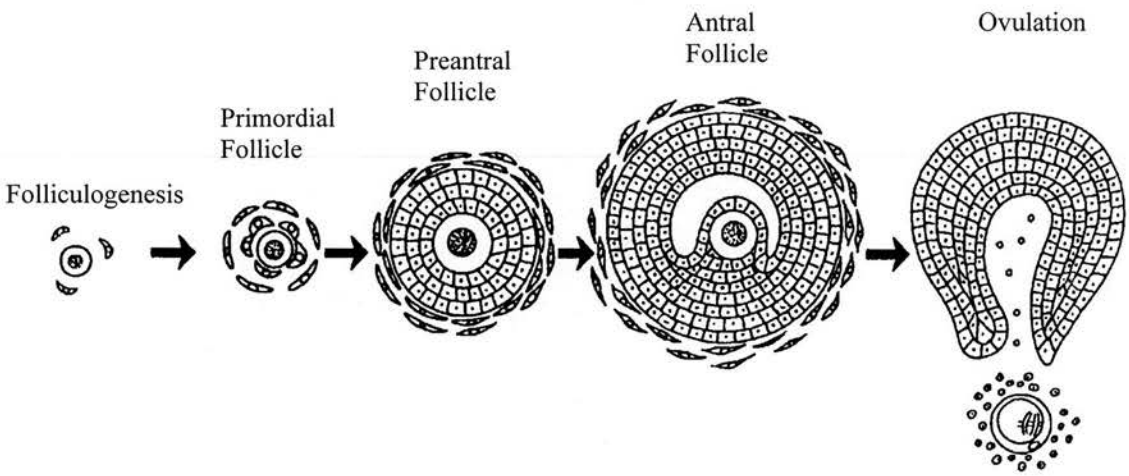


Figure 1.1 Stages of follicular growth and development.

(Adapted from Webb *et al.*, 1999).

Real-time ultrasonography has enabled the tracking of follicular and luteal dynamics. In the bovine oestrous cycle large antral follicle growth occurs in two or three waves. Each wave is characterised by the simultaneous growth of a group of antral follicles, one of which is selected to become dominant, while other cohort follicles regress (Figure 1.2). The follicle that is dominant at luteolysis progresses to become the ovulatory follicle (Fortune, 1994; Webb *et al.*, 1999). Ovulation releases the oocyte, and the corpus luteum forms from the granulosa and theca interna cells of the collapsed follicle.

The length of the bovine oestrous cycle is 21 days for cows and 20 days for heifers, with a normal range of 17-24 days (Hawk and Bellows, 1980). The corpus luteum is the dominant structure on the ovary for approximately 17 days, and the follicular phase lasts 3-4 days. The oestrus period of sexual receptivity is of short duration, with a mean length of 12-16 hours, ranging in individual animals from 3-28 hours (Allrich, 1994) and is designated day 0 of the cycle. Ovulation usually occurs 18-48 hours after the onset of oestrus, or 10-12 hours after the end of oestrus.

1.1.3 Formation and structure of the corpus luteum

1.1.3.1 Gross morphology of the corpus luteum

The corpus luteum is formed from the ruptured follicle following ovulation (Donaldson and Hansel, 1965a), and exhibits rapid growth, to reach its maximal size around mid-cycle (Ireland *et al.,* 1980; Reynolds *et al.*, 1994). Indeed, the dramatic growth of the CL is comparable to that of the fastest growing tumours (Jablonka-Shariff *et al.,* 1993; Zheng *et al.*, 1994).

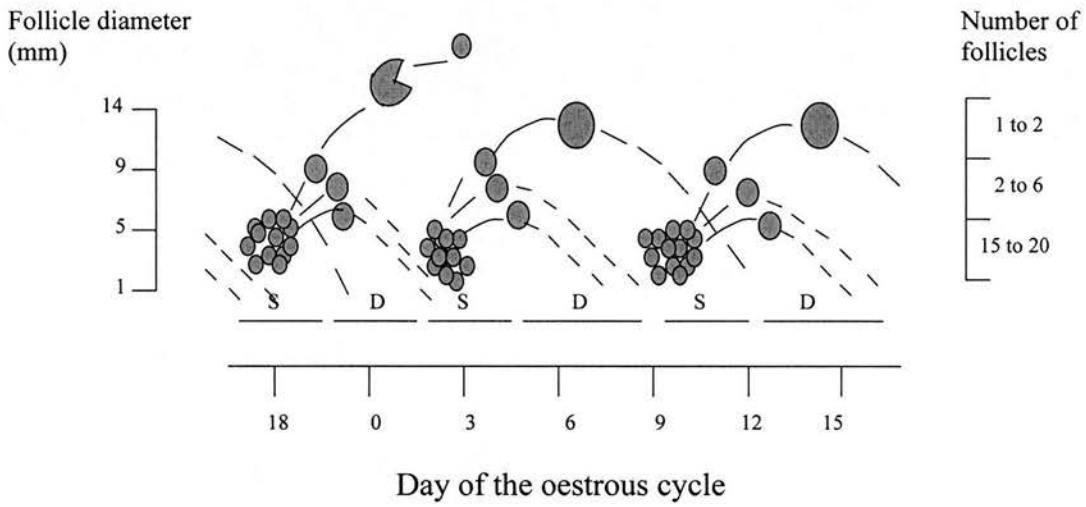


Figure 1.2 Waves of follicular development in cattle during the oestrous cycle.

A three wave pattern of follicular development is illustrated. S and D represent phases of follicular selection and dominance respectively.

(Adapted from Webb *et al.*, 1999).

Gross changes in the appearance of corpora lutea throughout the bovine oestrous cycle have been described in detail and are used to estimate the day of the cycle in animals of unknown reproductive history (Ireland *et al.*, 1980). In stage I (days 1-4) the CL is red, between 0.5 – 1.5 cm in diameter and the point of follicle rupture is not covered by epithelium. In stage II (days 5-10) the CL has increased in size to its maximal diameter of 1.6 – 2.0 cm, the ovulation point is now covered and red or brown in colour, whilst the remainder of the CL is orange. In stage III (days 11-17) the apex of the CL appears tan or orange, and has a visible vasculature, on bisection the CL is bright orange or yellow. In the final stage of luteal development (stage IV, days 18-20) the CL undergoes regression, decreasing in size to less than 1 cm in diameter, and becomes paler in colour (Ireland *et al.*, 1980).

1.1.3.2 Angiogenesis

The female reproductive organs undergo cyclical growth and regression, which is accompanied by extensive changes in vasculature and blood flow. Few other tissues in the adult exhibit the growth of new blood vessels (angiogenesis) unless they are undergoing tissue repair. Irregularities in the control of angiogenesis, either by the over or under production of vascular growth, are associated with a number of pathological conditions, therefore ovarian angiogenesis is expected to be tightly controlled (Redmer and Reynolds, 1996).

The vascular bed of the Graafian follicle is confined to the theca cell layer by a basement membrane, which separates the avascular granulosa layers of the follicular interior from the network of blood vessels. Around the time of ovulation, the basement membrane breaks down, allowing rapid capillary outgrowth. The subsequent formation

of the luteal vasculature is both rapid and extensive. Indeed, tissue growth is dependent upon the growth of new blood vessels and the establishment of a functional blood supply (Folkman and Klagsbrun, 1987). During early luteal development capillary sprouts invade the granulosa-derived tissue, and by mid-cycle an abundant capillary network has formed within the mature CL. Indeed, the vascular network is so dense that most steroidogenic cells appear in immediate contact with at least one capillary (Zheng *et al.*, 1993). Endothelial cells, which line blood vessels, are a prominent cell-type within the corpus luteum, occupying around 15% of luteal tissue volume, and representing around 50% of all cells at mid-cycle (O'Shea *et al.*, 1989). In addition, the mature corpus luteum receives one of the highest rates of blood flow to any organ. Blood flow to the CL-bearing ovary is correlated with progesterone concentrations, reaching maximal levels during the mid-cycle and showing a rapid decrease associated with luteolysis (Niswender *et al.*, 1976).

Corpora lutea produce angiogenic factors throughout the oestrous cycle and pregnancy (Grazul-Bilska *et al.*, 1992b; Ricke *et al.*, 1995). Heparin binding growth factors, namely the fibroblast growth factors (FGF) and the vascular endothelial growth factors (VEGF) are considered key mediators of luteal neovascularisation, and are the subject of recent review (Reynolds and Redmer, 1998).

Acidic FGF (aFGF/ FGF-1) and basic FGF (bFGF/ FGF-2) are known to stimulate angiogenesis (Klagsbrun and D'Amore, 1991), and both proteins have been detected in the bovine CL (Zheng *et al.*, 1993). In addition luteal bFGF mRNA expression is correlated with angiogenic activity (Stirling *et al.*, 1991). Immunoneutralisation of aFGF and bFGF markedly reduced luteal angiogenic activity, and demonstrated that

bFGF was responsible for stimulating the majority of the luteal vascular response (Grazul-Bilska *et al.*, 1992a).

VEGF mRNA expression has been detected in the ovine CL, and was maximal early in the cycle (Redmer *et al.*, 1996). The temporal association between high luteal VEGF expression and extensive neovascularisation supports an important role for VEGF in luteal vascular development. In addition, endothelial cell proliferation and migration, stimulated by the products of luteal explants in culture, was reduced by anti-VEGF antibodies, and the greatest reduction in angiogenic activity was observed in the early CL (Doraiswamy *et al.*, 1995).

Specific receptors for FGF and VEGF have been demonstrated in the corpus luteum of sheep, rats and women (Asakai *et al.*, 1993; Doraiswamy *et al.*, 1998; Otani *et al.*, 1999). Basic-FGF binding capacity and mRNA encoding the FGF receptor-1 (FGFR-1) which is activated by bFGF decreases with advancing luteal age (Asakai *et al.*, 1993). FGFR-1 and –2 have been detected in vascular and parenchymal cells of the ovine CL. Parenchymal FGFR-1 concentrations are reduced in the late luteal phase, whilst vascular FGFR-1 was detected in endothelial cells throughout the oestrous cycle. In contrast, FGFR-2 was localised to luteal parenchymal cells at all stages of oestrous cycle, and vascular expression was limited to large microvessels and was only detectable during the late luteal phase (Doraiswamy *et al.*, 1998). Messenger RNA encoding the VEGF receptor Flt-1 is expressed in the human corpus luteum. Flt-1 protein was localised to human granulosa and theca lutein cells and luteal endothelial cells during the menstrual cycle and early pregnancy (Otani *et al.*, 1999). In addition, treatment with truncated soluble Flt-1 receptors to inhibit VEGF bioactivity caused

near absolute suppression of luteal angiogenesis, demonstrating that VEGF is essential for neovascularisation (Ferrara *et al.*, 1998).

Luteal angiogenic activity appears to be regulated by luteotrophic and luteolytic factors. Luteinising hormone (LH) stimulated bFGF mRNA expression in cultured bovine luteal cells, whilst prostaglandin (PG)$F_{2\alpha}$ reduced bFGF mRNA (Stirling *et al.*, 1991). VEGF is also subject to modulation by LH; LH significantly increased VEGF production and mRNA expression by luteinising granulosa cells (Christenson and Stouffer, 1996; Garrido *et al.*, 1993).

1.1.3.3 Cellular components of the corpus luteum

The corpus luteum consists of a number of different cell types. These include steroidogenic cells, endothelial cells and pericytes, fibroblasts and immune cells (Farin *et al.*, 1986; O'Shea *et al.*, 1989; Parry *et al.*, 1980). The most numerous cells are those associated with the luteal vasculature, whilst the steroidogenic cells occupy most of the volume of the CL.

At least two populations of steroidogenically-active luteal cells can be distinguished. These are characterised by size and referred to as "large" and "small" luteal cells. Both luteal cell types possess ultrastructural features characteristic of steroid secreting cells, including a well-developed smooth endoplasmic reticulum (SER), abundant mitochondria containing tubular cristae, an extensive Golgi apparatus and cytoplasmic lipid droplets (Parry *et al.*, 1980). Lipid droplets, which may serve as steroid precursor stores, are inversely related to progesterone synthesis (Parry *et al.*, 1980).

In addition to their difference in size, other distinguishing structural features have been attributed to the small and large luteal cells (Fields and Fields, 1996; Parry *et al.*, 1980). The small cells characteristically display whorls of SER and adherens-type junctions, whilst large cells contain a notable abundance of membrane-bound electron-dense secretory granules within their cytoplasm which are extruded from the cell by exocytosis.

It was initially proposed that these granules contained progesterone, based on the correlation between progesterone secretion and granule formation and release (Sawyer *et al.*, 1979), although an autoradiographic study suggested that labelled progesterone-precursors synthesised into progesterone did not concentrate in the granules (McClellan *et al.*, 1979). Immunocytochemistry has subsequently localised oxytocin and neurophysin to the same secretory granule (Fields *et al.*, 1992). Secretory granules have also been reported to contain tissue inhibitor of metalloproteinases (TIMP)-1, which plays an important role in regulating extracellular matrix (ECM) remodelling (McIntush *et al.*, 1996).

Whilst both large and small luteal cells possess the cellular machinery associated with steroid synthesis, they are also well equipped for protein secretion (Niswender *et al.*, 1985; Parry *et al.*, 1980).

In addition to displaying structural differences, the two cell populations appear to be functionally distinct. Whilst both large and small cells secrete progesterone, steroidogenesis appears to be controlled by different regulatory pathways. *In vitro* studies have shown that large luteal cells produce higher amounts of progesterone in the absence of LH stimulation than small cells (Brannian *et al.*, 1993; Ursely and

Leymarie, 1979). However, physiological doses of LH stimulate small luteal cells to synthesise progesterone whilst eliciting only a modest response from the large luteal cells.

1.1.4 Function of the corpus luteum

1.1.4.1 Steroidogenesis

The first reaction in steroidogenesis is the conversion of cholesterol to pregnenolone. This conversion is catalysed by the cytochrome P450 side chain cleavage (P450scc) enzyme in complex with adrenodoxin and adrenodoxin reductase. P450scc has been immunolocalised to both large and small luteal cells in the bovine CL (Rodgers *et al.*, 1986a) and luteal content of P450scc has been characterized throughout the bovine oestrous cycle. Levels of P450scc and its electron donor adrenodoxin in the developing CL are not different from follicular concentrations. However, subsequent luteal maturation leads to a substantial increase in the tissue content of luteal P450scc in parallel with changing progesterone production, followed by a decline associated with luteolysis (Rodgers *et al.*, 1986b). Expression of mRNA encoding P450scc and adrenodoxin has been demonstrated in the bovine corpus luteum during luteal growth and development, and was undetectable during luteal regression (Rodgers *et al.*, 1987).

Steroid hormone biosynthesis is regulated by both the levels of regulatory enzymes and substrate availability. Steroid secreting cells may derive cholesterol from the uptake of lipoprotein cholesterol extracted from the circulation, from intracellular cholesterol ester stores, or by *de novo* synthesis. Bovine luteal cells have been shown to depend on lipoproteins as the cholesterol source for progesterone synthesis (O'Shaughnessy and Wathes, 1985). Low density (LDL) or high-density (HDL)

lipoproteins transport the majority of blood cholesterol, and the lipoprotein class utilised appears species-specific. In the cow both LDL and HDL are able to stimulate progesterone production (Carroll *et al.*, 1992), although LDL may be the preferred lipoprotein (O'Shaughnessy and Wathes, 1985).

P450scc activity was considered the rate-limiting step in steroid hormone biosynthesis. However, recent observations have indicated that the acute regulation of steroids is indispensably regulated by the actions of a protein involved in substrate delivery. P450scc is located within the inner mitochondrial membrane: therefore, steroid precursors must firstly be transported to the outer mitochondrial membrane and then across the intermembrane space to the inner membrane. This intra-mitochondrial transport is facilitated by the actions of a protein namely the steroidogenic acute regulatory (StAR) protein (Clark and Stocco, 1996). StAR mRNA and the protein product have been detected in bovine luteal homogenates. StAR mRNA abundance is low in developing bovine CL, elevated in the mid-to late-luteal phase and virtually absent in regressing CL (Pescador *et al.*, 1996).

Pregnenolone produced by the actions of P450scc must travel from the mitochondria to be converted to progesterone in the SER. The final steps in progesterone biosynthesis are performed by 3-β-hydroxysteroid dehydrogenase/ Δ 4-5 isomerase (3β-HSD). This enzyme is bi-functional, catalysing the dehydrogenation and isomerisation of Δ-5, 3-β hydroxysteroid precursors into Δ-4, 3-ketosteroids (Penning, 1997).

3β-HSD mRNA, protein product and enzymatic activity have been characterised in the bovine ovary. Prior to ovulation, bovine follicles display low and constant 3β-HSD

activity. However, shortly after oestrus 3β-HSD levels undergo a marked increase, reaching maximal levels on days 8-11, before declining dramatically coincident with luteal regression (Couet *et al.*, 1990).

The changing levels of steroidogenic enzymes may offer an explanation for the marked changes in progesterone secretion during the oestrous cycle. However, studies following induced luteolysis do not support the hypothesis that the decline in progesterone associated with regression is due to a reduction in tissue concentrations of P450scc or 3β-HSD (Rodgers *et al.*, 1995). Whilst mRNA levels have been shown to decline, the authors argued that since P450scc and 3β-HSD enzymes have long half lives, changes in their mRNA levels do not cause a sufficiently rapid reduction in the tissue content of the enzymes to be responsible for the decline in plasma progesterone concentrations.

Whilst both large and small luteal cells have been shown to possess the necessary regulatory enzymes for progesterone production, they appear to vary in their secretory ability. In the absence of hormonal stimulation, basal progesterone production is much higher in large bovine luteal cells than small luteal cells (Ursely and Leymarie, 1979). However, the large cells appear less sensitive to LH, requiring supraphysiological concentrations of LH to achieve stimulation *in vitro* (Alila *et al.*, 1988; Ursely and Leymarie, 1979). Ovine large luteal cells possess LH receptors, but at considerably lower levels than small luteal cells (Fitz *et al.*, 1982). Messenger RNA encoding the LH receptor was highly expressed in small bovine luteal cells and theca-derived luteal cells luteinised in culture (Mamluk *et al.*, 1998; Yuan and Lucy, 1996b). Intracellular levels of cyclic adenosine monophosphate (cAMP) were significantly elevated in ovine

small luteal cells stimulated by LH. However, LH did not stimulate cAMP levels in large luteal cells. In addition, treatments that dramatically increased cAMP levels failed to stimulate large luteal cell progesterone production. This suggests that large luteal cell steroidogenesis is independent of elevated cAMP levels, despite the similarity in protein kinase A activity observed in ovine small and large cells (Hoyer *et al.*, 1984; Wiltbank *et al.*, 1989).

1.1.4.2 Biological functions of progesterone

The primary function of the corpus luteum is to secrete progesterone, which has a range of effects on the reproductive tract that are essential for pregnancy (Graham and Clarke, 1997).

Progesterone influences events from early in gamete transport, regulating oviductal contractility and secretory activity that support early development of the conceptus and the timing of its delivery to the uterus. Once within the uterus progesterone has an important, if incompletely understood, role in the preparation and support of implantation, with actions on both the uterine environment and the blastocyst. In the event of successful fertilisation, transport and implantation, progesterone secreted by the rescued corpus luteum continues to support uterine growth and function. In addition, it inhibits contractions of the myometrium, which if unopposed could result in the expulsion and subsequent loss of the growing foetus.

1.1.5 Hormonal control of luteal function

Regulation of luteal function and lifespan involves a number of hormonal factors produced outside of the ovary. The adenohypophysis, or anterior pituitary gland, in response to GnRH signals from the hypothalamus, secretes LH which is considered

the primary luteotrophic hormone in most species. If pregnancy occurs the corpus luteum is maintained in response to the presence of the conceptus. However, in the absence of conception the corpus luteum must undergo morphological and functional regression, so releasing the inhibition on follicular development and ovulation, allowing the next cycle of follicle development to begin, and so creating the next chance for fertilisation. In non-primate species the uterus produces this luteolytic signal.

1.1.5.1 Luteinising hormone

Early experiments in the ewe suggested that pituitary hormones were not obligatory in the formation and maintenance of corpora lutea. However subsequent studies have shown that the removal of pituitary gland early after ovulation can prevent CL formation, and hypophysectomy on day 5 causes luteolysis (Kaltenbach *et al.*, 1968), therefore supporting a positive role for one or more pituitary products.

The pituitary hormones LH and prolactin are both candidate luteotrophs. Prolactin has been shown to be an important luteal regulator in several rodent species. The maintenance of luteal function following prolactin therapy in hypophysectomised-hysterectomised ewes suggested that prolactin may also be an important regulator in ruminants (Denamur *et al.*, 1973). However, subsequent prolactin depletion studies showed no concurrent diminishment of luteal function in sheep and cattle (Hoffman *et al.*, 1974; Niswender, 1973). In addition, prolactin infusions could not stimulate luteal steroidogenesis or lifespan, and were unable to overcome the inhibitory effects of oxytocin (Donaldson *et al.*, 1965).

In contrast, a range of evidence supports the role of LH as the primary luteotroph in ruminants (Hansel and Seifart, 1967). Administration of LH can prolong the lifespan of the corpus luteum (Donaldson and Hansel, 1965b; Karsch *et al.*, 1971), whilst administration of antiserum to LH reduces luteal weight and progesterone content and can induce luteolysis (Fuller and Hansel, 1970; Snook *et al.*, 1969). In addition, LH stimulates luteal progesterone *in vivo* (Carlson *et al.*, 1971) and *in vitro* when incubated with luteal cells or slices (Seifart and Hansel, 1968).

1.1.5.1.1 Mechanism of action of LH

Binding of LH to its membrane-bound receptor causes the activation of two second-messenger systems leading to a biological response (Davis *et al.,* 1996). Cyclic AMP is considered the primary intracellular mediator of LH action in the corpus luteum, and is synthesised from adenosine triphosphate (ATP) by the enzyme adenylate cyclase. The gonadotrophin receptors are members of the super-family of G-protein-coupled, seven transmembrane receptors (McFarland *et al.*, 1989). Functionally these receptors are characterised by interactions with a guanosine triphosphate (GTP)-binding regulatory protein (or G-protein) upon hormone binding which couples the activated receptor to adenylate cyclase.

In its inactive form the stimulatory G-protein (Gs) binds guanosine diphosphate (GDP). When activated by binding to a receptor-hormone complex the guanyl-nucleotide binding site is altered, allowing GDP to be replaced by GTP. The binding of GTP causes a further conformational change, resulting in the dissociation of heterotrimeric G proteins into α subunits and $\beta\gamma$ dimers. The GTP-bound α-subunit then binds to the catalytic component of adenylate cyclase, which is subsequently

activated to produce cAMP. Following a short period of G-protein activation, Gs hydrolyses bound GTP to GDP, dissociates from adenylate cyclase, reassociates with the βγ dimer and returns to its inactive form (Taussig and Gilman, 1995).

Intracellular cAMP, the product of adenylate cyclase activation, in turn stimulates protein-kinase A (PKA). It is also rapidly destroyed by one or more cAMP phosphodiesterases which hydolyze cAMP to adenosine monophosphate. The binding of cAMP causes the dissociation of the regulatory (R) subunits of protein kinase A from its catalytic (C) subunits, enabling the free C-subunits to phosphorylate target proteins. C-subunits phosphorylate serine and threonine residues when recognised as part of a target sequence present in a number of cytosolic and nuclear proteins: the serine/threonine residue must be downstream from a pair of basic amino acids, separated by a single neutral amino acid (Spaulding, 1993). A major role of protein kinase phosphorylation is the regulation of transcription factor activity. Active PKA modulates nuclear factors which bind to cAMP-responsive elements (CREs) in the promoter regions of cAMP-inducible genes, resulting in either the activation or inhibition of transcription. CRE-binding factors, which include CRE-binding proteins (CREB), CRE modulator (CREM) and activating transcription factor-1 (ATF-1) are members of the basic region/ leucine zipper (bZIP) transcription factor class, some of which can also interact with components of the protein kinase C pathway (Lalli and Sassone-Corsi, 1994; Spaulding, 1993).

LH stimulates multiple signal transduction pathways. In addition to adenylate cyclase mediated events, LH also activates inositol phospholipid-specific

phospholipase C (PLC). PLC generates two second-messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 acts to release Ca^{2+} from intracellular calcium stores. DAG can be cleaved to release arachidonic acid which can itself act as a messenger or be utilised in eicosanoid synthesis. However, its major role is the activation of a crucial serine/threonine protein kinase, Ca^{2+} dependent protein kinase C (PKC) (Newton, 1995). Jun and Fos proteins are targets of activated PKC phosphorylation, and can dimerize to form the nuclear factor AP-1. AP-1 in turn binds to TPA (12-*O*-tetradecanoylphorbol-13-acetate) responsive elements (TREs) present in the promoters of various genes to regulate their expression (Karin and Hunter, 1995).

1.1.5.2 Follicle Stimulating Hormone

LH is secreted in a pulsatile manner, and causes the pulsatile release of progesterone in response. However, the frequency of progesterone pulses exceeds that of LH, and led to the suggestion that progesterone secretion was not solely under LH-control (Hixon *et al.*, 1983). Indeed, almost all pulses of Follicle Stimulating Hormone (FSH) were associated with progesterone release, suggesting that FSH may be a principle regulator of progesterone secretion (Walters *et al.*, 1984). In addition to this temporal relationship, binding characteristic of FSH receptors has been detected in the bovine CL (Manns *et al.*, 1984). However, whilst FSH stimulated progesterone release by granulosa cells, FSH has not been shown to have a direct luteotrophic effect (Hansel *et al.*, 1973). Indeed, early studies of FSH receptor binding and activation which suggested a possible role for FSH in stimulating luteal function utilised preparations of FSH that were contaminated with LH. More recent studies suggest that the corpus luteum is not under the influence of FSH. Low levels

of mRNA encoding the full-length FSH-receptor have been detected in the bovine CL on day 1. However, by day 3 no full-length transcripts were detectable. Splicing of the FSH-receptor primary transcript was also observed in granulosa cells luteinised *in vitro*. The loss of full-length FSH-receptor transcripts associated with luteinisation is likely to prevent FSH action (Rajapaksha *et al.*, 1996).

1.1.5.3 Prostaglandins

Corpora lutea have been shown to produce prostaglandins PGI_2 and PGE_2 (Rodgers *et al.*, 1988), which stimulate progesterone secretion by luteal cells *in vitro* (Alila *et al.*, 1988; Milvae and Hansel, 1980). A regulatory role for these prostaglandins is further supported by the demonstration of PGI_2 and PGE_2 binding sites in the bovine CL (Chegini *et al.*, 1991).

Bovine luteal cells also secrete $PGF_{2\alpha}$ and production is greatest in the early luteal phase (Del Vecchio and Sutherland, 1997; Milvae and Hansel, 1983; Rodgers *et al.*, 1988). Binding sites for $PGF_{2\alpha}$ have been detected in the bovine corpus luteum and were predominantly localised to large luteal cells (Chegini *et al.*, 1991). In ovine corpora lutea, mRNA encoding the $PGF_{2\alpha}$ (FP) receptor was also largely limited to large steroidogenic cells (Juengel *et al.*, 1996; Tsai *et al.*, 1998). In the cow, mRNA encoding $PGF_{2\alpha}$ was present in both large and small steroidogenic luteal cells and endothelial cells, with greatest expression detected in large luteal cells (Mamluk *et al.*, 1998). Whilst the role of uterine $PGF_{2\alpha}$ in luteolysis is established (see section 1.1.6.2), $PGF_{2\alpha}$ has also been shown to stimulate progesterone release, suggestive of a luteotrophic role for luteal $PGF_{2\alpha}$ (Miyamoto *et al.*, 1993).

Administration of the prostaglandin synthetase inhibitor indomethacin, early in the cycle reduced progesterone secretion and luteal lifespan, indicating that prostaglandins are required for early luteal development and function (Milvae and Hansel, 1985). This correlates with the observation that luteal biosynthesis of prostaglandins is highest during the early luteal phase (Rodgers *et al.*, 1988).

The vasodilatory effects of prostaglandins may also influence luteal function, by altering blood flow and luteotrophin supply.

1.1.5.4 Oxytocin

Oxytocin is a major luteal peptide product in cattle and sheep. Whilst there is considerable evidence to support a role for oxytocin in control of luteal regression, the evidence indicating a role for oxytocin in luteinisation and luteal steroidogenesis remain inconclusive.

Bovine granulosa cells isolated from the preovulatory follicle show a dramatic increase in oxytocin mRNA expression in response to the LH surge (Voss and Fortune, 1992). Indeed, oxytocin gene expression is considered a marker of luteinisation (Smith *et al.*, 1994c). Oxytocin mRNA and protein have been localised to the large steroidogenic cells of the bovine corpus luteum (Fehr *et al.*, 1987; Fields *et al.*, 1992). Luteal oxytocin mRNA peaks around days 1-3, whilst oxytocin concentrations are maximal in the mid-luteal phase (Fehr *et al.*, 1987; Jones and Flint, 1988).

Oxytocin has been suggested to have direct effects on luteal progesterone release. An inhibitory effect of high doses of oxytocin on steroidogenesis in bovine luteal cells of early pregnancy has been demonstrated (Tan *et al.*, 1982), and oxytocin infusions during the early luteal phase inhibited luteal development (Wathes *et al.*, 1991). Others

have reported limited effects of oxytocin on luteal cell progesterone production (Hansel and Dowd, 1986; Rodgers *et al.*, 1985). In further contrast, intraluteal administration of oxytocin using microdialysed bovine corpora lutea stimulated progesterone in a dose dependant manner, and was most effective during the early to mid-luteal phase (Miyamoto and Schams, 1991). A luteotrophic role for oxytocin is supported by the detection of oxytocin receptors in the bovine CL, which display maximal binding in the mid-luteal stage (Okuda *et al.*, 1992).

1.1.6 Luteolysis

Luteal regression or "luteolysis" occurs at the end of the luteal phase of the non-pregnant ovarian cycle, and also at the end of pregnancy. This loss of luteal function is characterised by decreased progesterone secretion, followed by breakdown of luteal tissue.

1.1.6.1 Role of the uterus

The importance of the uterus in luteolysis was first recognised by Loeb, who demonstrated that hysterectomy resulted in extension of luteal lifespan in guinea pigs (cited by McCracken *et al.*, 1999). Hysterectomy has since been shown to cause maintenance of luteal function in the cow, pig, sheep, mare and the pseudopregnant hamster, rat and rabbit (Andersen *et al.*, 1969; Wiltbank and Casida, 1956). However, in a number of other species the uterus is not required for luteolysis to occur. Hysterectomy does not cause luteal maintenance in a number of primate species, including women and rhesus and cynomologous monkeys, the dog, the opossum or the ferret (Andersen *et al.*, 1969; Beling *et al.*, 1969; Castracane *et al.*, 1979; Neill *et al.*, 1969).

In most species where hysterectomy demonstrated a uterine involvement in luteolysis, the effect was unilateral. Removal of the uterine horn adjacent (ipsilateral) to the ovary bearing the CL prevented luteal regression. However, removal of the uterine horn contralateral to the CL did not lengthen luteal lifespan, demonstrating that uterine effects on the ovary were via local rather than systemic pathways (Andersen *et al.*, 1969). In addition, autotransplantation of either the ovary or the uterus to the neck, extended luteal function in ewes, whilst transplantation of the one uterine horn and the adjacent ovary with associated blood system maintained normal ovarian cyclicity (Harrison *et al.*, 1968). In the rabbit and the mare, the uterine luteolysin may be mediated systemically (Ginther and First, 1971; Hunter and Casida, 1967).

The local transport of a uterine luteolysin to the corpus luteum in the sheep and cow is via a veno-arterial countercurrent system. The uterine factor appears to pass from the uterine vein to the ovarian artery directly, despite a lack of vascular connections between the two vessels. The ovarian artery is highly convoluted, and is closely apposed to the uterine vein, creating extensive regions of contact. Surgical manipulations of the veno-arterial system support the theory that a factor borne by the uterine venous output causes regression of the ipsilateral corpus luteum. In addition, ovine uterine venous plasma collected around the time of luteolysis reduces progesterone secretion following infusion into the ovarian artery (Caldwell and Moor, 1971).

1.1.6.2 Nature of the luteolytic factor

Elevated $PGF_{2\alpha}$ concentrations in uterine venous output (Baird *et al.*, 1976), endometrial tissue (Shemesh and Hansel, 1975) and uterine flushings (Bartol *et al.*, 1981) are temporally associated with falling progesterone and expected luteolysis. Exogenous administration of $PGF_{2\alpha}$ causes premature luteal regression in cattle and sheep (McCracken *et al.*, 1970; Rowson *et al.*, 1972; Thorburn and Nicol, 1971), whilst inhibition of uterine prostaglandin synthesis prevents spontaneous luteolysis, and maintains luteal weight and plasma progesterone concentrations (Lewis and Warren, 1977). In addition, immunisation of cyclic ewes against $PGF_{2\alpha}$ prolongs luteal maintenance (Scaramuzzi and Baird, 1976), suggesting that prostaglandin, in particular $PGF_{2\alpha}$ is required for luteolysis, thus providing further evidence that $PGF_{2\alpha}$ is the natural luteolysin.

1.1.6.3 Secretion of $PGF_{2\alpha}$

In the ewe, the first significant increases in $PGF_{2\alpha}$ concentrations in utero-ovarian venous blood occur on days 12-14 (Baird *et al.*, 1976), and the primary $PGF_{2\alpha}$ metabolite 15-keto-13, 14 dihydro $PGF_{2\alpha}$ (PGFM) is detectable in bovine plasma and uterus, at increasing concentrations from around day 14 (Parkinson and Lamming, 1990; Shemesh and Hansel, 1975). $PGF_{2\alpha}$ secretion is pulsatile, and $PGF_{2\alpha}$ is released from the uterus in a series of pulses which are typically 6-8 hours apart. Low level PGFM episodes can be detected on day 14 (Parkinson and Lamming, 1990), the amplitude of pulses of PGFM are markedly increased during luteolysis and in contrast are inhibited in the pregnant animal (Kindahl *et al.*, 1984).

1.1.6.4 Regulation of uterine secretion of $PGF_{2\alpha}$

1.1.6.4.1 The role of oxytocin

Uterine secretion of $PGF_{2\alpha}$ can be stimulated by oxytocin (Lafrance and Goff, 1990; Roberts and McCracken, 1976). Oxytocin is released primarily from the corpus luteum (Hooper *et al.*, 1986) where it is stored in large luteal cell secretory granules. Around the time of luteolysis, spontaneous episodes of $PGF_{2\alpha}$ secretion occur concurrently with elevated oxytocin concentrations (Hooper *et al.*, 1986; Vighio and Liptrap, 1986). In addition, immunoneutralisation of oxytocin in ewes significantly extended the oestrous cycle, reflecting prolonged luteal lifespan (Sheldrick *et al.*, 1980), and exogenous administration of oxytocin causes premature luteolysis (Armstrong and Hansel, 1959), providing support for a regulatory role for oxytocin in luteolysis. Furthermore, the observation that ovarian secretion of oxytocin is stimulated by prostaglandin analogue (Flint and Sheldrick, 1982) has led to the suggestion that a positive feedback loop exists between the two hormones (Flint and Sheldrick, 1983). Uterine $PGF_{2\alpha}$ initiates luteal oxytocin release (Moore *et al.,* 1986), which subsequently stimulates further $PGF_{2\alpha}$ release from the uterus. Pulsatile secretion may be established by a combination of effects, with the interval between episodes determined by transient uterine refractoriness to further oxytocin stimulation, luteal desensitisation to $PGF_{2\alpha}$, and depletion of oxytocin stores or $PGF_{2\alpha}$ precursors (Flint *et al.*, 1990; Silvia *et al.*, 1991).

However, others have failed to demonstrate both a role for oxytocin in modulating luteal function *in vivo,* as determined by cycle length, luteal weight and progesterone concentration (Milvae *et al.*, 1991), and a link between $PGF_{2\alpha}$ and oxytocin *in vitro*

(McCann and Flint, 1990). Furthermore, frequent sampling of ovine uterine-ovarian venous output demonstrated that whilst most pulses of $PGF_{2\alpha}$ coincided with a pulse of oxytocin, a number of oxytocin pulses occurred in the absence of a $PGF_{2\alpha}$ pulse (Hooper *et al.*, 1986). In the ewe, surgical lutectomy did not affect the frequency of pulsatile $PGF_{2\alpha}$ release, although pulse amplitude was reduced (Mann and Lamming, 1995), and depletion of oxytocin stores did not affect luteolysis or length of the oestrous cycle in cattle (Kotwica and Skarzynski, 1993).

Oxytocin is synthesised *de novo* in the ruminant corpus luteum. Messenger RNA encoding oxytocin is expressed at high levels in the early luteal phase and declines thereafter (Fehr *et al.*, 1987; Ivell *et al.*, 1990; Jones and Flint, 1988), whilst oxytocin concentrations peak at mid-cycle, and decline during the late luteal phase, prior to functional regression (Jones and Flint, 1988; Wathes *et al.*, 1984;). Indeed the lack of temporal association between maximal oxytocin levels and luteolysis seems paradoxical in light of oxytocins' expected role in luteolysis.

In order that oxytocin can stimulate $PGF_{2\alpha}$ release, the uterus must acquire responsiveness to oxytocin. Uterine sensitivity develops during the late luteal phase (Roberts *et al.*, 1976; Silvia and Taylor, 1989) and is the likely product of increased oxytocin receptors (Fuchs *et al.*, 1990; Roberts *et al.,* 1976), increased $PGF_{2\alpha}$ precursor utilisation (Silvia *et al.*, 1991), and $PGF_{2\alpha}$ synthetic capacity (Basu and Kindahl, 1987).

1.1.6.4.2 The role of steroids

The timing and extent of $PGF_{2\alpha}$ production are thought to be further regulated by the ovarian sex steroids oestradiol and progesterone (McCracken *et al.*, 1999; Silvia *et al.*, 1991).

1.1.6.4.2.1 Progesterone

Supplemental progesterone administration in the early luteal phase induces premature $PGF_{2\alpha}$ release and subsequent luteolysis in sheep and cows (Ginther, 1970; Ottobre *et al.*, 1980), whilst luteal regression is delayed following progesterone antagonist treatment. Furthermore, progesterone priming is required in order for oxytocin to stimulate uterine $PGF_{2\alpha}$ secretion in ovariectomised cows or ewes (Homanics and Silvia, 1988; Lafrance and Goff, 1988).

Progesterone regulates the capacity of the uterus to synthesise prostaglandins, by increasing precursor accumulation (Brinsfield and Hawk, 1973) and prostaglandin synthase/ cyclooxygenase concentration and activity (Raw *et al.*, 1988). Whilst progesterone increases the synthetic capacity of the uterus to produce prostaglandins, luteal phase levels may prevent the premature release of $PGF_{2\alpha}$. This effect is proposed to be the result of progesterone inhibiting oestradiol receptor formation, and hence oestradiol-dependant up-regulation of the oxytocin receptor. The inhibitory effect of progesterone is thought to be lost in the late luteal phase, allowing increased uterine responsiveness to oxytocin at an appropriate time (McCracken *et al.*, 1999). Indeed, progesterone withdrawal during the late luteal phase has been shown to stimulate uterine $PGF_{2\alpha}$ secretion, and oxytocin receptor numbers increase following the loss of progesterone action (Leavitt *et al.*, 1985).

1.1.6.4.2.2 Oestradiol

Oestradiol also appears to regulate uterine $PGF_{2\alpha}$ secretion, and the stimulatory effects of oestradiol can be further enhanced by a prior period of progesterone priming (Homanics and Silvia, 1988; Lafrance and Goff, 1988).

Oestradiol may up-regulate prostaglandin synthesis by increasing both the release of arachidonic acid from phospholipids by the enzyme phospholipase A_2 (PLA_2) and the conversion of arachidonic acid to $PGF_{2\alpha}$ by PG synthase (Raw *et al.*, 1988). However, the treatment of ovariectomised ewes with oestradiol-17β markedly reduced mRNA encoding prostaglandin synthase in the ovine endometrium (Salamonsen *et al.*, 1991). In addition, oestradiol is thought to enhance the uterine response to oxytocin stimulation, an effect that may be mediated by increased oxytocin receptors (Nissenson *et al.*, 1978).

Whilst both progesterone and oestradiol have been implicated in the control of uterine $PGF_{2\alpha}$ secretion, and are thought to regulate oxytocin receptor concentration, there is little evidence for direct steroid stimulation of the oxytocin receptor gene (Ivell and Walther, 1999). Indeed, detailed analysis of the oxytocin receptor gene in a range of species has failed to find functional response elements for either oestrogen or progesterone.

The expression of oxytocin receptors may also be regulated by specific non-genomic actions of sex steroids. In the rat uterus, progesterone directly inhibits oxytocin-binding and signal transduction, without interacting with nuclear receptors (Grazzini *et al.*, 1998). However, this effect may prove to be highly species-specific (Ivell and Walther, 1999).

1.1.6.5 Mechanism of $PGF_{2\alpha}$ action

1.1.6.5.1 Receptors

Prostaglandin binding sites have been identified on luteal cells of both cattle (Powell *et al.*, 1975) and sheep (Balapure *et al.*, 1989), and two classes of receptor can be

distinguished on the basis of affinity and capacity for $PGF_{2\alpha}$. Large ovine luteal cells possess predominantly high affinity binding sites, whilst $PGF_{2\alpha}$ binds small luteal cells with low affinity (Balapure *et al.*, 1989). $PGF_{2\alpha}$ binding sites are also more numerous in large luteal cells (Chegini *et al.*, 1991). High affinity binding $PGF_{2\alpha}$ activity is increased during the late luteal phase in bovine corpora lutea (Rao *et al.*, 1979) and luteal cells (Chegini *et al.*, 1991), and $PGF_{2\alpha}$ receptor mRNA follows the same pattern (Sakamoto *et al.*, 1995).

The identification of specific luteal receptors for $PGF_{2\alpha}$ supports a role for $PGF_{2\alpha}$ acting directly on luteal cells. However, whilst $PGF_{2\alpha}$ is clearly luteolytic in ruminants *in vivo*, studies *in vitro* have provided inconsistent results. Indeed, $PGF_{2\alpha}$ has been shown to both stimulate (Speroff and Ramwell, 1970) and inhibit (Wiltbank *et al.*, 1990) luteal progesterone production.

1.1.6.5.2 Multiple sites of action

$PGF_{2\alpha}$ is thought to have multiple sites of action (Auletta and Flint, 1988; McCracken *et al,.* 1999; Michael *et al.*, 1994), and can inhibit a range of steroidogenic responses to LH. LH receptor numbers are reduced following luteal regression (Garverick *et al.*, 1985), however, the $PGF_{2\alpha}$-stimulated reduction in progesterone has been shown to occur before a decline in occupied or unoccupied LH receptors (Diekman *et al.*, 1978). The LH receptor was thought to become "uncoupled" from adenylate cyclase in response to $PGF_{2\alpha}$ as demonstrated by decreased adenylate cyclase activity in day 19 bovine CL (Garverick *et al.,* 1985). It has also been proposed that the decrease in adenylate cyclase activity during luteolysis may be the result of altered interactions of the enzymes regulatory and catalytic subunits (Agudo *et al.*, 1984). The concurrent

increase in phosphodiesterase activity may additionally reduce cAMP levels, and hence alter the response to LH stimulation (Garverick *et al.,* 1985). Alterations in membrane fluidity associated with luteolysis and loss of cell function may also influence receptor-mediated events (Carlson *et al.*, 1982; Goodsaid-Zalduondo *et al.*, 1982).

$PGF_{2\alpha}$ may also exert actions subsequent to cAMP generation, and a primary target may be cholesterol transport. $PGF_{2\alpha}$ causes inhibition of luteal steroidogenesis that results in part from reduced utilisation of lipoprotein substrate (Wiltbank *et al.*, 1990). Messenger RNA encoding StAR, which facilitates cholesterol transport across the mitochondrial membrane, was significantly reduced within 12 hours of induced regression in ewes (Juengel *et al.*, 1995). A similarly rapid decline in StAR mRNA was observed in the bovine CL, with levels becoming undetectable after 24 hours, whilst levels of P450scc mRNA decreased more slowly (Pescador *et al.*, 1996). In addition to effects prior to pregnenolone production, $PGF_{2\alpha}$ has also been shown to markedly reduce 3β-HSD mRNA concentrations (Hawkins *et al.*, 1993), which is in agreement with the dramatic fall observed around the time of natural luteolysis (Couet *et al.*, 1990).

An alternative means of action for $PGF_{2\alpha}$ to induce luteolysis was thought to be alterations in luteal blood flow, and subsequent hypoxia. Decreased luteal blood flow has been demonstrated during natural and $PGF_{2\alpha}$-induced luteolysis (Ford and Chenault, 1981; Nett *et al.,* 1976). However, blood flow reduction is not thought to initiate functional luteal regression, but may play a role in structural luteolysis. (McCracken *et al.*, 1999).

The cellular mechanisms of $PGF_{2\alpha}$ inhibition are thought to be mediated by an increase in intracellular calcium concentration, which can be cytotoxic to luteal cells (Hansel *et al.*, 1991), and can also cause activation of the calcium dependant PKC (Michael *et al.*, 1994; Wiltbank *et al.*, 1991).

1.1.6.6 Local $PGF_{2\alpha}$ production in the corpus luteum

The corpus luteum contains substantial levels of arachidonic acid (Lukaszewska and Hansel, 1980) and has the ability to convert arachidonic acid to PGFs (Milvae and Hansel, 1983). However, luteolysis is not temporally associated with high levels of luteal $PGF_{2\alpha}$ production (Milvae and Hansel, 1983). In contrast, some cytokine products of immune cells which increase around the time of luteolysis such as interferon (IFN)-γ, tumour necrosis factor (TNF)-α, and interleukin (IL)-1β, can stimulate luteal cell $PGF_{2\alpha}$ production, and may play autocrine/ paracrine regulatory roles (Fairchild and Pate, 1991; Fairchild Benyo and Pate, 1992; Nothnick and Pate, 1990).

Interest in the ability of accessory cells of the corpus luteum such as endothelial cells, fibroblasts, pericytes and macrophages to modulate corpus luteum function is increasing. Luteal cells cultured in the presence of accessory cells show elevated $PGF_{2\alpha}$ synthesis (Del Vecchio and Sutherland, 1997). In addition, there is considerable evidence to support a role for an endothelial cell product, endothelin-1 (ET), in mediating the luteolytic actions of $PGF_{2\alpha}$. ET content in the bovine CL was highest on days 17-21 of the cycle, and ET-1 mRNA was significantly increased around the time of luteolysis (Girsh *et al.*, 1996b). $PGF_{2\alpha}$ rapidly increased luteal ET-1 mRNA, when administered to heifers, or luteal slices *in vitro*, and the endothelial cells of bovine

luteal microvessels respond to $PGF_{2\alpha}$ with increased ET production and expression (Girsh *et al.*, 1996a, 1996b). ET-1 production was also elevated in response to oxytocin (Girsh *et al.*, 1996b). ET-1 is thought to mediate the response of CLs to $PGF_{2\alpha}$, since ET-1 suppressed progesterone release from both bovine corpus lutea microdialysed *in vitro* (Miyamoto *et al.*, 1997), and granulosa and theca cells luteinised in culture (Girsh *et al.*, 1996a). In addition, the inhibitory effect of $PGF_{2\alpha}$ on LH-stimulated progesterone secretion was prevented by the addition of an ET receptor antagonist (Girsh *et al.*, 1996a). The ET-1 receptor gene is expressed by small and large steroidogenic luteal cells, and luteal endothelial cells, and is maximal during luteal regression (Mamluk *et al.*, 1999). ET-1 may act directly to reduce the progesterone production by steroidogenic cells, and may in addition contribute to a reduction in luteal blood flow, since ET-1 is a potent vasoconstrictor.

1.1.6.7 Role for immune cells in luteal regression

A potential role for immune cells in luteal regression has long been recognised, following the detection of an influx of leukocytes into the corpus luteum around the time of luteolysis. However, whilst immune cells were thought to be primarily phagocytotic, involved in the removal of cell debris associated with regression, they are now thought to have more active luteolytic functions (Pate, 1995).

Immune cell populations have been detected in corpora lutea from a range of species including guinea pig, rabbit, pig, mare, human and cattle (Bagavandoss *et al.*, 1988; Duncan *et al.*, 1998b; Lawler *et al.*, 1994; Paavola, 1979; Spanel-Borowski *et al.*, 1997; Standaert *et al.*, 1991), and a number of studies have demonstrated increased immune cell numbers prior to luteolysis. The stimulus for macrophage migration is

undefined. However there is increasing interest in a specific chemoattractant for monocytes/ macrophages namely monocyte chemoattractant protein (MCP-1). MCP-1 mRNA is expressed in the corpora lutea of pigs, rats, sheep, cattle and women (Haworth *et al.*, 1998; Hosang *et al.*, 1994; Penny *et al.*, 1998; Senturk *et al.*, 1999; Townson *et al.*, 1996) and is increased during functional luteolysis. In addition to this temporal correlation, the administration of exogenous luteolytic stimuli induces MCP-1 expression in the corpora lutea of sheep, rats and cattle (Bowen *et al.*, 1996; Tsai *et al.*, 1997).

Changes in the expression of cell surface glycoproteins, the major histocompatibility complex (MHC) molecules, may be important for immune cell activation within the corpus luteum. Luteal cells of the early bovine CL (day 6) express Class I MHC molecules, but exhibit limited Class II MHC expression. However, Class II MHC expression increases during the oestrous cycle, and maximal expression was observed prior to luteolysis. Class II MHC expression can be induced following $PGF_{2\alpha}$ administration and is significantly reduced in corpora lutea of pregnancy (Fairchild Benyo *et al.*, 1991).

The expression of Class II MHC molecules is largely restricted to antigen-presenting cells, and is involved in antigen recognition. Hence, it is thought that the luteal cell expression of Class II MHC molecules may stimulate an immune response, despite the non-immune cell location. Indeed, bovine luteal cells can stimulate T-cell proliferation in a Class II-restricted manner, and the proliferative response was greatest in the presence of cells from regressing corpora lutea (Petroff *et al.*, 1997).

Secreted products of activated immune cells can have important effects on luteal cell function. Cytokines can be directly cytotoxic, can inhibit progesterone production and can stimulate luteal cell prostaglandin production (Pate and Townson, 1994).

Immune cells have also been implicated in the regulated production of reactive oxygen species (ROS) which may further mediate luteolytic events. Indeed activated leukocytes are among the most potent generators of ROS. ROS such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), and lipid peroxides (formed by the actions of ROS on polyunsaturated fatty acids) are generated in the rat CL at luteolysis (Riley and Behrman, 1991; Sawada and Carlson, 1991; Shimamura *et al.*, 1995). In addition, levels of the protective antioxidant ascorbic acid (Vitamin C) are reduced in response to $PGF_{2\alpha}$. This rapid depletion of ascorbic acid is the result of $PGF_{2\alpha}$ inhibiting ascorbic acid uptake from the circulation, combined with increased luteal cell ascorbic acid secretion (Musicki *et al.*, 1996). The corpus luteum has other mechanisms to protect against ROS damage, that include degradative enzyme activity, antioxidant Vitamin E, and radical scavengers such as the carotenoids (Aten *et al.*, 1992; Chew *et al.*, 1984).

ROS have several luteal sites of action, damaging the plasma membranes of luteal cells (Wu *et al.*, 1993), and inhibiting rat luteal cell steroidogenesis. The anti-steroidogenic effect may result from the inhibition of adenylate cyclase activation, impaired delivery and utilisation of steroid precursors, and inhibition of protein synthesis (Behrman and Aten, 1991; Behrman and Preston, 1989; Kodaman *et al.*, 1994; Musicki *et al.*, 1994). Whilst ROS can clearly influence important aspects of luteal function directly, they may stimulate other effector systems with luteolytic results.

The heat shock proteins (Hsp) may form such a system, and are produced in response to ROS in other cells (Heufelder *et al.*, 1992). Hsp 70 is induced in the corpora lutea of rats and sheep following either exogenous $PGF_{2\alpha}$ or natural luteolysis (Khanna *et al.*, 1995a; Murdoch, 1995). A role for Hsp 70 in luteal regression is further supported by its ability to inhibit rat luteal progesterone secretion, an effect that is reversed by blocking Hsp synthesis (Khanna *et al.*, 1995b).

1.1.6.8 Apoptosis in luteolysis

Apoptosis is a form of cell death, distinct from pathological or necrotic cell death, which serves an important physiological role in the maintenance of tissue homeostasis (Martin *et al.*, 1994; Steller, 1995; Ueda and Shah, 1994).

Cells undergoing physiological cell death by apoptosis display a series of characteristic changes. Typically cells shrink and lose cell-cell contacts. Nuclear condensation creates dense aggregates of chromatin and membrane alterations result in the fragmentation of the cell into membrane-bound "apoptotic bodies". Apoptotic cells are commonly phagocytosed, and the removal of cellular debris does not evoke an inflammatory reaction. Cell death caused by apoptosis is commonly associated with marked deoxyribonucleic acid (DNA) fragmentation, caused by endonuclease-mediated internucleosomal chromatin cleavage. The resultant "DNA laddering" is often used as a marker.

The morphological changes characteristic of the process now known as apoptotic cell death, were first observed in the ovary over a century ago, and apoptosis is now thought to be important in perinatal germ cell loss, follicular atresia and luteal

regression (Amsterdam and Selvaraj, 1997; Tilly, 1993). Indeed, apoptotic cells have since been detected in regressing corpora lutea of many species, and endonuclease-mediated DNA fragmentation has been identified at the time of structural luteal degeneration (Bacci *et al.*, 1996; Boone and Tsang, 1998; Juengel *et al.*, 1993; McCormack *et al.*, 1998; Murdoch, 1995; Young *et al.*, 1997; Yuan and Giudice, 1997; Zeleznik *et al.*, 1989).

The fate of ovarian cells is thought to be determined by the complex interplay of survival factors and apoptotic signals. The gonadotrophins are crucial to the growth and development of follicles. Reduced gonadotrophin concentrations induce follicular apoptosis, whilst gonadotrophin treatment is anti-apoptotic, suggesting that FSH and LH act as follicle survival factors (Chun *et al.*, 1994, 1996). The treatment of ovarian cells in culture with serum deprivation has identified numerous additional factors that can regulate cell survival when replaced *in vitro*. Ovarian steroids, glucocorticoids, ECM components and a range of growth factors and cytokines (e.g. IGF-I, epidermal growth factor (EGF), transforming growth factor (TGF)α, bFGF, IL-1β, IL-6) may all influence apoptotic pathways (Amsterdam *et al.*, 1999; Hsu and Hsueh, 1997).

Apoptotic pathways (White, 1996) appear to be well-conserved across species from the nematode *Caenorhabditis elegans* to mammals, and among different cell types. One such well-conserved gene with an important regulatory role in apoptosis is the proto-oncogene *bcl-2*. The protein product of *bcl-2* inhibits apoptosis resulting from a range of stimuli. Several genes related to *bcl-2* also encode proteins with key roles in mediating cell death. BAX interacts with BCL-2 and potentially antagonises its

protective activity. The BAX: BCL-2 ratio is thought to be critical in determining susceptibility to cell death. A further member of the *bcl-2* gene family *bcl-x*, is uniquely processed via alternative splicing, to produce a long and short form with markedly different regulatory potential: BCL-X_{LONG} has death suppressor activity, whereas BCL-X_{SHORT} inhibits cell survival (Adams and Cory, 1998).

Cell fate is further influenced by the products of tumour suppressor genes. The p53 protein can arrest cell-cycle progression following cellular DNA damage, via alterations in kinase activity. Alternatively, when DNA damage is too extensive to be repaired, high levels of p53 can induce apoptosis. The mechanism of p53 action may involve cross-talk with *bcl-2*-related genes, since p53 protein can directly activate *bax* gene transcription and repress *bcl-2* expression. In addition, alterations in murine double minute-2 (mdm-2) oncogene expression may be important in controlling a cell's entry into apoptosis, based on the ability of mdm-2 to complex with the p53 protein and to neutralise p53-mediated transactivation (Evan and Littlewood, 1998; Oren, 1999).

A family of cytoplasmic proteases are thought to be important executors of cell death, promoting apoptosis through the degradation of proteins crucial to cellular integrity. This family comprises of at least ten cysteine proteases or "caspases", related to the interleukin-1β converting enzyme (ICE), which are structurally and functionally homologous to the *C.elegans* death gene ced-3 (Thornberry and Lazebnik, 1998). Pro-survival proteins such as BCL-X_{LONG} may function by inactivating caspase activity.

Known regulators of cell death have been localised to corpora lutea undergoing regression, in addition to the identification of physiological markers of apoptosis. For example, luteal cells of several species express a number of *bcl-2*-related genes. In the bovine corpus luteum, pro-apoptotic *bax* mRNA expression was significantly elevated during luteal regression and was accompanied by increased p53 mRNA levels (Rueda *et al.*, 1997). In the rabbit, withdrawal of luteotropic oestradiol results in luteal cell apoptosis and a coincident increase in *bax* mRNA (Goodman *et al.*, 1998). In contrast, in women luteal BAX production was not altered at luteolysis (Rodger *et al.*, 1998). Anti-apoptotic *bcl*-2 family members have also been localised within the regressing CL, and *bcl-x* mRNA levels decreased in the rabbit CL coincident with apoptosis (Goodman *et al.*, 1998). However, limited changes in BCL-2 protein and longform *bcl*-x were observed in the human and bovine CL respectively (Rodger *et al.*, 1995; Rueda *et al.*, 1997). Caspase mRNA and protein activity has also been detected in luteolytic tissue. A $PGF_{2\alpha}$-initiated increase in both caspase-3 mRNA expression and activity was detected in ovine CL (Rueda *et al.*, 1999) and high levels of caspase-3 have been detected in the human corpus luteum (Krajewska *et al.*, 1997). Studies in the rat found no difference in the distribution or intensity of caspase-3 immunostaining in CL that were positive or negative for apoptosis, however this study did not distinguish between the proenzyme and the mature activated form of caspase-3 (Boone and Tsang, 1998). Whilst in the cow, ICE/caspase-1 mRNA levels were significantly higher in day 21 regressed CL than day 21 functional pregnant CL (Rueda *et al.*, 1997). A role for additional regulatory pathways such as Fas and Fas ligand (Roughton *et al.*, 1999) and ubiquitin (Murdoch

et al., 1996) in luteal apoptosis has also been suggested, although their mechanisms of action are not well characterised.

1.1.6.9 Structural luteal regression

Luteolysis is characterised by both functional and structural changes. In the absence of pregnancy the ECM undergoes extensive remodelling to enable the removal of non-functional luteal tissue. The ECM not only provides a physical structure for cellular attachment, it can also influence aspects of cellular function. The ECM can sequester growth factors and their binding proteins and hence influence their mobilisation. In addition, cell-matrix interactions can directly influence cell behaviour. The dissolution of matrix components therefore has considerable regulatory potential (Streuli, 1999).

There is limited information on the composition and organisation of the luteal ECM. However, the ruminant luteal ECM is thought to be composed primarily of collagen (types I and IV), fibronectin and laminin (Silvester and Luck, 1999; Zhao and Luck, 1995). Indeed, collagen is a major component of the bovine corpus luteum, accounting for up to one sixth of luteal dry weight (Luck and Zhao, 1993).

A number of enzymes are responsible for ECM remodelling. These include the plasminogen activator (PA)/ plasmin system, and the large family of matrix metalloproteinases (MMPs).

1.1.6.9.1 Plasminogen activators

Two plasminogen activators, the tissue-type PA (tPA) and urokinase-type PA (uPA), are responsible for converting the zymogen plasminogen into the proteolytically active enzyme plasmin. Plasmin has fibrinolytic activity, can activate other proteases

involved in matrix breakdown, and can degrade both laminin and fibronectin. To prevent uncontrolled tissue destruction, the proteolytic activity of the plasminogen activator-plasmin system is regulated by the actions of two specific protease inhibitors, PA inhibitor type 1 (PAI-1) and type 2 (PAI-2).

Plasminogen activators have been identified as luteal secretory products of the rhesus monkey (Feng *et al.*, 1993), rat (Liu *et al.*, 1995, 1996) and sheep (Smith *et al.*, 1997), and luteolysis was temporally associated with increased expression of mRNA encoding rat tPA (Liu *et al.*, 1996). Luteal PA activity is controlled by the local production of PAI-1, and unexpectedly mRNA encoding PAI-1 also increased following both prostaglandin induced (Smith *et al.*, 1997) and natural luteolysis (Liu *et al.*, 1996). However, despite the increase in inhibitor expression, net PA activity was elevated at the time of luteolysis in the rat and monkey supporting a role for PA activity in the dissolution of matrix components (Feng *et al.*, 1993; Liu *et al.*, 1995, 1996). It has also been suggested that tPA may play a role in functional luteal regression, since the addition of tPA to rat luteal cells in culture decreased progesterone production (Liu *et al.*, 1995), and immunoneutralisation of endogenous tPA increased progesterone production in the rat and monkey (Feng *et al.*, 1993; Liu *et al.*, 1995).

1.1.6.9.2 Matrix Metalloproteinases

Considerable attention has focused on the role of matrix metalloproteinases in ECM degradation in the ovary (Duncan, 2000; McIntush and Smith, 1998; Smith *et al.*, 1999). The family of MMPs has at least 17 members, which display different specificities for ECM proteins. MMPS are largely secreted in a latent proenzyme

form and undergo proteolytic cleavage to produce the active enzyme. Association with specific inhibitors, the TIMPs, further regulates MMP activity.

Structural luteolysis induced by prolactin or GnRH agonist treatment caused an increase in total proteinase activity in the rat CL, and was associated with increased activated MMP-2 (Endo *et al.*, 1993) and MMP-2 mRNA expression (Goto *et al.*, 1999). Others have shown that mRNA encoding MMP-13 is uniquely expressed in the regressing rat CL (Liu *et al.*, 1999). Evidence from human corpora lutea also supports a role for MMPs in regulating structural luteolysis. MMP-2 activity and mRNA expression peaked in the late luteal phase. MMP-9 activity was also high around the time of luteolysis, however MMP-9 levels were also raised in the early luteal phase suggesting an additional role in luteinisation (Duncan *et al.*, 1998a). Gelatinase activity has been detected in the developing bovine corpus luteum, and attributed to MMP-2 and MMP-9 (Goldberg *et al.*, 1995), however, the potential role of MMPs in luteal regression has not been addressed.

TIMPs, most notably TIMP-1, have been detected in bovine (Goldberg *et al.*, 1996), ovine (Smith *et al.*, 1994a), porcine (Smith *et al.*, 1994b), murine (Bagavandoss, 1998), marmoset (Duncan *et al.*, 1996), and human (Duncan *et al.*, 1998a) luteal tissue. TIMPs are important regulators of MMP activity, and the ratio of TIMPs to active MMPs largely determines the extent of ECM degradation. Therefore luteal regression might be expected to be associated with reduced inhibitor levels. In the marmoset corpus luteum induced luteal regression was correlated with a significant reduction in mRNA encoding TIMP-1, which may allow for increased ECM degradation by MMPs (Duncan *et al.*, 1996). In addition, levels of TIMP-1 protein were reduced in the ovine CL following $PGF_{2\alpha}$ treatment (McIntush and Smith,

1997). However, others have failed to demonstrate a correlation between luteolysis and reduced TIMP concentrations or mRNA expression. In the cow CL concentrations of mRNA encoding both TIMP-1 and TIMP-2 showed transient increases following induced luteal regression (Juengel *et al.*, 1994). In the pseudopregnant rat, luteal TIMP-1 mRNA expression was highest on day 1, decreased from days 2 to 12, and increased again around the time of luteolysis (Nothnick *et al.*, 1995). Whilst the proteolytic activity of MMPs must be tightly regulated, it is unclear why in some species the regressing CL is a site of abundant TIMP production. However, it has been suggested that since MMPs and TIMPs occupy different cellular sites, that MMP activity can occur despite the high levels of inhibitor (Bagavandoss, 1998; Duncan *et al.,* 1998a). Alternatively, the balance of active MMPs to TIMPs may be further regulated by the destruction of TIMPs, which may favour ECM degradation. TIMP-1 has been shown to be subject to inactivation by proteolytic degradation or reaction with oxygen radicals (Frears *et al.*, 1996; Itoh and Nagase, 1995).

Whilst TIMPs are responsible for much of the MMP-inhibitory activity, broad spectrum protease inhibitors such as the α-macroglobulin–type inhibitors have also been shown to contribute to the regulation of MMP activity in the ovary (Nothnick *et al.*, 1995). Indeed, α2-macroglobulin protein content and mRNA expression declines in the pregnant rat CL when functional luteolysis occurs at parturition (Gaddy-Kurten *et al.*, 1989).

1.1.6.10 Maternal recognition of pregnancy

In the event of pregnancy, the corpus luteum must be maintained. The inhibition of luteolysis requires the 'maternal recognition of pregnancy' in response to the products of conception, and subsequent interruption or modification of luteolytic prostaglandin release (Thatcher *et al.*, 1997).

The critical period for recognition of pregnancy in the cow is around day 16 after conception. Luteal lifespan is extended despite the removal of bovine embryos on day 17 but not day 15. Furthermore, infusion of day 17 or day 18 embryo homogenates into the uterine lumen of non-pregnant heifers has an anti-luteolytic effect (Northey and French, 1980).

The embryos of ruminants regulate luteal maintenance via the secretion of antiluteolytic interferons. In the sheep and cow, the embryonic signal is provided by ovine (o) or bovine (b) trophoblast protein-1 (TP-1), which has since been characterised as a type I IFN, and subsequently renamed IFN-τ (Roberts *et al.*, 1992). Homologous proteins have also been identified as products of goat and red deer conceptuses (Demmers *et al.*, 1999; Guillomot *et al.*, 1998). Intrauterine infusion of purified trophoblast proteins or recombinant IFN-τ extends luteal function in cyclic cattle (Helmer *et al.*, 1989b; Meyer *et al.*, 1995), ewes (Godkin *et al.*, 1984; Martal *et al.*, 1990), goats (Ott and Newton, 1993) and red deer (Demmers *et al.*, 2000). In addition, uterine administration of IFN-τ is associated with attenuation of both basal and oxytocin-induced endometrial prostaglandin secretion, both *in vivo* and *in vitro* (Danet-Desnoyers *et al.*, 1994; Helmer *et al.*, 1989a; Meyer *et al.*, 1995).

Embryonic antiluteolysin production is further supported by the detection of messenger RNA encoding bTP-1 and bTP-1 protein in the trophectoderm of bovine conceptuses (Farin *et al.*, 1990; Lifsey *et al.*, 1989). In addition, bTP-I mRNA and protein levels were highest at blastocyst elongation, coincident with maternal recognition of pregnancy around days 15-17 of gestation (Bartol *et al.*, 1985; Farin *et al.*, 1990).

IFN-τ is thought to prevent the development of the luteolytic mechanism, and to act on the uterus rather than the corpus luteum. The primary targets of the inhibitory effects of IFN-τ are endometrial oxytocin receptors. Intrauterine injection of ovine conceptus secretory proteins decreased the number and binding affinity of oxytocin receptors (Mirando *et al.*, 1993), and inhibited subsequent second messenger generation (Mirando *et al.*, 1990). IFN-τ may regulate the oxytocin receptor directly or indirectly by down-regulating endometrial oestrogen receptors and/or maintaining endometrial progesterone receptors.

In cyclic cows, endometrial oestrogen receptor numbers increased dramatically from day 14 to day 16, to reach levels ninefold higher than in pregnant ewes on day 16 (Ott *et al.*, 1993). Furthermore, intrauterine injection of ovine conceptus secretory proteins from day 11 to day 15 post-oestrus reduced endometrial oestrogen receptor concentration and mRNA (Mirando *et al.*, 1993). Oestrogen receptor mRNA and protein levels were also suppressed by infusion of recombinant oIFN-τ into the uterus of cyclic ewes (Spencer *et al.*, 1995). However, in cows the presence of an embryo was not associated with any alteration in oestrogen receptor mRNA expression (Robinson *et al.*, 1999).

Interferon-τ does not appear to regulate oxytocin receptor number via maintenance of progesterone receptors in the cow, since progesterone receptor mRNA and protein concentrations were not different between pregnant and non-pregnant animals at the time of luteolysis (Robinson *et al.*, 1999). The influence of IFN-τ on regulation of progesterone receptor levels in sheep is unclear, although the loss of endometrial progesterone receptors may be prevented during pregnancy (Ott *et al.*, 1993).

In addition to inhibiting oxytocin reception, the embryo may also inhibit prostaglandin synthesis, by down-regulation of cycloxygenase-2 and prostaglandin F synthase expression (Xiao *et al.*, 1999).

1.2 Overview of the IGF System

The insulin-like growth factors, IGF-I and IGF-II, are homologous polypeptide growth factors with widespread roles in growth and development. IGF-I binds preferentially to the type 1 IGF receptor, a tyrosine kinase receptor related to the insulin receptor. The type 1 IGF receptor also binds IGF-II with slightly lower affinity than IGF-I, and binds insulin with still lower affinity. The IGF-II receptor, which is identical to the mannose-6-phosphate receptor, binds IGF-II with high affinity, binds IGF-I with lower affinity and does not recognise insulin. In addition, at high concentrations both IGF-I and –II can stimulate the insulin receptor. The IGFs are regulated by the specific interaction with high affinity IGFBPs, which have been shown to both inhibit and enhance IGF actions. IGFBPs are subject to post-translational modifications including proteolytic cleavage, which can alter binding of the IGFs to the IGFBPs, and further modulate the biological actions of the IGFs.

1.2.1 IGFs

1.2.1.1 Discovery

The growth promoting activities of pituitary–derived somatotrophin or growth hormone (GH) are well established *in vivo* and *in vitro*. The administration of GH to hypohysectomised rats was found to stimulate the release of a factor in serum, which mediates GH actions on cartilage growth and sulphate incorporation (Salmon and Daughaday, 1957). Subsequently, this sulphation factor was renamed somatomedin, due to its generalised ability to mediate the actions of somatotrophin on target tissues (Daughaday *et al.*, 1972). At around the same time, studies were underway to characterise serum-borne factors with insulin-like effects. Distinct from insulin, the bioactivity of these factors *in vitro* would be unaffected by the addition of an anti-insulin antibody and was hence described as non suppressible insulin-like activity (NSILA). Following the observation that both the NSILA extract and somatomedin stimulated sulphate incorporation into cartilage and affected glucose metabolism, similarity of identity was proposed. The amino acid sequence of purified NSILA was deduced and it was shown that its insulin-like activity was accompanied by insulin-like structure, with NSILA showing 48% homology with human proinsulin, and was renamed IGF-I. A second insulin-like factor was shown to be structurally homologous, but not identical to IGF-I, and was designated IGF-II. Subsequently the similarity of identity between NSILA and somatomedin was confirmed by amino acid sequencing, which showed that somatomedin-C was identical to IGF-I (Klapper *et al.*, 1983).

1.2.1.2 Peptide structure

The IGFs are both low molecular weight, single chain polypeptides, with structural similarity to proinsulin. Human IGF-I was first purified and sequenced by Rinderknect and Humbel in 1978 (Rinderknect and Humbel, 1978a). It consists of 70 amino acids, has a molecular weight of 7646 Da, and a basic (8.5) isoelectric point. The amino acid sequences of both IGF-I and IGF-II are highly conserved (Rotwein, 1991). Bovine (Honegger and Humbel, 1986) and porcine (Francis *et al.*, 1989b) IGF-I sequences are identical to human IGF-I, whilst ovine IGF-I has a single substitution at residue 66 (Francis *et al.*, 1989a). There are three amino acid differences between rat and human IGF-I (Shimatsu and Rotwein, 1987), and an additional fourth substitution in the mouse sequence (Bell *et al.*, 1986).

Human IGF-II is a 67-amino acid polypeptide, with a molecular weight of 7470 Da and an acidic (<6.5) isoelectric point (Rinderknecht and Humbel, 1978b). IGF-II is approximately 70% homologous with IGF-I and 50% homologous with proinsulin. The IGF-II peptide sequence is also highly conserved with sixty of sixty seven residues being invariant for the human, cow, pig, sheep, rat and mouse (Rotwein, 1991). Porcine IGF-II differs from human IGF-II by just one substitutive change (Francis *et al.*, 1989a), while bovine (Honegger and Humbel, 1986) and ovine IGF-II (Francis *et al.*, 1989b) have three and four amino acid changes respectively.

1.2.1.3 Gene structure

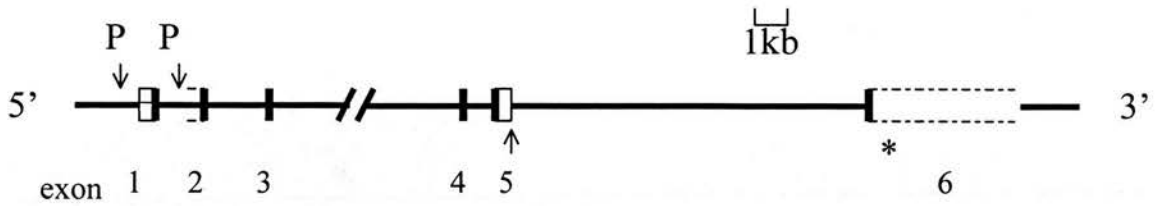
The human IGF-I gene spans more than 90 kilobases, and comprises six exons, five introns and a number of promoter sites (Figure 1.3). Exons 1 and 2 encode part of the signal peptide. Exons 3 and 4 encode the mature IGF-I peptide, plus a portion of

the signal peptide and E domain. Exons 5 and 6 encode the E domain and are the site of potential polyadenylation (Rotwein, 1991; Sussenbach *et al.*, 1992).

The mature IGF peptides are the products of complex processing at both the peptide and mRNA levels. The primary IGF-I transcript undergoes alternative splicing to produce precursor peptides IGF-IA and -IB, which have different carboxy-termini (Jansen *et al.*, 1983; Rotwein, 1986). The two precursors have 16 residues of the E domain in common, the remaining amino acids of the human IGF-IA precursor are derived from exon 5, whilst IGF-IB is derived from exon 6. Divergent IGF precursors have also been reported for the rat and mouse (Bell *et al.*, 1986; Shimatsu and Rotwein, 1987). The heterogeneity of IGF-I transcripts is further increased by the use of alternative promoters and variable polyadenylation. The mechanism for post-translational cleavage of the mature peptide from its precursors is unknown.

The processing of IGF-II is similarly complicated (Figure 1.3). The IGF-II gene in the mouse, rat and human contains a number of untranslated 5' exons. The regions coding for the signal peptide, the mature IGF-II peptide, and E domain are found within the last 3 carboxy-terminal exons in all three species. Multiple IGF-II mRNAs are produced by the use of alternative promoters, transcription start sites, and polyadenylation. The combination of variables enables the production of at least 8 different human IGF-II mRNAs. However, the positioning of promoters adjacent to non-coding exons and the absence of alternative splicing of coding exons produces multiple mRNAs that encode the same precursor (Rotwein, 1991).

a)



b)

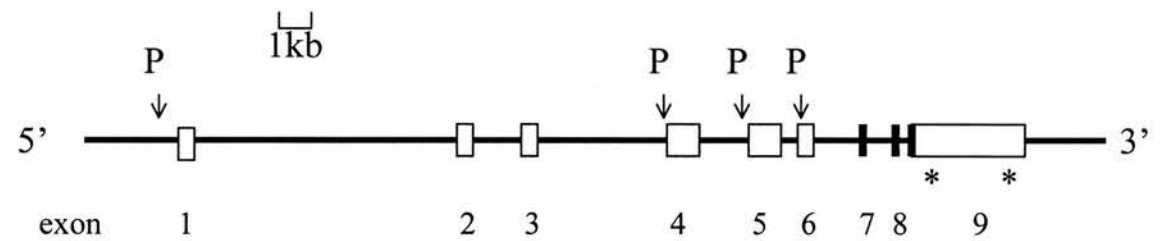


Figure 1.3. Structure of human IGF-I and IGF-II genes.

The exon-intron organization of the human IGF-I (a) and IGF-II (b) genes are shown.

Exons are represented by boxes, with coding regions in black and non-coding regions in white. Asterix represents polyadenylation sites, P represents promoter regions and dotted lines represent regions yet to be determined.

(Adapted from Rotwein, 1991).

1.2.1.4 Regulation of IGF-I expression

IGF-I and –II mRNAs are synthesised at multiple sites. A range of factors regulate IGF-I expression, and for many tissues GH is the principal modulator. GH administration increases both circulating IGF-I levels (Lemal *et al.*, 1989) and IGF-I mRNA abundance (Hynes *et al.*, 1987; Murphy *et al.*, 1987; Roberts *et al.*, 1987). The increase in IGF-I mRNA is the product of enhanced transcription (Mathews *et al.*, 1986) and differential processing (Lowe *et al.*, 1988) and has been demonstrated in the primary site of IGF-I synthesis, the liver, and in extra-hepatic tissues. In addition, cattle with a GH receptor deficiency have reduced blood IGF-I (Chase *et al.*, 1998). Furthermore, the response to GH stimulation occurs rapidly, nuclear IGF-I transcripts are induced within 15 minutes of hormone treatment (Gronowski and Rotwein, 1995), and IGF-I mRNA levels in hypophysectomised rats are restored to normal within 4 hours (Hynes *et al.*, 1987). The molecular mechanisms by which GH stimulates IGF-I gene expression remain incompletely understood (Carter-Su *et al.*, 1996; Gronowski and Rotwein, 1995; Thomas, 1998).

A number of other factors are involved in the regulation of IGF gene expression (Daughaday and Rotwein, 1989; Rotwein, 1991; Simmen, 1991). In reproductive tissues IGF-I mRNA abundance can be modulated by steroid hormones. In the pig, uterine IGF-I mRNA levels are increased by administration of both oestradiol and progesterone, and IGF-I levels secreted into uterine luminal fluid are also raised (Simmen *et al.*, 1990). In the rat ovary, IGF-I mRNA is localised to the granulosa cell layer, and oestradiol treatment doubles ovarian IGF-I transcripts in immature hypophysectomised animals (Hernandez *et al.*, 1989).

IGF-I production may also be regulated by other growth factors, including platelet-derived growth factor (PDGF), FGF, EGF, and TGF-α and β-1 although understanding of the complexity of growth factor interactions remains limited (Simmen, 1991).

Nutrition is an important modulator of IGF-I. The restriction of dietary energy or metabolizable protein, adversely affects IGF-I synthesis and action (Thissen *et al.*, 1994; Wester *et al.*, 1995). A reduction in serum IGF-I levels following food restriction has been demonstrated in a range of species including swine (Buonomo and Baile, 1991), dog (Maxwell *et al.*, 1998), cow (Yambayamba *et al.*, 1996), sheep (Hua *et al.*, 1995), rat (Goldstein *et al.*, 1991; Straus and Takemoto, 1990) and human (Thissen *et al.*, 1994). Hepatic IGF-I mRNA is co-ordinately reduced following fasting and protein restriction. The reduction in mRNA abundance following such nutritional regulation has been proposed to be the result of changes at the level of gene transcription (Hayden *et al.*, 1994; Straus and Takemoto, 1990) and/or post-transcriptional mechanisms such as altered RNA splicing or stability (Zhang *et al.*, 1997). IGF-I expression has also been shown to be induced during development, and in a number of tissues undergoing growth or regeneration.

1.2.1.5 Regulation of IGF-II expression

The regulation of gene expression is less well defined for IGF-II than for IGF-I (Daughaday and Rotwein, 1989; Rotwein, 1991). IGF-II has been implicated as an important modulator of embryonic development. Targeted mutagenesis in mice has demonstrated that IGF-II has a major post-implantation growth-promoting role, and mutation of the IGF-II gene results in a 40% reduction in birth weight (Baker *et al.*,

1993). Investigations into this developmental role have demonstrated that the IGF-II gene undergoes parental imprinting, such that the gene is expressed from only one parent-specific allele (Bartolomei and Tilghman, 1997; Lyle, 1997), and is subject to tissue-specific regulation. The paternal IGF-II gene is transcriptionally active in most mouse embryonic tissues, whereas the maternal gene is silent, with the exception of the choroid plexus and leptomeninges of the brain, where both maternal and paternal alleles are expressed (DeChiara *et al.*, 1991). Imprinting of the IGF-II gene is evolutionarily conserved from mouse to man (Ohlsson *et al.*, 1993). Ovine parthenogenetic embryos, which lack a paternal genome, display growth retardation and subsequent foetal lethality. In addition, whilst normal sheep foetuses express IGF-II, parthenogenetic embryos do not (Feil *et al.*, 1998). Subsequent molecular studies have confirmed that IGF-II is subject to genomic imprinting in the sheep, and that most embryonic tissues express the paternal allele (McLaren and Montgomery, 1999). IGF-II is implicated in the promotion of carcinogenesis, and is highly expressed in a variety of tumours, including embryonal tumours (Scott *et al.*, 1985). It has been suggested that disruption of the normal pattern of imprinting of IGF-II may be important in the regulation of tumorigenesis (Lyle, 1997).

1.2.2 IGF reception

The IGF-I receptor, or type 1 IGF receptor, mediates most of the effects of both IGF-I and IGF-II. The IGF-II receptor, or type 2 IGF receptor, specifically binds both IGF-I and –II, and is identical to the cation-independent-mannose 6-phosphate receptor (MPR), which is involved in targeting lysosomal enzymes to lysosomes (Oshima *et al.*, 1988). However, the type 2 IGF receptor is not thought to have an IGF signalling function. In addition, the insulin receptor has low binding affinity for

the IGFs and hybrid IGF/ insulin receptors may also have IGF-mediated signalling functions (Nissley and Lopaczynski, 1991; Oh *et al.*, 1993a).

1.2.2.1 The type 1 IGF receptor

1.2.2.1.1 The type 1 IGF receptor: structure

The type 1 IGF receptor and the insulin receptor are heterotetramers, comprised of two classes of subunits, α and β, which are linked in a β-α-α-β conformation (Figure 1.4). The α- and β- subunits are the product of a common precursor, which is proteolytically cleaved to yield the subunits. They are subsequently linked by disulphide bonds, to form an αβ- pro-receptor. The mature receptor is then assembled by the dimerisation and linking of two pro-receptors (Treadway *et al.*, 1989; Ullrich *et al.*, 1985, 1986).

Each α- subunit is entirely extracellular, and contains the ligand binding domain, whilst the β- subunit has a transmembrane domain and a cytoplasmic domain which confers the receptor's tyrosine kinase activity.

Structural similarity of the insulin receptor and the IGF-I receptor has been indicated by a range of functional studies, and subsequently confirmed by sequencing of the complementary DNA (cDNA) encoding the human IGF-I receptor. The two receptors show considerable homology. Overall the two sequences exhibit 50-60% sequence identity, with the greatest homology (84%) being located in the tyrosine kinase domain of the β-subunit (Ullrich *et al.*, 1986).

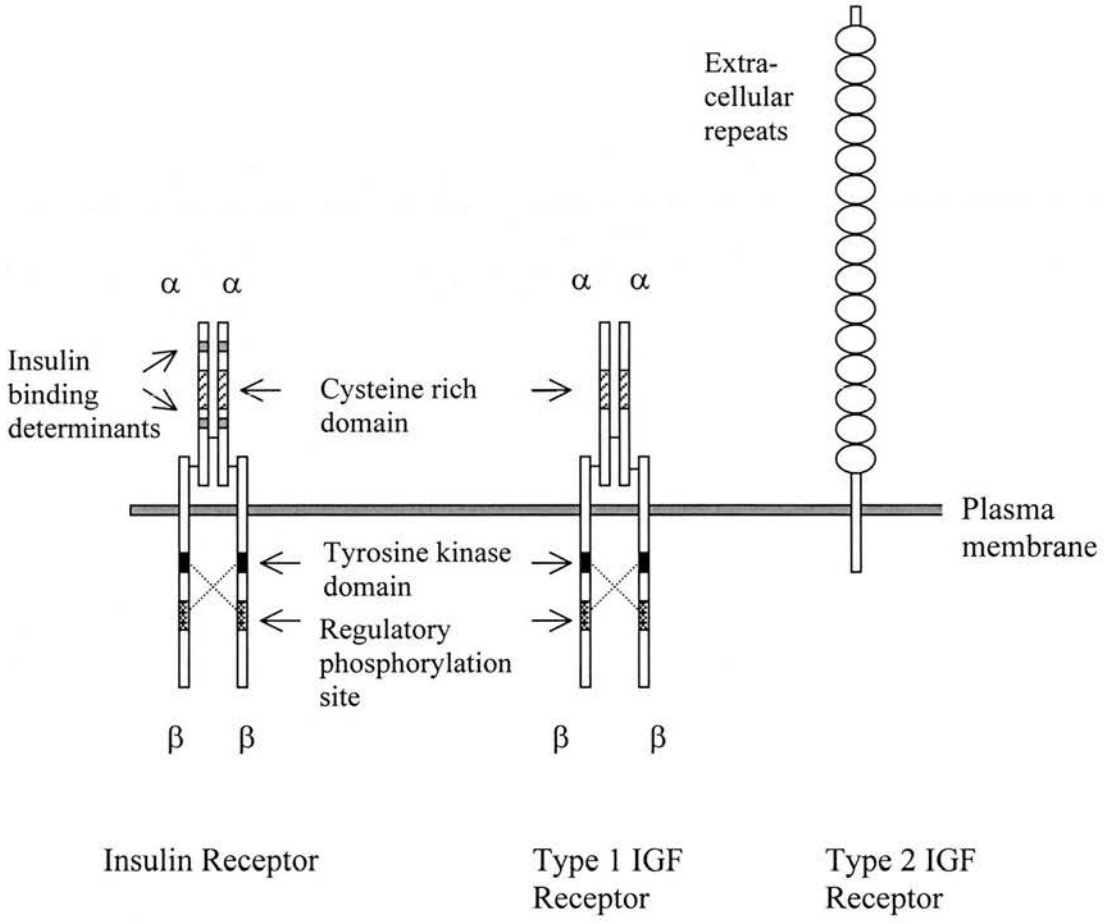


Figure 1.4. Receptor structure of the insulin receptor, type 1 IGF receptor and type 2 IGF receptor.

The insulin and type 1 IGF receptor are structurally related heterotetramers, composed of extracelluar α-subunits which confer ligand binding specificity and transmembrane β-subunits that contain cytoplasmic tyrosine kinase activity. The type 2 IGF receptor is structurally distinct from both the insulin and type 1 IGF receptor, lacks an intrinsic tyrosine kinase domain and is characterised by fifteen extracellular repeats.

1.2.2.1.2 The type 1 IGF receptor: signalling

The type 1 receptor preferentially binds IGF-I with high affinity, IGF-II with up to 15-fold lower affinity, and insulin with low affinity (Steele-Perkins *et al.*, 1988). IGF-I can also bind to the insulin receptor, albeit with 100-fold lower affinity than insulin. A cysteine-rich domain of the IGF-I receptor confers IGF-I binding specificity, whilst the determinants of insulin recognition appear more complex, and are discontinuous (Fabry *et al.*, 1992; Gustafson and Rutter, 1990; Kjeldsen *et al.*, 1991; Schumacher *et al.*, 1991). Ligand occupancy of the α-subunit causes phosphorylation of specific β-subunit tyrosine residues (Jacobs *et al.*, 1983). This ligand-stimulated autophosphorylation occurs as a "trans" intra-molecular reaction between β-subunits within an $\alpha_2\beta_2$ receptor, in both the IGF-I and insulin receptor (Fratalli and Pessin, 1993).

The β subunit contains a tyrosine kinase catalytic domain, which when disrupted by mutagenesis abrogates IGF action, suggesting that IGF-I receptor signalling requires tyrosine kinase activity (Kato *et al.*, 1993). Intrinsic tyrosine kinase activity has also been shown to be important in mediating most, if not all, of the functions of insulin (Debant *et al.*, 1988; Murakami and Rosen, 1991). Subsequent signal transduction also appears similar for the IGF-I and insulin receptors (Figure 1.5).

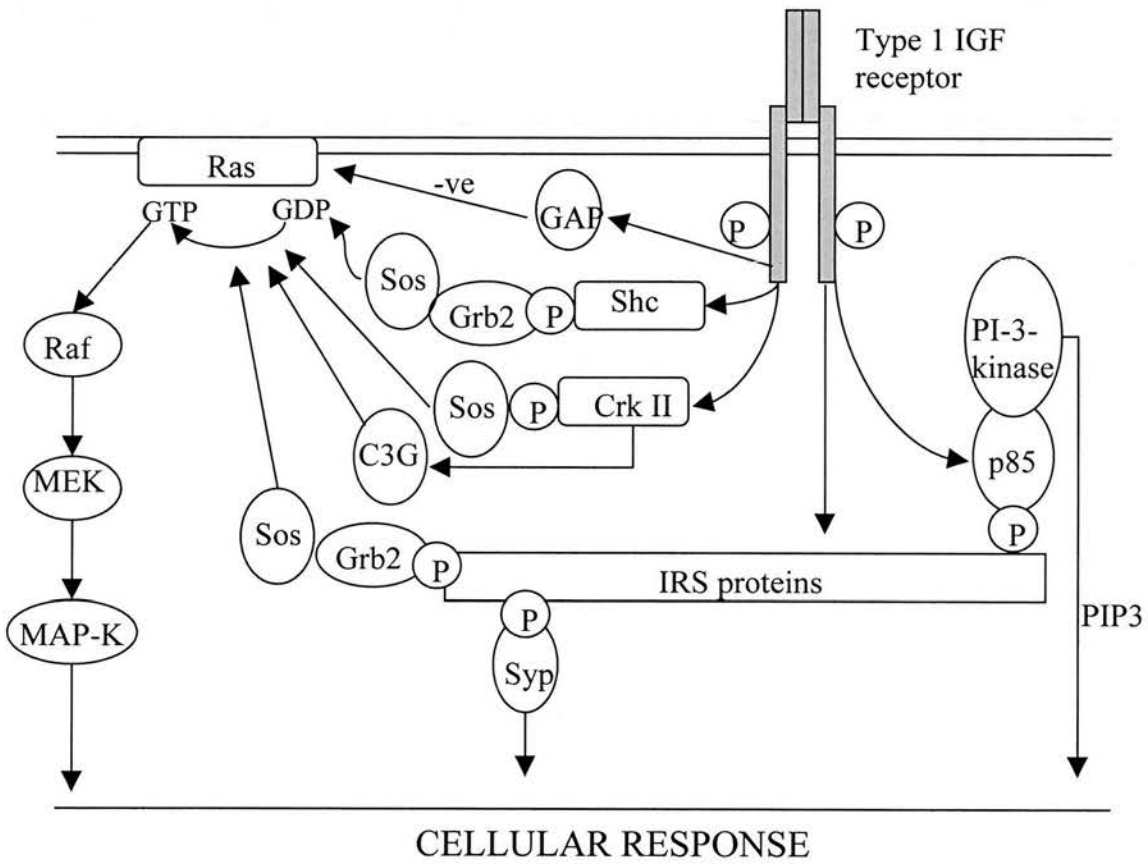


Figure 1.5 Proposed signalling pathways for the type 1 IGF receptor.

A protein with a key role in insulin receptor signalling, the insulin receptor substrate (IRS-1) and its homologues IRS-2 and IRS-3, are also phosphorylated in response to IGF-induced receptor activation (He *et al.*, 1996; Myers *et al.*, 1993; Xu *et al.*, 1999). IRS-1, –2 and -3 contain a number of potential tyrosine phosphorylation sites, and a recognition sequence for interaction with src homology-2 (SH2) domain-containing proteins. Following activation, they act as docking molecules linking the insulin and IGF receptors to signalling pathways.

Despite the high level of conservation between the IRS proteins, and common functional features, evidence now suggests that they are not functionally interchangeable: rather they may play important distinct roles in IGF-I and insulin signalling. Targeted gene knockout of IRS-1 in mice reduces growth by up to 80 % (Brunig *et al.*, 1997), and alters glucose homeostasis (Tamemoto *et al.*, 1994). IRS-2 disruption causes type-2 diabetes in mice, and indicates that IRS-2 is of particular importance in glucose regulation (Withers *et al.*, 1998).

SH2 domain binding by the IRS proteins activates several signalling cascades (Sun *et al.*, 1993). The phosphatidylinositol-3 kinase (PI-3 kinase) is activated via binding of its p85 regulatory subunit, leading to production of the cell-growth signal phosphatidylinositol-3 phosphate (PIP3) (Myers *et al.*, 1993).

The growth factor receptor-bound protein 2 (Grb2) contains Src homology domains. Binding of Grb2 to phosphorylated IRS leads to the eventual activation of mitogen activated protein (MAP) kinase, which signals to the nucleus, and acts as an important intermediate in cellular differentiation and growth (Davis, 1993). Grb2 binding induces the guanine nucleotide releasing factor, Son of Sevenless (Sos), to

activate the GTP-binding protein Ras. In turn, Ras activates the MAP-kinase kinase kinase Raf, which activates the MAP-kinase kinase Mek, leading to the phosphorylation of MAP-kinase on threonine and tyrosine residues and its subsequent activation (LeRoith *et al.*, 1995).

The tyrosine phosphatase Syp can mediate growth factor-stimulated signal transduction, including responses to insulin and IGF-I (Xiao *et al.*, 1994). Syp contains two SH2 domains, and can associate with IRS-1. In addition, direct interaction with the insulin and IGF-I receptors has been suggested (Seely *et al.*, 1995).

The GTPase-activating protein (GAP) also has an SH2 domain, and an association with tyrosine kinase receptor signal transduction. GAP negatively regulates Ras activation, and can also directly interact with the IGF-I receptor via its SH2 domain (Seely *et al.*, 1995).

IGF-I-induced autophosphorylation of the IGF-I receptor can also result in the tyrosine phosphorylation of a cytosolic protein termed Shc (Src-homology 2/ collagen), which is considered a major mediator of IGF-I receptor signalling. Phosphorylated Shc can then complex with Grb2/Sos and subsequently activate the Ras/Raf/MAP kinase pathway, independent of IRS binding (Giorgetti *et al.*, 1994).

IGF-I and insulin receptor signalling appear therefore to share many features, whilst resulting in markedly different physiological outcomes. Signal divergence could be achieved in many ways. The identification of an IGF-I receptor specific substrate, Crk-II (Beitner-Johnson and LeRoith, 1995), suggests potential mechanisms for specificity. Proteins of the Crk family contain SH2 and SH3 domains, and are

considered to be important modulators of growth factor-stimulated signal transduction.

Phosphorylated Crk-II binds guanine nucleotide-releasing factors, which can activate the Ras/Raf/MAP kinase pathway (Tanaka *et al.*, 1994) and/or the Jun kinase pathway (Tanaka *et al.*, 1997). Indeed, the suggestion that IGF-I inducible signalling pathways must exist in addition to PI-3-kinase and MAP kinase activation (Scrimgeour *et al.*, 1997) supports a role for Crk-mediated Jun kinase activation.

1.2.2.2 The type 2 IGF receptor

1.2.2.2.1 The type 2 IGF receptor: structure

The type 2 IGF receptor has a large extracellular domain, composed of 15 repeats, each of around 150 amino acids (Lobel *et al.*, 1988). Within each repeat, there is a conserved pattern of 8 cysteine residues. Repeat number 13 is characterised by a 43 amino acid insertion with homology to fibronectin. The intracellular domain is short, contains sequences known to be potential protein kinase substrates and can be phosphorylated at a number of these sites. The MPR was first described for its role in the recognition of newly synthesised lysosomal enzymes and their subsequent transport to lysosomes from the Golgi apparatus and cell surface (Kornfeld and Mellman, 1989).

The MPR was shown to be multifunctional, following the demonstration that the human IGF-II receptor and the bovine cation-independent MPR had a similar general structure, and 80% amino acid identity (Morgan *et al.*, 1987). The receptor has distinct sites for IGF-II and lysosomal enzyme binding (MacDonald *et al.*,

1988), and can bind both ligands simultaneously (Waheed *et al.*, 1988). However, simultaneous binding may cause noncompetitive alterations in binding.

1.2.2.2.2 The type 2 IGF receptor: signalling

Cell surface type 2 IGF receptors bind IGF-II with high affinity. The ligand-bound receptor is then rapidly internalised, segregated from IGF-II and recycled back to the cell surface, whilst IGF-II undergoes degradation (Oka *et al.*, 1985). This scavenging role for the type 2 IGF receptor is well established, however the receptor's signalling potential remains in question (Nielsen, 1992; Nissley and Lopaczynski, 1991). IGF-II receptor-blocking antibodies fail to affect the response to IGF-II stimulation in a range of cell types, including ovarian granulosa cells (Adashi *et al.*, 1990). In addition, inhibition of IGF-I receptor activation has demonstrated that many of the biological responses to IGF-II are mediated by the type 1 IGF receptor. In contrast, others have demonstrated that blocking the type 1 IGF receptor does not alter IGF-II stimulation (Minniti *et al.*, 1992). The IGF-II receptor, despite the absence of the seven transmembrane helices characteristic of the G-protein-coupled receptor family, may mediate signal transduction via G-protein coupling to the receptor (Nishimoto *et al.*, 1989), which can then activate the MAP-kinase dependant pathway (Groskopf *et al.*, 1997). IGF-II receptor coupling may be cell-type specific, as others have failed to demonstrate functional coupling to G-proteins (Korner *et al.*, 1995).

The type 2 IGF receptor has also been implicated in binding additional mannose-6-phosphate-containing ligands, such as latent TGF-β (Dennis and Rifkin, 1991), leukemia inhibitory factor (LIF) (Blanchard *et al.*, 1999), and proliferin (Lee and

Nathans, 1988). In addition, recent reports have extended the potential functions of the IGF-II receptor by the demonstration that the IGF-II receptor binds retinoic acid and urokinase-type plasminogen activator receptor at sites distinct from the IGF-II and mannose-6-phosphate binding sites (Kang *et al.*, 1998; Nykjaer *et al.*, 1998).

1.2.3 IGF binding proteins

1.2.3.1 Structure and function

In addition to binding their cognate receptors with high affinity, the IGFs bind a family of structurally related secreted proteins, the IGFBPs with comparable affinity (Clemmons, 1997; Jones and Clemmons, 1995; Rechler and Clemmons, 1998). Six high affinity IGFBPs have been identified and characterised, and are numbered according to the order of their discovery. In addition, several related proteins comprise a family of low affinity IGFBPs, designated IGFBP-related proteins (IGFBP-rPs) (Hwa *et al.*, 1999; Kim *et al.*, 1997). The IGFBPs are synthesised and secreted by many tissues, and they can act locally as autocrine or paracrine factors, or as endocrine factors effecting targets distant from the site of production. The IGFBPs transport IGFs, regulating their half-life and clearance. In addition, the high affinity of binding prevents interaction with cell surface receptors, hence regulating IGF bioavailability, and inhibiting IGF activity.

Approximately 75% of IGFs are present as a complex of IGF-I or IGF-II, IGFBP-3 and a glycoprotein termed the acid labile subunit (ALS). Association of IGFs into this ternary complex of approximately 150kDa limits efflux of IGFs out of the vascular compartment, so increasing their half lives, and creating a pool of IGFs unable to exert undesirable insulin-like metabolic effects.

The IGFBPs display considerable structural similarities. Consisting of around 2-300 amino acids, they show between 47 to 67% overall amino acid similarity in the rat, and 49 to 62% in the human. The highest regions of similarity are the N and C terminal domains. In particular, all IGFBPs except IGFBP-6 have 18 conserved cysteines, 12 at the N terminal and 6 at the C terminal. These are conserved both in number and position (Shimasaki and Ling, 1991), and may be important in ligand binding (Lalou *et al.*, 1996).

Whilst the IGFBPs are structurally and functionally homologous, they also exhibit distinctive characteristics. Human IGFBP-1 contains a C-terminal Arg-Gly-Asp (RGD) integrin recognition sequence, which is conserved in the IGFBP-1 sequence of the rat, cow and mouse, (Luthman *et al.*, 1989; Murphy *et al.*, 1990; Schuller *et al.*, 1994; Sneyers *et al.*, 1991b) and in human, mouse, rat, sheep and chick IGFBP-2 (Binkert *et al.*, 1989; Delhanty and Han, 1992; Margot *et al.*, 1989; Schuller *et al.*, 1994).

Integrins mediate adhesion of cells to the ECM. In addition, whilst they lack intrinsic tyrosine kinase activity, there is increasing evidence that integrins are important in intracellular signal transduction. Indeed, integrins can generate signals following ligand-binding, or can mediate signals originating from growth factor and cytokine receptors (Giancotti, 1997; Howe *et al.*, 1998).

IGFBP-1 has been shown to interact with the $\alpha5\beta1$ integrin receptor. In Chinese hamster ovary cells IGFBP-1 stimulates cell migration via specific binding to the $\alpha5\beta1$ integrin receptor, an effect that was abrogated by mutation of the RGD

sequence (Jones *et al.*, 1993c). In the rabbit, IGF-1-stimulated wound healing requires activation of the α5β1 integrin receptor by IGFBP-1 (Galiano *et al.*, 1996).

Whilst the RGD sequence is conserved in IGFBP-2, there is limited information on the mediation of its actions via association with integrin receptors. IGFBP-3 and IGFBP-5 lack an RGD sequence, but can adhere to the surface of cells in culture (Booth *et al.*, 1995), and IGFBP-5 additionally binds to the ECM (Jones *et al.*, 1993b). Glycosaminoglycans such as heparin and heparan sulphate are abundant components in ECM, and both IGFBP-3 and IGFBP-5 contain C-terminal heparin-binding sequences, and exhibit heparin binding affinity (Booth *et al.*, 1995). IGFBP-5 binding to ECM proteoglycans markedly reduces IGF-I binding affinity, and the resultant dissociation of the IGF/IGFBP complex may then potentiate IGF action by increasing potential IGF receptor activation. Binding of IGFBP-3 to cell surfaces also results in reduced IGF-I binding affinity. The cellular response to IGFBP-3 binding is further complicated by the recent discovery of an IGFBP-3 receptor (Leal *et al.*, 1997), identified as the type V TGF-β receptor (TβR-V) which mediates a growth inhibitory IGF-independent signalling pathway (Leal *et al.*, 1999).

1.2.3.2 Post-translational modification

1.2.3.2.1 Glycosylation and phosphorylation

The IGFBPs also vary in the extent to which they are post-translationally modified. Structural analysis has demonstrated that IGFBP-3 and IGFBP-4 can be subject to N-glycosylation, IGFBP-5 and IGFBP-6 have potential O-glycosylation sites, and IGFBP-1, IGFBP-3 and IGFBP-5 occur as phosphoproteins. The functional significance of glycosylation is unknown, although it has been suggested that

glycosylation slows the rate of proteolytic degradation of IGFBP-3 (Kubler *et al.*, 1998). Functional consequences of phosphorylation have been described for IGFBP-1. *In vitro*, the affinity for IGF-I of IGFBP-1 phosphorylated on serine residues is up to six fold higher than non-phosphorylated IGFBP-1 (Jones *et al.*, 1991). IGFBP-1 mutated at a primary site of phosphorylation displayed reduced IGF-I affinity (Jones *et al.*, 1993a). In addition, physiological significance of phosphorylated IGFBP-1 is suggested by the presence of phosphorylated isoforms *in vivo* (Jones *et al.*, 1991).

1.2.3.2.2 Proteolysis

In order for interaction with the cell surface receptors the IGFs must be released from the IGFBPs. In general terms, this is achieved by a reduction in binding affinity of IGFBPs for IGFS. As described above, association of IGFBPs with cell surfaces or ECM can alter IGFBP binding affinity, as can the degree of phosphorylation. However, the major mechanism for regulating IGF bioavailability may be proteolytic degradation of the IGFBPs (Fowlkes, 1997; Maile and Holly, 1999).

IGFBP proteolysis was first described in human pregnancy serum (Guidice *et al.*, 1990). IGFBP-degrading protease activity has since been described in a wide range of biologic fluids such as amniotic fluid (Claussen *et al.*, 1994), seminal plasma (Lee *et al.*, 1994), skin interstitial fluid (Xu *et al.*, 1995a), synovial fluid (Matsumoto *et al.*, 1996) and follicular fluid (Cwyfan Hughes *et al.*, 1997) under normal physiological conditions. In addition, IGFBP-proteolysis has been identified in clinical conditions such as arthritis (Matsumoto *et al.*, 1996) and has been shown to be elevated in patients suffering an acute catabolic state associated with severe

illness (Davies *et al.*, 1991), in patients suffering from advanced cancer (Frost *et al.*, 1996) and in cases of untreated diabetes (Bang *et al.*, 1994).

IGFBP-1 to 6- all appear susceptible to proteolytic degradation: however, this is not thought to be the result of the activity of a common enzyme. A number of candidate enzymes have been identified which are capable of IGFBP proteolysis, and in general are characterised as serine proteinases, matrix metalloproteinases or cathepsins (Fowlkes, 1997).

Numerous studies have demonstrated that the major consequence of IGFBP proteolysis is a reduction in affinity of the binding protein for the IGFs. The addition of prostate specific antigen (PSA), an IGFBP-3 protease, to prostatic epithelial cells reversed the inhibitory effects of IGFBP-3 on IGF mitogenesis, by the generation of IGFBP-3 fragments with a ten-fold reduction in IGF-I binding affinity (Cohen *et al.*, 1994). In human pregnancy plasma, limited proteolysis of IGFBP-3 resulted in diminished affinity for IGF, thus accelerating dissociation of the IGF-IGFBP complex and increasing free-form IGF-I (Lassare and Binoux, 1994). Porcine aortic smooth muscle cells secrete an IGFBP-2 specific serine protease, which cleaves IGFBP-2 into two fragments that do not bind the IGFs (Gockerman and Clemmons, 1995). Degradation of IGFBP-4 is associated with reduced IGF-I binding capacity and increased IGF-I stimulated DNA synthesis (Cohick *et al.*, 1993). In human osteosarcoma cells, plasmin caused dissociation of IGF-I and IGF-II from IGFBPs and reversed the inhibitory effects of IGFBP-1 on IGF-I stimulated DNA synthesis (Campbell *et al.*, 1992). The creation of an IGFBP-5 mutant with IGF-I binding affinity and protease resistance, demonstrated that proteolysis prevents the inhibition

of IGF-I-stimulated events by IGFBP-5, and regulates the responsiveness of porcine smooth muscle cells to IGF-I (Imai *et al.*, 1997).

However, whilst most IGFBP fragments display limited IGF binding affinity, in some circumstances proteolysis may generate biologically active fragments. Plasmin-induced proteolysis of recombinant human IGFBP-3 *in vitro* generated two major fragments, of 22/25 kDa and 16 kDa respectively. The 22/25 kDa fragment displayed a marked reduction in affinity for IGFs. Furthermore, inhibition of IGF-I stimulation of chick embryo fibroblast DNA synthesis (Lalou *et al.*, 1996) and prostate carcinoma cell (PC-3) proliferation (Angelloz-Nicoud *et al*, 1998) were diminished in comparison to intact IGFBP-3. In contrast, the 16 kDa fragment lost all ability to bind radiolabelled IGF, but remained a potent inhibitor of IGF-I activity. The anti-mitogenic effects of the 16 kDa proteolytic fragment of IGFBP-3 were also demonstrated in mouse fibroblasts carrying a targeted disruption of the type 1 IGF receptor, confirming that the action was IGF-independent (Zadeh and Binoux, 1997). The fragments of IGFBP-3 generated by plasmin digestion have also been shown to block insulin-stimulated mitogenesis of chick embryo fibroblasts (Lalou *et al.*, 1996), insulin-receptor auto-phosphorylation (Vorwerk *et al.*, 1998) and activation of the FGF signalling pathway (Zadeh and Binoux, 1997).

In contrast to the inhibitory effects of IGFBP fragments, others have demonstrated stimulatory activity following proteolysis. A 23- kDA fragment of IGFBP-5 purified from human osteosarcoma cell conditioned media, stimulated mitogenesis in the absence of IGF, and further enhanced cell proliferation when coincubated with IGF-I or IGF-II (Andress and Birnbaum, 1992).

1.3 The IGF system and the ovary

1.3.1 Actions of IGFs in the ovary

IGFs influence critical aspects of ovarian function in follicular and luteal cells of many species (Poretsky *et al.*, 1999; Spicer and Echternkamp, 1995).

1.3.1.1 Follicular growth

IGF-I stimulates cell proliferation and DNA synthesis in human (Yong *et al.*, 1992), rodent (Bley *et al.*, 1992), porcine (May *et al.*, 1988), ovine (Monniaux and Pisselet, 1992) and bovine (Spicer *et al.*, 1993) granulosa cells in culture, and this potent mitogenic effect can be further enhanced in synergy with gonadotrophins. FSH enhances IGF-I–stimulated granulosa cell mitosis in the rat and human. Similarly, the proliferation of bovine granulosa cells from small follicles (< 5mm) is increased following stimulation by IGF-I plus FSH or LH. The mitogenic effects of IGF-I do not appear to be granulosa cell specific; IGF-I has also been shown to enhance DNA synthesis and/or proliferation in human and rat theca cells (Duleba *et al.*, 1997, 1998), and in bovine luteal cells (Chakravorty *et al.*, 1993).

In vivo studies also support a role for IGF-I in promoting follicle growth. In cattle and pre-pubertal gilts, somatotropin treatment increased both circulating IGF-I concentrations and the number of small follicles, without alterations in gonadotrophin levels (Gong *et al.*, 1991; Spicer *et al.*, 1992). In addition, superovulated heifers treated with somatotropin showed an increase in ovulation rate correlated with raised serum IGF-I concentrations (Gong *et al.*, 1993).

Whilst studies of the growth promoting effects of the IGFs on ovarian cells in culture have concentrated primarily on IGF-I, IGF-II also stimulates cell proliferation and

DNA synthesis in porcine granulosa (Kamada *et al.*, 1992) and human granulosa-luteal cells (Di Blasio *et al.*, 1994).

1.3.1.2 Steroidogenesis

The IGFs have been shown to be important promoters of steroidogenesis with the potential to act at multiple sites.

1.3.1.2.1 Progesterone

IGF-I and –II stimulate granulosa cell progesterone production in numerous species. In the human, IGF-I augments progesterone production by granulosa-luteal cells in synergy with gonadotrophins (Erickson *et al*,. 1991), whilst IGF-II stimulates basal progesterone secretion (Kamada *et al.*, 1992). In the rat, IGF-I enhances FSH-stimulated progesterone secretion, but has limited effect alone (Adashi *et al.*, 1985b). IGF-I stimulates progesterone production by granulosa and theca cells of domestic animals, which is further increased by FSH treatment (Spicer and Echternkamp, 1995). In addition, LH receptor acquisition is enhanced in rat granulosa cells in culture, in synergy with FSH (Adashi *et al.*, 1985c), an effect that may be mediated via increased LH receptor mRNA stability (Hirakawa *et al.*, 1999).

Increased progesterone release in response to IGF stimulation is the result of actions at a number of loci. In porcine granulosa cells IGF-I augments the metabolism of the steroid precursors LDL and HDL (Veldhuis, 1989). IGF-I can enhance the subsequent delivery of cholesterol from the outer to inner mitochondrial membrane, by up-regulation of the mitochondrial transport protein StAR (Balasubramanian *et al.*, 1997). Once within the mitochondria, cholesterol is converted to pregnenolone by the side chain cleavage enzyme complex comprised of cytochrome P450scc,

adrenodoxin and adrenodoxin reductase, which are all regulated by IGF-I (deMoura *et al.*, 1997; Magoffin *et al.*, 1990). The final conversion of pregnenolone to progesterone is performed by 3β-HSD, which increases in concentration following IGF-I stimulation (Magoffin and Weitsman, 1993).

Whilst the cellular actions of IGF-II are less well defined, IGF-II has been shown to increase progesterone production. In porcine granulosa cells the potent stimulatory effects of IGF-II on progesterone biosynthesis are the result of enhanced sterol delivery, via increased lipoprotein binding, internalisation and utilisation, and P450scc enzyme activity (Garmey *et al.*, 1993).

Direct stimulatory effects of IGFs on luteal cells have been demonstrated, and consistently result in enhanced progesterone production (Devoto *et al.*, 1995; Khan-Dawood *et al.,* 1994; McArdle and Holtorf, 1989; Sauerwein *et al.*, 1992; Talavera and Menon, 1991; Yuan and Lucy, 1996a). Whilst the sites of IGF action on luteal steroidogenesis are undefined, it is clear from follicular studies that the IGFs have great potential to modulate critical components of progesterone production.

1.3.1.2.2 Oestradiol

IGF-I stimulates basal oestradiol secretion in human granulosa cells and granulosa-luteal cells, and this is enhanced in synergy with gonadotrophins (Erickson *et al.*, 1989). IGF–II also stimulates oestradiol secretion by human granulosa cells, and is most effective following a period of insulin pre-incubation (Mason *et al.*, 1994). In rat granulosa cell cultures IGF-I stimulates oestradiol production in co-treatment with FSH, and aromatase activity is significantly greater than following FSH treatment alone (Adashi *et al.*, 1985a). Both IGF-I and IGF-II stimulate porcine

granulosa cell oestradiol production (Howard and Ford, 1994; Kamada *et al.*, 1992). In cattle IGF-I stimulated the secretion of oestradiol by granulosa cells from small, medium and large follicles (Gong *et al.*, 1994). However, it has been suggested that insulin is a more potent promoter of oestradiol biosynthesis than IGF-I. In addition, changes in follicular fluid oestradiol content have not always been correlated with changes in IGF-I concentrations *in vivo*, although this may be influenced by concurrent changes in IGFBPs and hence IGF-bioavailability (Spicer and Echternkamp, 1995).

1.3.1.2.3 Androgen

IGF-I can stimulate androgen biosynthesis induced by LH in the theca cells of rats (Cara and Rosenfield, 1988; Hernandez *et al.*, 1988), pigs (Caubo *et al.*, 1989), and women (Bergh *et al.*, 1993). In contrast, IGF-I was unable to stimulate basal androstenedione secretion by bovine theca cells obtained from large follicles (Meidan *et al.*, 1990). In the rat the stimulatory effect of IGF-I on androgen production is mediated via increased cytochrome P450 17α-hydroxylase/C_{17-20} lyase (P450c17) enzyme content and mRNA expression (Magoffin *et al.*, 1990).

1.3.2 The intra-ovarian IGF system

Ovarian patterns of expression and production for components of the IGF system show marked variation between species.

1.3.2.1 Ovarian IGF production and reception

1.3.2.1.1 Human

Limited IGF-I mRNA expression has been detected in the human ovary (Zhou and Bondy, 1993). Follicular IGF-I mRNA expression was restricted to the theca cells of small follicles and was absent from dominant follicles (El-Roeiy *et al.*, 1993). Immunohistochemistry also localised IGF-I peptide to the thecal compartment (Hernandez *et al.*, 1992), although others failed to detect IGF-I immunoreactivity in the human ovary (El-Roeiy *et al.*, 1993).

Follicular fluid IGF-I levels did not vary with stage of the menstrual cycle, and were not correlated with follicular fluid oestradiol levels or follicular size (Van Dessel *et al.*, 1996). Clinical observations also suggest that IGF-I may not play an obligatory role in follicular development. Women with Laron-type dwarfism exhibit GH receptor deficiency, elevated peripheral GH concentrations and very low levels of both circulating and follicular fluid IGF-I. However, despite these hormonal alterations, there are cases of fertility and response to gonadotrophin stimulation (Dor *et al.*, 1992).

In contrast, abundant IGF-II mRNA and protein was detected in the granulosa cells of dominant follicles, and follicular fluid IGF-II levels were correlated with follicle diameter and oestradiol levels, leading the authors to suggest that IGF-II may have

greater influence in the regulation of human follicular growth than IGF-I (El Roiey *et al.*, 1993).

In the human, abundant type 1 IGF receptor mRNA was localised by *in situ* hybridisation to oocytes, and the granulosa cells of antral follicles, where expression increased with follicular dominance (El-Roeiy *et al.*, 1993; Zhou and Bondy, 1993).

1.3.2.1.2 Rodent

In the rat, IGF-I gene expression is exclusively localised to the granulosa cell layer of healthy follicles, and is absent from follicles undergoing atresia. In addition, mRNA encoding IGF-I was undetectable in the corpus luteum (Oliver *et al.*, 1989; Zhou *et al.*, 1991). In contrast, the expression of mRNA encoding IGF-II is theca cell-specific (Hernandez *et al.*, 1990). Whilst IGF-II mRNA expression was detected in the immature and mature rat ovary by RNase protection assay, others have failed to detect ovarian IGF-II mRNA expression in the adult rat by Northern analysis (Murphy *et al.*, 1987). In addition, others have suggested that IGF-II must play a limited role in the regulation of ovarian function, based on the decline in ovarian IGF-II levels observed postnatally (Levy *et al.*, 1992).

In the rat, the type 1 IGF receptor protein is present in both the granulosa and theca cells, does not vary according to the maturational status of the follicle and was present in both healthy and atretic follicles (Hernandez *et al.*, 1990; Zhou *et al.*, 1991).

Whilst most rodent work has focused on the rat, the IGF-I system has also been characterised in the mouse. As in the rat, mouse ovarian IGF-I mRNA expression is granulosa-specific and restricted to healthy growing follicles. Messenger RNA

encoding the type 1 IGF receptor was also concentrated in granulosa cells and was detected in small, large, healthy and atretic follicles (Adashi *et al.*, 1997).

1.3.2.1.3 Livestock species

The ovaries were first suggested to be a local site of IGF-I production following studies in the pig, which demonstrated that IGF levels in follicular fluid from pre-ovulatory follicles were greater than those in serum or in immature follicles (Hammond *et al.*, 1985). In addition, following gonadotropin-induced follicular development, follicular fluid IGF-I levels were correlated with oestradiol levels (Hammond *et al*,. 1988). IGF-I has since been detected in the follicular fluid of mares (Spicer *et al.*, 1991), cows (Spicer *et al.*, 1988) and sheep (Monget *et al.*, 1993).

In the porcine ovary IGF-I mRNA expression was granulosa cell-specific and restricted to healthy follicles (Yuan *et al.*, 1996; Zhou *et al.*, 1996). In porcine granulosa cell culture, FSH and GH increased levels of IGF-I mRNA and protein (Samaras *et al.*, 1996). IGF-II mRNA has also been localised to granulosa cells where expression was independent of follicle health (Zhou *et al.*, 1996). However, others have found IGF-II mRNA expression to be theca specific (Yuan *et al.*, 1996). Messenger RNA encoding the type 1 IGF receptor has been detected in porcine granulosa cells (Zhou *et al.*, 1996).

IGF-I mRNA expression patterns in the bovine follicle remain controversial. IGF-I mRNA was localised by *in situ* hybridisation to the granulosa cell layer of developing follicles in heifers, and was greatest in the dominant follicle (Yuan *et al.*, 1998). Low and inconsistent follicular IGF-I mRNA expression has also been

demonstrated (Perks *et al.*, 1999). In contrast, others have failed to detect both IGF-I protein in bovine granulosa cell conditioned medium (Gutierrez *et al.*, 1997) and follicular IGF-I mRNA expression (Armstrong *et al.*, 2000a). Reports of follicular IGF-II expression show more agreement. IGF-II mRNA expression is theca-specific, and was detected throughout the growth of antral follicles, from antrum formation to the development of dominance (Armstrong *et al.*, 2000a; Yuan *et al.*, 1998). Messenger RNA encoding the type 1 IGF receptor was detected in both granulosa and theca cells of preantral and antral bovine follicles (Armstrong *et al.*, 2000a), whilst others have shown that receptor expression was restricted to the granulosa layer (Perks *et al.*, 1999).

In sheep, IGF-II as opposed to IGF-I is thought to have a major role in regulating ovarian function. IGF-I mRNA was undetectable in the ovine follicle, whilst mRNA encoding IGF-II was detected in the theca cell layer and varied significantly with the size and health of the follicle. IGF-I receptor mRNA was detected in both granulosa and theca cells (Perks *et al.*, 1995).

1.3.2.2 Ovarian IGF binding proteins

IGFBPs also appear to have important roles in regulating ovarian function, and one or more IGFBPs are produced by the ovary of the rabbit (Ricciarelli *et al.*, 1991), sheep (Perks and Wathes, 1996), human and non-human primate (El-Roiey *et al.*, 1994; Fraser *et al.*, 1998), cow (Armstrong *et al.*, 1998), pig (Zhou *et al.*, 1996), mouse (Adashi *et al.*, 1997) and rat (Nakatani *et al.*, 1991).

The addition of IGFBPs to ovarian cells in culture, or to whole ovaries perfused *in vitro,* has resulted in the attenuation of IGF action. IGFBPs have been shown to

block ovarian steroidogenesis (Mason *et al.*, 1998; Spicer *et al.*, 1997; Ui *et al.*, 1989), inhibit cell proliferation (Bicsak *et al.*, 1990), decrease ovulation rate and oocyte maturation (Yoshimura *et al.*, 1996), and remove IGF-I-mediated suppression of apoptosis (Chun *et al.*, 1994).

A number of studies have demonstrated that follicular fluid IGFBP concentrations are correlated with the size and health of the follicle, thus suggesting a physiological significance of IGFBP action *in vivo*. In cattle, the establishment of follicular dominance is associated with a reduction in low molecular weight IGFBPs (IGFBP-2, -4 and -5) (Echternkamp *et al.*, 1994: Stewart *et al.*, 1996), whilst follicular atresia is associated with increased IGFBP levels (Echternkamp *et al.*, 1994). IGFBP profiles in follicular fluid from women demonstrated similar increases in IGFBP concentrations during atresia (Cataldo and Guidice, 1992). Messenger RNA encoding IGFBPs has been detected in the ruminant follicle and the patterns of expression have been related to follicular development (Armstrong *et al.*, 1998; Perks and Wathes, 1996). Follicular fluid IGFBP-2 concentrations are reduced in large healthy bovine follicles in parallel with decreased granulosa cell IGFBP-2 mRNA expression (Armstrong *et al.*, 1998; Perks and Wathes, 1996).

Changes in follicular fluid IGFBP profiles may be attributed to alterations in local production or degradation of IGFBPs, and/or transudation from the serum. The resultant changes in IGF availability are thought to influence follicular sensitivity to gonadotrophins, and hence regulate follicle selection and dominance. In addition, alterations in bovine follicular IGFBP mRNA expression observed following increased dietary energy regimes is suggestive of a role for IGFBPs in mediating the effect of nutrition on follicular growth (Armstrong *et al.*, 2000b).

Enzymes capable of cleaving IGFBPs have been reported in follicular fluid and ovarian cell conditioned media, and such proteolytic activity is thought to be an important mechanism for increasing IGF bio-availability at critical points during follicle growth and development (Besnard *et al.*, 1996, 1997; Cwyfan Hughes *et al.*, 1997; Grimes and Hammond, 1994; Mason *et al.*, 1996).

Chapter 2. Materials and Methods

The composition of buffers are detailed in the appendix. The sources of individual chemicals are detailed within the experimental protocols and suppliers listed in the appendix. Frequently used materials and chemicals including solvents, chemicals for buffers and plastic or glassware were obtained from the Sigma-Aldrich Company Ltd

.2.1 Total RNA isolation

2.1.1 Controlling ribonuclease activity

To obtain good preparations of RNA it is necessary to minimise the actions of RNases. Polypropylene centrifuge tubes (50 ml) used throughout the isolation procedure were filled with 0.1% (v/v) diethyl pyrocarbonate (DEPC), allowed to stand for 1 hour at room temperature and then autoclaved. Wherever possible solutions were treated with 0.1% (v/v) DEPC overnight at room temperature and then autoclaved. Solutions not suited to DEPC (e.g. Tris) were prepared in DEPC-treated water (0.1% v/v) and autoclaved. Solutions not suited to autoclaving (e.g. dithiothreitol; DTT) were prepared using DEPC-treated water (0.1% v/v), baked glassware and sterile filtered. RNA was isolated in an area with restricted air movement and all surfaces were treated with an RNase decontamination solution (RNase Zap; AMS Biotechnology (UK) Ltd). Disposable gloves were worn throughout the preparation of materials and solutions and during the isolation and analysis of RNA.

2.1.2 RNA isolation

Total cellular RNA was prepared according to the method of Chomczynski and Saachi (1987) from bovine ovarian and liver tissue collected from the local abattoir. Denaturing solution (2.5ml) containing the potent RNase inhibitor guanidine thiocyanate was dispensed into 50ml DEPC-treated centrifuge tubes, 17.9µl of β-mercaptoethanol (β-ME) was added, mixed and held on ice. Frozen tissue (250mg) was placed into the solution and immediately dissociated using a high-speed polytron homogeniser for 3x 10 sec. To the homogenate was added 62.5µl of 20% (w/v) sodium lauryl sarcosine (SLS) to aid in the breakdown of nucleoprotein complexes. RNA was isolated from contaminants by phenol: chloroform extraction performed at low pH. The acid environment was created by the addition of 250µl of 2M NaAc (pH 4). Aquaphenol (2.5ml) (Appligene Oncor) was added and mixed by inversion, followed by 500µl of chloroform:isoamyl alcohol (24:1 v/v). The tube was then vigorously shaken for 10 sec and placed on ice for 15 min. The sample was then centrifuged at 9100rpm (Beckman J-20) for 20 min at 4°C. RNA is selectively partitioned to the aqueous phase, which was carefully removed avoiding the interphase and transferred to a fresh DEPC-treated tube. An equal volume of isopropanol was added, mixed and placed at -20°C for at least 1hr. The RNA was pelleted by centrifugation at 9100rpm (Beckman J-20) for 20 min at 4°C and then re-dissolved in 750µl of denaturing solution plus 18.75µl of 20% (w/v) SLS and 5.35µl of β-ME. An equal volume of isopropanol was added, mixed and placed at -20°C for at least 1hr. The sample RNA was again pelleted by centrifugation (9100rpm Beckman J-20) for 10 min at 4°C. The pellet was washed with 75% (v/v) ethanol,

vortexed and spun for a further 10 min (9100rpm Beckman J-20) at 4°C. The pellet was then air dried, and resuspended in 100µl of DEPC water.

2.1.3 Assessing total RNA yield

The optical density of a suitable dilution (typically 1/200) was measured at 260nm using a Beckman Du-63 Spectrophotometer and a 1cm-pathlength spectrophotometric cuvette and the concentration calculated using the formula;

optical density of 1 at 260nm=40µg RNA.

Thus the concentration of RNA = optical density at 260nm x 40 x dilution factor.

RNA was stored at -80°C in 50µg aliquots until required.

2.1.4 Assessing RNA integrity

The integrity of the purified RNA was determined following denaturing gel electrophoresis. Typically 10-15µg of RNA was mixed with 12.5µl of deionised formamide (AMS Biotechnology (UK) Ltd), 4µl of formaldehyde (37% v/v) and 2.5µl of 10x MOPS in a total volume of 25µl, and incubated at 65°C for 10 min. The mixture was chilled on ice for 20 sec and 2.5µl of loading buffer (0.01% (w/v) bromophenol blue in 50% (v/v) glycerol) added. The RNA was electrophoresed through a 1.1% (w/v) standard agarose gel containing 1x MOPS, and 17% (v/v) formaldehyde (37% stock) using 1x MOPS as running buffer. If electrophoresis was performed overnight, buffer was constantly recirculated, and the gel run at 20V. After electrophoresis, the gel was stained with dilute ethidium bromide (0.5µg/ml; 30 min), destained in water (30 min) and photographed under UV transillumination (Polapan 55PN film, Polaroid, supplied by HA West). RNA resolved by denaturing

gel electrophoresis characteristically exhibits 28s and 18s ribosomal RNAs, which in the absence of gross degradation display a near 2:1 ratio by ethidium bromide staining.

2.2 Synthesis of cDNA

Total RNA extracted as described above was used as the template for cDNA synthesis. Total RNA (1μg) was mixed with random hexamers (125pmol; Promega) in the presence of 20 units of ribonuclease inhibitor (RNasin, Promega) to a total volume of 11μl. This RNA/primer mix was incubated for 10 min at 70°C and then held on ice. Following the annealing of primers to template RNA, cDNA synthesis was initiated by the addition of 9μl of reverse transcription mix, incubated at 20°C for 10 min, followed by 42°C for 1hr. The reaction was terminated by incubation at 95°C for 5 min. Prior to cDNA amplification the synthesis reaction was diluted with 4μl of 50mM $MgCl_2$ (Life Technologies) and 8μl of 10x RTase buffer (Promega) to a total volume of 100μl and stored at -70°C.

Reaction tubes were prepared without RNA or without reverse transcriptase to assess contamination of reagents with DNA and genomic contamination of RNA samples respectively. These control tubes were included in subsequent polymerase chain reactions.

2.3 Polymerase Chain Reaction (PCR)

Primers were designed to amplify target sequences of bovine IGF-I, IGF-II, the type 1 IGF receptor, and the insulin receptor using published DNA sequences available in the European Molecular Biology Laboratory (EMBL) database (http://srs.ebi.ac.uk) and the Prime programme of the GCG Wisconsin sequence analysis package (GCG).

Details of the primer pairs, their target sequences and subsequent PCR conditions are shown in Table 2.1.

Plasmids containing porcine IGF-I, bovine IGFBP-2, -3 and –4, human P450arom and ovine LH receptor probes were already available and target sequences are detailed in the relevant experimental chapters.

A PCR reaction mixture (20µl) for each sample was prepared by adding 6µl of RTase reaction, 1µl of 10x PCR buffer (Promega) and 12µl of dH_20. A reaction tube was prepared in the absence of the cDNA synthesis reaction to assess reagent contamination. The tubes were vortexed and then placed in the thermocycler (Personal Cycler, Biometra Ltd), and heated to 93°C. At this point 1µl of Taq/primer mix was added to each sample. Taq/primer mix (25µl) contained 5µl of Taq polymerase (5U/µl; Roche Diagnostics Ltd), and 100-200pmol of both the 5' and 3' primers (Cruachem). The tubes were held at 93°C for 5 min. This was followed by 30 cycles of denaturing (93°C, 30 sec), annealing (55-65°C (table 2.1), 30 sec) and primer extension (72°C, 30 sec). The final cycle was completed by an extended final stage at 72°C for 5 min. On completion of the reaction, samples were cooled to 4°C prior to analysis by agarose gel electrophoresis. A 5µl aliquot of each reaction was mixed with 1µl of 6x loading buffer (Promega) and electrophoresed on a 3% (w/v) agarose gel (2:1 mix of standard agarose (Appligene Oncor) and NuSieve GTG (Flowgen) dissolved in 1x TAE) with 1x TAE running buffer. A DNA ladder (PCR Markers; Promega) was included as a marker lane to confirm amplified fragment size. After electrophoresis, the gel was stained with dilute ethidium bromide

(0.5μg/ml; 30 min), destained in water (30 min) and photographed under UV transillumination (Polapan 55PN film, Polaroid, supplied by HA West) (Figure 2.1).

Target sequence (EMBL)	Primer Sequences	Annealing temperature	Product size (bp)
IGF-I (BTILGF1A)	5'-CCTCTGCGGGGCTGAGTTGGT-3' 5'-CGACTTGGCGGGCTTGAGAGGC-3'	65°C	196
IGF-II (BTILGF2)	5'-TCTGTGCGGCGGGGAGCTGGT-3' 5'-AGTCTCCAGCAGGGCCAGGTCG-3'	65°C	154
Type 1 IGF receptor (BTIGF1B)	5'-CCAAGCTAAACCGGCTCAAC-3' 5'-TTATAACCAAGCCTCCCAC-3'	60°C	189
Insulin Receptor (HSINSR)	5'-AACTCTTCTTCCACTATAACCC-3' 5'-GCAATGTCGTTTCTCTCC-3'	55°C	100

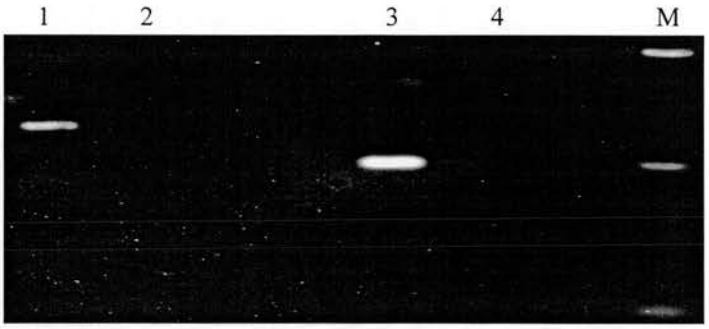
Table 2.1

Details of PCR primers are shown for IGF-I, IGF-II, the type 1 IGF receptor and the insulin receptor. (EMBL) refers to the 'ID' of each sequence in the EMBL database.

Figure 2.1

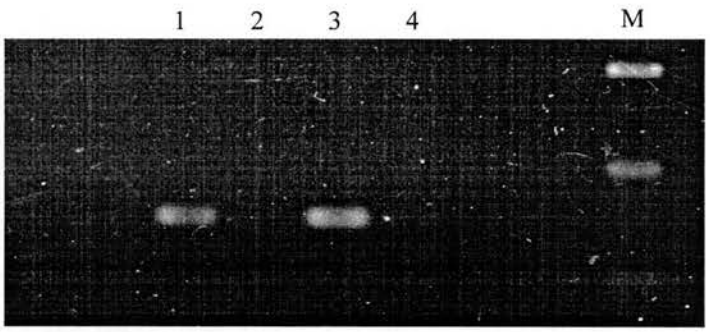
Agarose gel electrophoresis of Reverse Transcription-PCR products.

a)



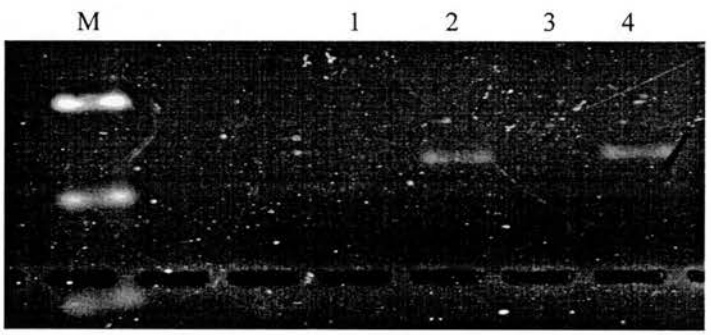
Amplified bands following RT-PCR of bovine luteal RNA, using primers designed against bovine IGF-I (1, 2) and IGF-II (3, 4). Blanks (minus reverse transcriptase) (2, 4). PCR Markers (M).

b)



Amplified bands following RT-PCR of bovine luteal (1, 2) and liver (3, 4) RNA, using primers designed against human insulin receptor. Blanks (minus reverse transcriptase) (2, 4). PCR Markers (M).

c)



Amplified bands following RT-PCR of bovine luteal RNA, using primers designed against bovine type1 IGF receptor. Blanks (minus reverse transcriptase) (1, 3). PCR Markers (M).

2.4 Primer design

2.4.1 IGF-I

Primers were designed to amplify positions 156-351 of bovine IGF-I mRNA (Fotsis *et al.*, 1990). The amplified product is within the coding region (1-462), and corresponds to the region encoding the mature peptide (145-354). A porcine IGF-I probe was kindly gifted by Dr M Lucy (University of Missouri). A comparison of the bovine and porcine (Tavakkol *et al.*, 1988) IGF-I mRNA sequences detailing the primer positions is shown in Figure 2.2.

2.4.2 IGF-II

Primers were designed to amplify positions 7-160 of a partial bovine IGF-II mRNA (Brown *et al.*, 1990). The amplified product is within the coding region (1-451), and by comparison to the human sequence (Rall *et al.*, 1987) corresponds to the region encoding the mature peptide. The human and bovine IGF-II mRNA sequences are compared in Figure 2.3.

A Bestfit comparison (GCG Wisconsin package, GCG) of bovine IGF-I and bovine IGF-II mRNA sequences demonstrates a 72% similarity as shown in Figure 2.4. Despite this level of homology, bovine IGF-I and IGF-II primers specifically amplify only their target mRNA, as determined by BLAST or FASTA analysis (GCG Wisconsin package, GCG). The amplified regions of the mRNA sequences encoding bovine IGF-I and -II are compared in Figure 2.4.

2.4.3 Type 1 IGF receptor

Primers for the type 1 IGF receptor were designed to avoid regions of homology with both the insulin receptor and insulin receptor related receptor. The lowest degree of similarity is shown in the signal peptide and transmembrane sequences. The available bovine mRNA sequence for the type 1 IGF receptor encodes only the β-subunit (Sneyers *et al.*, 1991a), whilst much more of the human mRNA sequence is published (Ullrich *et al.*, 1986). Therefore, initial searches were made using the human sequence. Suitable primers were identified and checked for homology. Since the most suitable primer pair was in the β-subunit the primers were then adapted to the bovine sequence and subjected to FASTA analysis (GCG Wisconsin package, GCG), which demonstrated specificity of amplification. Primers were designed to amplify positions 2717-2905 of human type 1 IGF receptor mRNA which corresponds to positions 491–679 of a partial bovine mRNA. A comparison of human type 1 IGF receptor mRNA and human insulin receptor mRNA is illustrated in Figure 2.5.

2.4.4 Insulin receptor

There is no published mRNA sequence data for the bovine insulin receptor. Primers were designed to amplify positions 1493-1592 of human insulin receptor mRNA (Figure 2.6) (Ebina *et al.*, 1985), which corresponds to part of the alpha subunit. FASTA analysis (GCG Wisconsin package, GCG) indicates that these primers specifically amplify the insulin receptor, and not the type 1 IGF receptor.

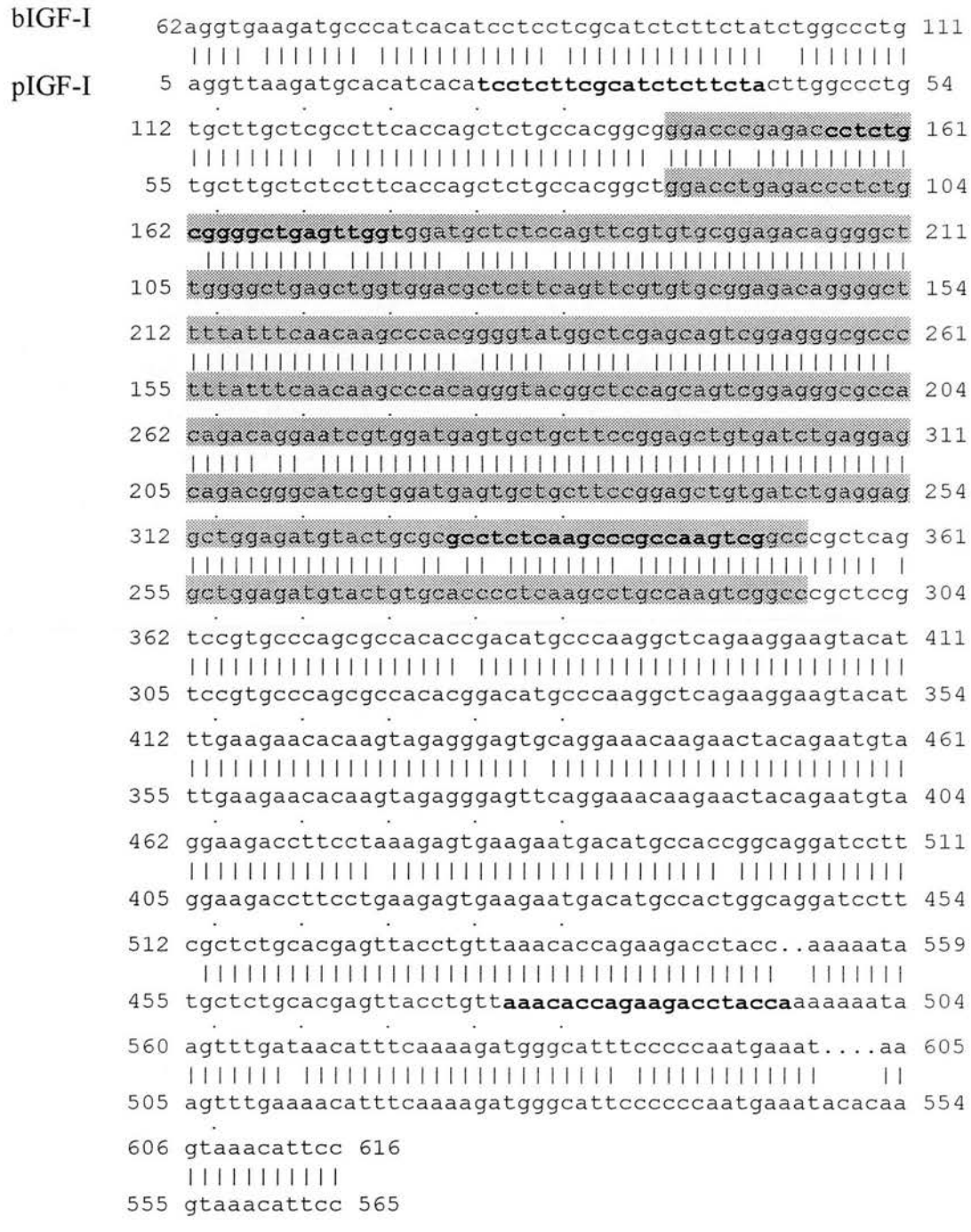


Figure 2.2

A Bestfit alignment of bovine and porcine IGF-I mRNA sequences. Primer positions are emboldened and the region coding for the mature peptide highlighted.

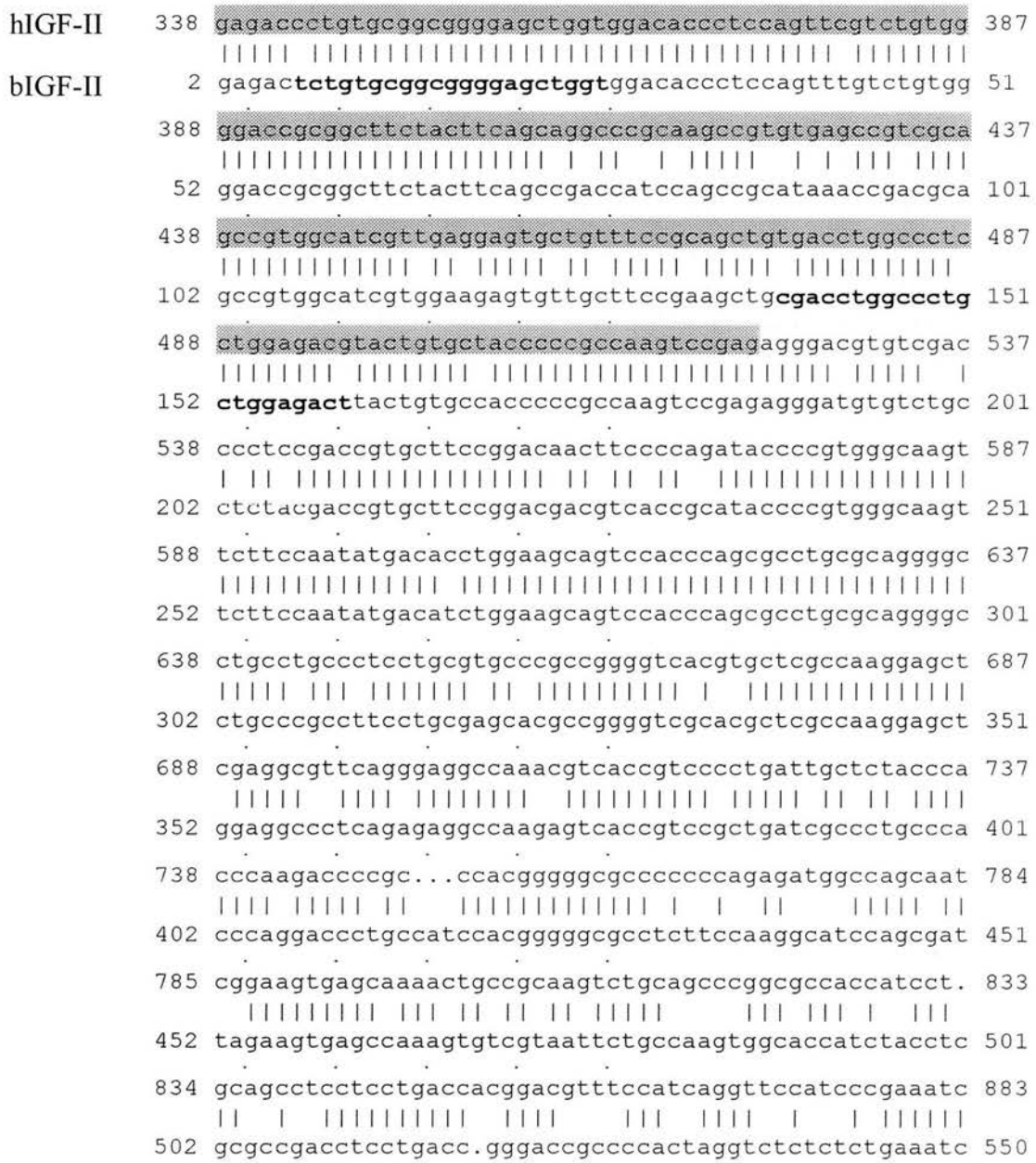


Figure 2.3

A Bestfit alignment of human and bovine IGF-II mRNA sequences. Primer positions are emboldened, and the region coding for the mature peptide of the human sequence is highlighted.

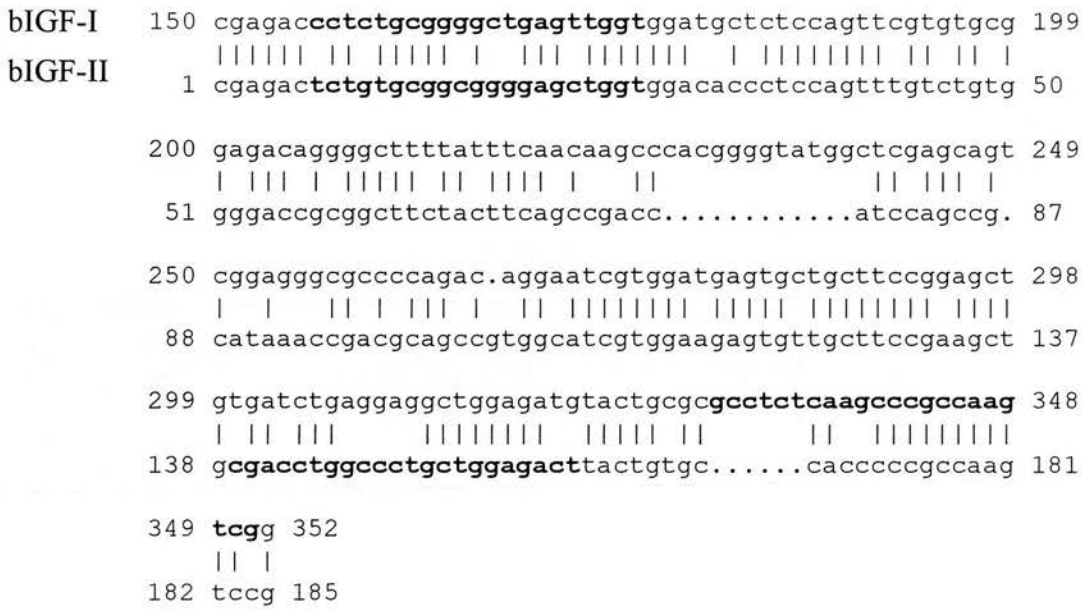


Figure 2.4

A Bestfit alignment of bovine IGF-I and IGF-II mRNA sequences. Primers are emboldened.

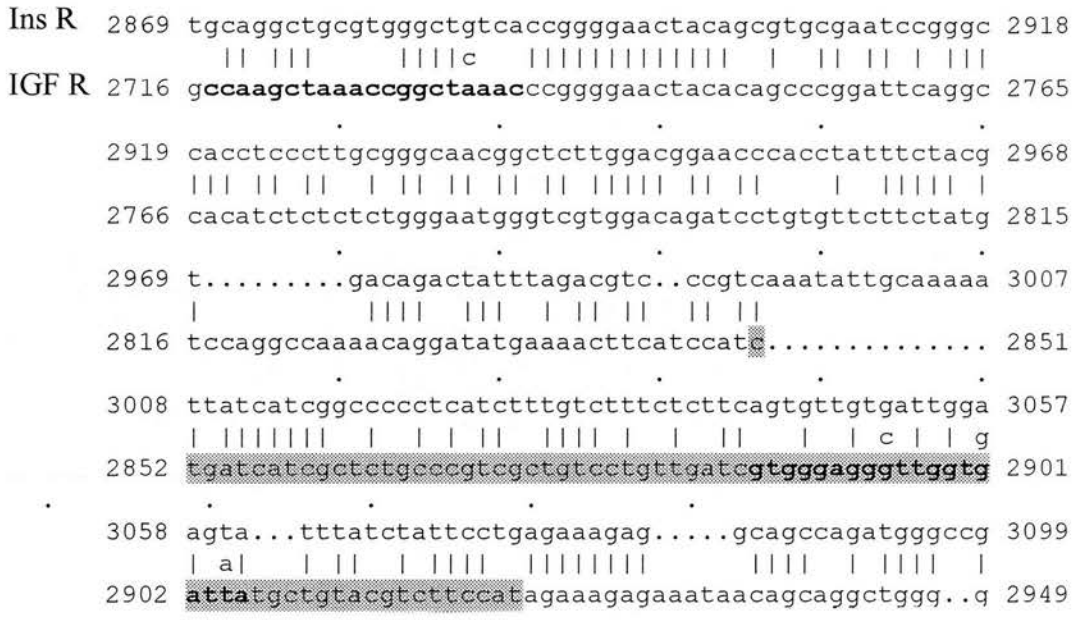


Figure 2.5

A Bestfit alignment of the human insulin receptor and type 1 IGF receptor mRNA sequences, to illustrate primer positions for the type 1 IGF receptor. Primer positions are emboldened, and the sequence coding for the transmembrane region is highlighted. Letters are shown between the aligned sequences to illustrate when the bovine type 1 receptor mRNA sequence varies from the human type 1 IGF receptor mRNA sequence.

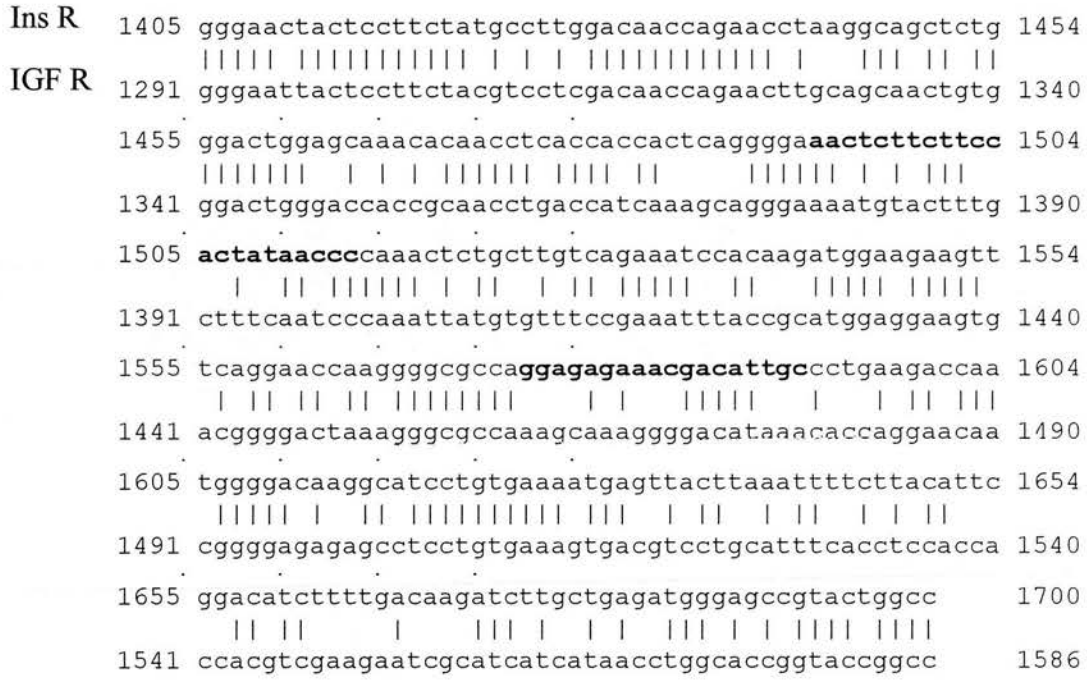


Figure 2.6

A Bestfit alignment of the human insulin receptor and type 1 IGF receptor mRNA sequences, to illustrate primer positions for the Insulin receptor.

2.5 Ligation of vector and insert DNA

pGEM-T (Promega) was used as the vector for PCR product cloning. pGEM-T is prepared from the pGEM-5Zf(+) vector, cut at the EcoRV site and with 3' terminal thymidines (3'-T overhangs) added to both cut ends for increased ligation efficiency. The plasmid contains T7 and Sp6 RNA polymerase transcription initiation sites, a sequence coding for the Lac α-peptide, interrupted by a multiple cloning site that enables blue/white colour selection for insertion, ampicillin resistance in addition to a number of restriction enzyme sites.

Aliquots of PCR product (0.5μl, 1.5μl or 4.5μl) or 2μl of pGEM-T vector control (Promega) were added to a ligation reaction (10μl) containing 1μl of T4 DNA Ligase (1U/μl; Promega), 1μl of T4 DNA ligase 10x buffer (Promega) and 1μl of pGEM-T vector (50ng). Reactions were mixed and incubated overnight at 4°C.

2.6 Bacterial transformation

2.6.1 Preparation of *Luria bertani* agar -ampicillin plates

Sterile *Luria bertani* (L.) agar was warmed to melting and mixed with antibiotic (ampicillin, 6mg/100ml) and poured into sterile petri dishes (Bibby Sterilin). Plates were allowed to set at room temperature. Prior to use L. agar-ampicillin plates were coated with a layer of Isopropyl β-D-Thiogalactopyranoside (IPTG) (4μl of 1M IPTG/plate; Promega) and 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (40μl of 50mg/ml X-Gal /plate; Promega) and allowed to absorb for approximately 30 min at 37°C, for subsequent blue/white colour screening.

2.6.2 Transformation of cells

Bacterial transformation of plasmids was performed using JM-109 High Efficiency Competent Cells (Promega). A 2μl aliquot of each ligation reaction was placed into a thin walled tube on ice. In addition, 0.5ng uncut pGEM-3Z plasmid was used as a positive control to determine transformation efficiency. JM-109 cells were thawed on ice and mixed by gentle flicking. A 50μl aliquot of cells was added to each pre-chilled ligation reaction and mixed during dispensing. The cells were placed on ice for 20 min, heat shocked for 45 sec at 42°C and returned to ice for 2 min. L. broth (900μl) was added and the cells incubated with shaking (225 rpm) at 37°C for 1hr. L.agar-ampicillin plates (see section 2.6.1) were spread with 100μl of each transformation culture and incubated overnight at 37°C.

2.6.3 Blue/ white colour screening

After overnight culture on IPTG/X-gal supplemented plates possible positive transformants were selected by colour. Bacterial colonies containing recombinant vector constructs are white due to the insertional inactivation of the Lac-α sequence, whilst in general non-recombinants produce functional β galactosidase as indicated by a blue colour.

Single colonies were lifted from the plates and used to inoculate 5ml of L. broth plus ampicillin (6mg/100ml). Cultures were incubated at 37°C overnight with shaking.

2.7 Plasmid DNA preparation

2.7.1 Mini plasmid preparation

Small-scale DNA preparation was carried out using the Wizard Miniprep system (Promega). A 2ml aliquot of each culture was removed and spun at high speed in a microcentrifuge (Beckman-E) for 2 min. The pellet of cells was resuspended in 200µl of cell resuspension solution, prior to cell lysis in 200µl of lysis solution. Neutralisation solution (200µl) was added to the mix and the resultant precipitate containing protein and chromosomal DNA was pelleted by high-speed centrifugation for 5 min. The supernatent was decanted to a fresh tube and mixed with 1ml of DNA purification resin. For each miniprep a 3ml-syringe barrel was attached to a Wizard minicolumn and placed on a vacuum manifold (Vac-man; Promega). The resin mix was then loaded on and drawn into the minicolumn under vacuum. Column wash (2ml) was then applied and the resin allowed to dry for a further 2 min under vacuum. The syringe barrel was removed and the minicolumn transferred to a microcentrifuge tube and spun at high speed for 20 sec to remove any residual column wash. The minicolumn was transferred to a fresh microcentrifuge tube and 50µl of water was applied to the minicolumn, and left for 1 min. To elute the plasmid DNA the minicolumn was spun at high speed for 20 sec and stored at 4°C.

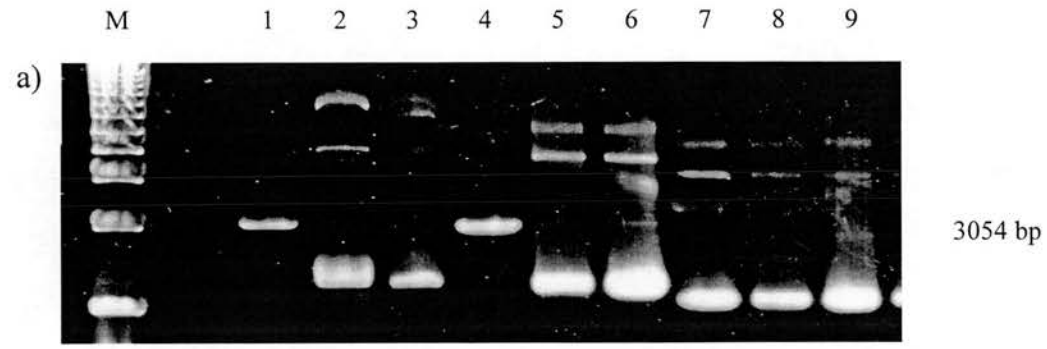
2.7.2 Restriction digestion

Initial confirmation of successful ligation was by restriction enzyme digestion. Restriction endonucleases were chosen for their ability to cleave DNA at specific recognition sequences present within the desired insert but not the plasmid vector. Potential target sites within the PCR product were analysed using the GCG Wisconsin Mapping programme (GCG). Restriction enzymes were purchased from New England Biolabs or Roche Diagnostics Ltd. Each restriction digestion reaction was performed to digest approximately 1μg of sample DNA in the presence of excess enzyme (20U) and the appropriate enzyme 10x buffer diluted to a final 1x strength. Samples were incubated at 37°C for 1hr. The result of restriction endonuclease cleavage was analysed by gel electrophoresis (Figure 2.7). It would be difficult to tell successful ligation by size difference alone. However, a single cleavage of circular DNA substrate will linearise the DNA, creating a difference in mobility through the gel that allows circular and cut to be resolved. Since the restriction enzyme sites were chosen to be present only within the insert, linearisation represents successful ligation.

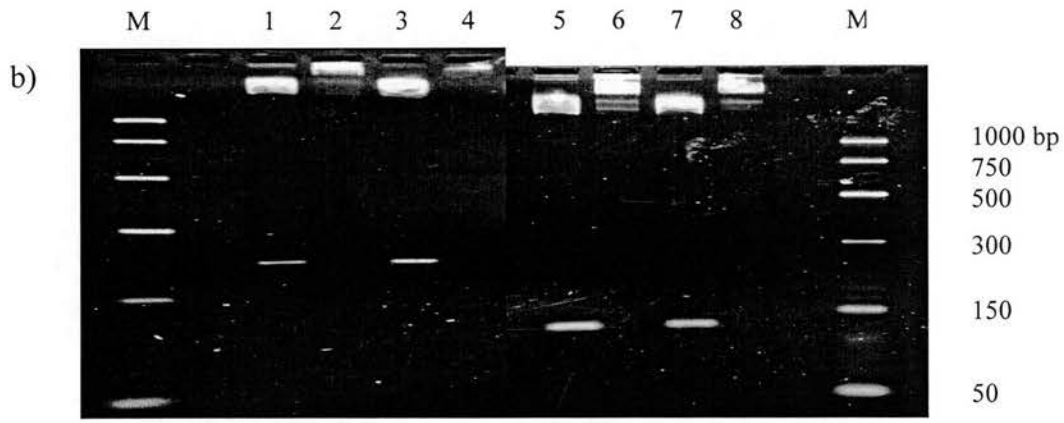
Digestion with two enzymes was also performed when possible, to confirm ligation by cutting out the insert from the plasmid cloning site. Digestion was carried out with both enzymes acting simultaneously if buffer and temperature conditions for both enzymes activity were compatible. However, when enzymes had different digestion requirements and were known to perform poorly in a generalised multicore buffer, digestion was carried out with first one of the enzymes and then the other, with each enzyme in its own buffer with the lowest salt concentration used first.

Figure 2.7

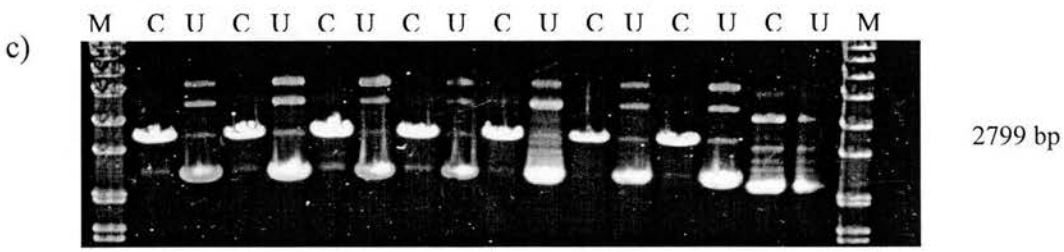
Agarose gel electrophoresis following single and double restriction enzyme digests.



Putative type1 IGF receptor plasmid cut with BamH1 (present in bovine sequence) (lanes 1, 4, 7), or Sma1 (present in human sequence) (2, 5, 8), or uncut (3, 6, 9). DNA Marker X (M; Roche Diagnostics Ltd).



Putative type1 IGF receptor plasmid (lanes 1-4) and insulin receptor plasmid (5-8) cut with Not1 and Nco1 (1, 3, 5, 7) or uncut (2, 4, 6, 8). PCR Marker (M; Promega).



Putative IGF-I plasmid cut with Xho1 (C) or uncut (U). DNA Marker VII (M; Roche Diagnostics Ltd)

2.7.3 PCR confirmation of ligation

PCR was also used for further confirmation of successful ligation. To 1µl of ligation reaction (10ng) was added 11µl of dH_2O, 2µl of deoxynucleotides (dNTPs) (Promega), 2µl of 10x PCR buffer (Roche Diagnostics Ltd), and 3µl of $MgCl_2$. The samples were mixed, placed in the thermocycler at 93°C and 1µl of taq/primer mix added prior to continued cycles of amplification.

Following initial confirmation of successful ligation, cultures of putative recombinants were used to streak out L. agar plus ampicillin plates (Section 2.7.1). Plates were incubated overnight at 4°C, single colonies removed and used to inoculate 5ml of L. broth plus ampicillin (6mg/100ml). Tubes were incubated at 37°C with shaking for approximately 8hr. The culture was then used to inoculate 500ml of L. broth plus ampicillin (6mg/100ml) grown overnight at 37°C with shaking.

2.7.4 Maxi plasmid preparation

Large scale preparation of plasmid DNA was carried out using the Wizard Maxiprep system (Promega). A 5ml aliquot of overnight culture was put aside for later use. The remainder of the culture was pelleted by spinning in a centrifuge bottle at 5,000g for 10 min at 22°C (Beckman JA-14). The bacterial pellet was resuspended in 15ml of resuspension solution and the pellet disrupted with a glass rod. Cell lysis solution (15ml) was added, gently mixed and allowed to stand for 20 min. Neutralisation solution (15ml) was then added, mixed gently by inversion and the precipitate pelletted by centrifugation at 14,000g for 15 min at 22°C. The supernatant was then filtered through 4 layers of muslin into a measuring cylinder and 0.5 volumes of

isopropanol added. After mixing by inversion the sample was spun at 14,000g for 15 min at 22°C. The supernatant was poured off and the pellet of DNA resuspended in 2ml of 1x TE buffer. DNA purification resin (10ml) was added and swirled to mix, and then loaded onto the Maxicolumn attached to a vacuum manifold. The centrifuge bottle was rinsed with column wash solution (13ml) which was then applied to the Maxicolumn under vacuum. A further 12ml of column wash was added, followed by 5ml of 80% (v/v) ethanol. Once the solutions had been drawn through the column, the vacuum was maintained for a further minute to dry the resin. The column was removed from the vacuum and placed inside a screw top tube. Residual column wash was then removed by centrifugation in a swinging bucket rotor at 2,500 rpm for 5 min and a further 5 min on the vacuum. The column was transferred to a fresh screw top tube and 1.5ml of preheated (65°C) water was applied to the column. After standing for 1 min, DNA was eluted by centrifugation in a swinging bucket rotor at 2,500 rpm for 5 min and stored at 4°C. Optical density of a suitable dilution was measured at 260nm using a 1-cm path-length spectrophometric cuvette. DNA concentration was calculated using the formula

optical density of 1 at 260nm = 50μg/ml of DNA.

Therefore, the concentration of DNA in μg/ml = optical density x 50 x dilution factor.

2.7.5 Glycerol stocks

Stocks of successful bacterial transformations were prepared for future use from 5ml of overnight culture previously set aside. Cells were pelleted by centrifugation at 4000 rpm for 15 min at room temperature, resuspended in 1ml of TM buffer and 1ml of glycerol, and stored in 0.5ml aliquots at -70°C.

2.8 DNA sequencing

DNA was sequenced using the Sequenase Version 2.0 kit (Amersham Pharmacia Biotech UK Ltd), based on a dideoxy-mediated chain-termination method. The DNA polymerase "Sequenase" initiates enzymatic synthesis of a DNA strand complementary to the template at the site where an oligonucleotide primer anneals specifically to the template. Deoxynucleotides are then added to the growing DNA chain until a competing dideoxynucleotide (ddNTPs) is incorporated, blocking further strand synthesis. Four separate reactions, each with a different ddNTP, generate chains of various lengths that terminate at each Adenosine, Cytidine, Guanosine or Thymidine in the template. The inclusion of a radiolabelled nucleotide enables the chains to be visualised by autoradiography after separation by high-resolution electrophoresis, and the template sequence deduced.

DNA sequencing was performed in five steps as detailed below.

2.8.1 Preparation of double stranded DNA

Double stranded DNA template was subjected to sequencing and required initial denaturation. An aliquot of DNA (5µg in 50µl) was denatured under alkaline conditions by the addition of 0.1 volume of 2M NaOH, 2mM EDTA (30 min, 37°C). The mixture was neutralised by adding 0.1 volume of 3M NaAc (pH 5.5) and the

DNA precipitated with 4 volumes of ethanol (-20°C, 30 min). The denatured DNA was then pelleted at high speed in a microcentrifuge at 4°C, washed with 70% (v/v) ethanol, air dried and resuspended in 7µl of dH_2O. A control reaction was also performed using single stranded DNA of known sequence (5µl of DNA + 2µl of water).

2.8.2 Anneal template and primer

To the DNA was added 1µl of oligonucleotide primer and 2µl of reaction buffer. The primer was annealed to the template by heating at 65°C for 2 min and cooling to room temperature over a period of approximately 30 min, before being placed on ice.

2.8.3 Labelling reaction

To the annealed template-primer mix was added 1µl of 0.1M DTT, 2µl of labelling mix (1.5µM of dGTP, dCTP, dTTP), 0.5µl of [α-^{35}S]dATP (Amersham Pharmacia Biotech UK Ltd) and 2µl of prediluted Sequenase (diluted 1:8 in ice cold enzyme dilution buffer). The contents were mixed thoroughly and incubated at room temperature for 5 min.

2.8.4 Termination reaction

Aliquots of labelling reaction (3.5µl) were transferred to one of four termination reactions containing prewarmed dideoxy GTP, dideoxy ATP, dideoxy TTP or dideoxy CTP (2.5µl). Contents were mixed and DNA synthesis continued at 37°C for 5 min, until halted by the addition of 4µl of stop solution. After thorough mixing samples were placed on ice prior to electrophoresis.

2.8.5 Acrylamide gel electrophoresis

A pair of glass plates, one slightly shorter in length, were repeatedly rinsed with distilled water and dried. The inner side of the small plate was coated with an acrylamide-releasing agent and both plates wiped with ethanol. The plates were placed together, separated by a spacer and then sealed together along the sides and bottom of the assembly using tape. A 6% monomer acrylamide gel was prepared using the Sequegel Sequencing System (National Diagnostics). Sequegel concentrate (24ml), diluent (66ml) and buffer (10ml) were mixed on ice and the polymerising agents N,N,N',N'-tetramethylethylenediamine (TEMED) (40µl) and ammonium persulphate (500µl of 10% v/v) were added. The solution was gently mixed and poured between the plates using a syringe. An inverted comb was inserted between the plates to form a flat upper surface, clamps were applied and the gel left to polymerise for at least 1hr. The clamps were then released and the comb removed. The plates were rinsed and the sealing tape removed, prior to placing in the vertical electrophoresis apparatus. Running buffer was poured into the top reservoir (1x TBE) and the apparatus checked for leaks prior to the addition of buffer to the bottom reservoir (1x TBE, 0.6M NaAc). Top buffer was used to rinse the gel surface and the sharks-tooth comb inserted to form sample wells. Stop solution (3µl) was added to alternate wells to check against leaks, and the gel pre-run for at least 15 min at 40W. Immediately before loading, samples were denatured by heating to 75°C for 2 min. Electrophoresis was then continued for approximately 2hr. Once the bromophenol band reached the bottom gel edge the power supply was stopped and the plates removed. The plates were levered apart, leaving the gel adhered to the large plate. The gel was transferred to a solution of 10% (v/v) acetic acid, 10% (v/v)

methanol in distilled water for 15 min before being lifted off onto filter paper (Whatman). The upperside of the gel was covered in clingfilm and the gel placed in a vacuum dryer (Bio-Rad Laboratories Ltd). Once the gel was dry, the clingfilm was removed and the gel exposed to X-ray film overnight in a cassette with intensifier screens. The film was developed using an automatic film developer (X-OGraph Compact X2, supplied by HA West).

2.9 Preparation of DNA template for transcription *in vitro*.

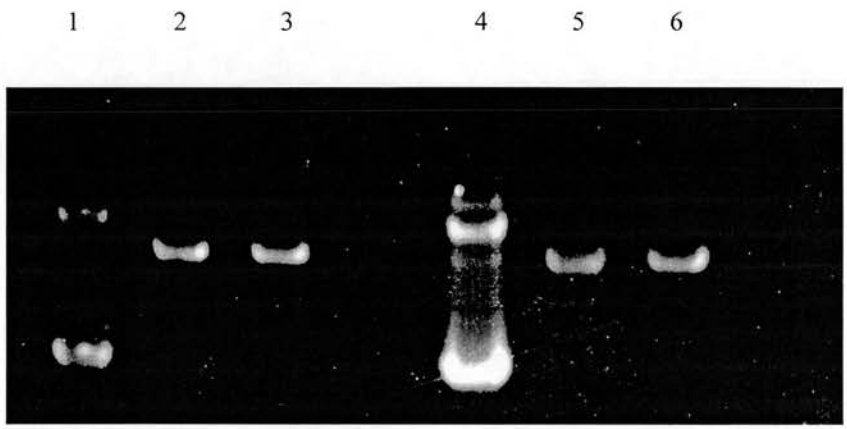
2.9.1 Plasmid linearisation

Following successful sequencing and comparison of insert to target, the DNA plasmid was linearised to enable production of "run-off" transcripts derived largely from the insert sequence alone. Restriction enzymes for linearisation were chosen to avoid the generation of 3' protruding ends, which are linked to extraneous transcript production, did not cut the insert and were present at only one site within the plasmid. Plasmids were cut in the presence of excess enzyme. A typical reaction (200μl) contained 50μg of template in the presence of 200 units of enzyme appropriately buffered, incubated at 37°C for 1hr and continued overnight at 4°C. In order for subsequent transcription to be successful, digestion for linearisation must be performed to completion. Aliquots of "cut" versus "uncut" were run on a 1% (w/v) low melting point agarose gel (SeaPlaque; Flowgen) to check for complete digestion (Figure 2.8). In the absence of complete linearisation following ethidium bromide staining (0.5μg/ml; 30 min), destaining in water (30 min) and UV visualisation, bands of cut

Figure 2.8

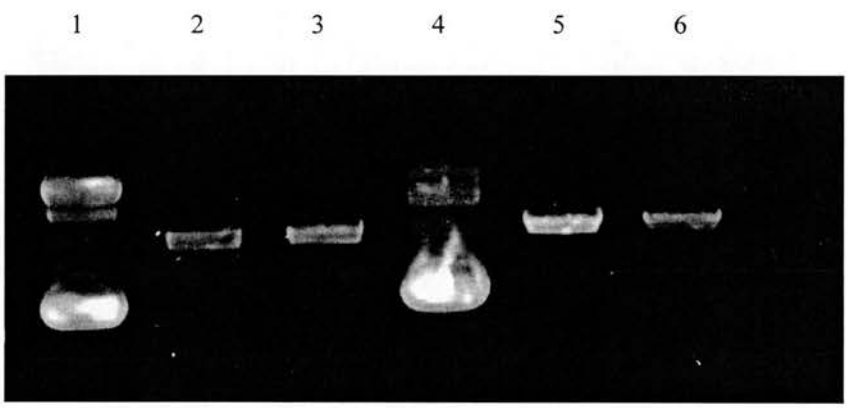
Agarose gel electrophoresis to check for plasmid linearisation.

a)



IGF-I plasmid uncut (1), cut with Nco1 (2) or Nsi1 (3).
IGF-II plasmid uncut (4), cut with Nco1 (5) or Nsi1 (6).

b)



Insulin receptor plasmid uncut (1), cut with Nco1 (2) or Not1 (3).
Type1 IGF receptor uncut (4), cut with Nco1 (5) or Not1 (6).

template were excised from the gel using a scalpel blade, placed into preweighed 1.5-ml tubes and weighed.

DNA was purified from the surrounding agarose either by phenol extraction or using the QIAquick gel extraction system (Qiagen).

2.9.2 Extraction of linearised plasmid

2.9.2.1 Phenol extraction.

To the weighed slice was added 3 volumes of water. The agarose was remelted at 65°C for 10 min and mixed well. An equal volume of phenol pH8 (Appligene Oncor) was added, well mixed and spun for 2 min. The supernatant was removed to a clean tube and phenol extraction repeated until the solution appeared clear. The solution was then subjected to phenol chloroform isoamyl alcohol (PCI), chloroform isoamyl alcohol (CI), ethanol precipitation.

2.9.2.2 Phenol chloroform isoamyl alcohol (PCI), chloroform isoamyl alcohol (CI), ethanol precipitation.

To the sample was added an equal volume of PCI (25:24:1 (v/v)). The sample was vortexed, spun and the top layer removed to a clean tube. To the supernatant was added 1 volume of CI, the sample was then vortexed, spun and the top layer removed to a clean tube. Then 0.1 volume NaAc (3M pH 5.5) was added, and then 2 volumes of 100 % (v/v) ethanol. The sample was then mixed and placed at -20°C for at least 30 min to precipitate the DNA. The DNA was recovered by centrifugation at 4°C for 20 min, washed in 70 % (v/v) ethanol, and centrifuged again. The supernatant was removed and the tube upturned and the pellet air dried for 10 min. The DNA was then resuspended in 50µl of DEPC water, and stored at -20°C in 1.5µl aliquots.

2.9.2.3 QIAquick Gel Extraction

The DNA fragment was removed from the agarose gel with a clean sharp scalpel, trimmed to remove excess agarose and placed in a pre-weighed tube. The gel slice was weighed and 3 volumes of buffer QX1 added to 1 volume of gel (where 100mg gel is equivalent to 100µl of buffer). The gel slice was dissolved by incubation at 50°C for 10 min, with vortexing every 2-3 min. After the gel slice had completely dissolved, 10µl of 3M NaAc (pH5) were added to ensure a pH suitable for adsorption of DNA to the QIAquick membrane (pH<7.5). One gel volume of isopropanol was added to the sample and mixed. The DNA sample was loaded onto a QIAquick column placed in a 2ml collection tube, microcentrifuged for 1 min and the flow through discarded. The column has a maximum volume of 800µl, sample volumes greater than 800µl required multiple loading. To ensure complete removal of agarose from the sample 0.5ml of buffer QX1 was added and centrifuged for 1 min. The column was washed with 0.75ml of buffer PE, centrifuged for 1 min and the flow through discarded. Residual column wash was removed by a further 1 min centrifugation. The QIAquick column was transferred to a clean 1.5ml centrifuge tube. To elute DNA, 50µl of H_2O was added to the centre of the column, allowed to stand for 1 min and then centrifuged for 1 min.

2.9.3 Proteinase digestion

Following the removal of agarose contaminants by either method, proteinase K digestion was performed. To the linearised plasmid sample was added 10µl of proteinase K (1mg/ml 30U/mg; Promega) in the presence of 10µl of 10x proteinase K buffer to a total volume of 100µl. The sample was incubated for 1hr at 65°C. The digestion was halted by PCI:CI:Ethanol precipitation (Section 2.9.2.2) and the DNA pellet resuspended in 50µl of DEPC-water. Template was stored in 2µl aliquots at -20°C for subsequent riboprobe synthesis.

2.10 Synthesis of high specific activity radiolabelled RNA probes

2.10.1 Transcription

RNA synthesis *in vitro* was performed to produce radio-labelled RNA transcripts, using ^{32}P-UTP for RNase protection assays and ^{35}S-UTP for *in situ* hybridisation studies. To prevent the precipitation of template DNA in the presence of cold spermidine from the 5x buffer, non-enzymatic components were allowed to equilibrate at room temperature for 1hr prior to transcription, and subsequently the mixture was held at room temperature during successive additions.

The transcription mix contained 4µl of 5x transcription buffer (Promega), 1µl of 100mM DTT (Promega), 1µl (20U) of RNasin ribonuclease inhibitor (Promega), 4µl of nucleotide mix (ATP, GTP, CTP each at 10mM; Promega), 1µl of linearised template (0.2-1mg/ml), 2.5µl of ^{32}P-UTP (20mCi/ml) or 5µl of ^{35}S-UTP (20mCi/ml) (Amersham Pharmacia Biotech UK Ltd), and 1µl (20U) of RNA polymerase

(typically Sp6, T7 or T3; Promega) in a volume of 20µl. The reaction mix was incubated at 37°C for 1hr.

Following transcription the DNA template was removed by digestion with DNase. To the mix was added 10µl of yeast transfer ribonucleic acid (tRNA) (11mg/ml), 8µl of 5x transcription buffer, 1µl of RNasin and 1µl (1U) of RNase-free DNase (Promega). A 1µl aliquot of labelled probe was removed and diluted 1:10 in water for subsequent determination of percent incorporation and specific activity. The remaining mix was incubated at 37°C for 15 min. In order to limit problems of high background and to give optimum sensitivity, unincorporated nucleotides were removed from the labelled probe by applying the transcription mix to a TE-Midi select G-50 spin column (CP Laboratories).

The gel contained within the column was resuspended by inversion, and shaken with a sharp downward motion to draw the resin from the top closure. The column closures were removed and the column placed into a collection tube, prior to microcentrifugation (90 sec). The collection tube was discarded and the column placed in a second collection tube. The 50µl sample was applied to the surface of the gel bed and allowed to stand for 2-3 min. The loaded column was then spun for 60 sec (Beckman E) to elute the labelled probe free of unincorporated nucleotides, into a solution of TE buffer.

2.10.2 Determination of percent incorporation and probe specific activity

Aliquots (1µl) of the 1:10 dilution of labelled probe removed previously were spotted onto duplicate (1cm diameter) glass fibre filters (Whatman International Ltd), allowed to air dry and used to determine total cpm per microlitre. In order to

calculate incorporation, a further 1µl of the 1:10 dilution was added to 10µl of yeast tRNA (11mg/ml) in a total volume of 100µl, mixed and 0.5ml of ice-cold 5% (w/v) trichloroacetic acid (TCA) added. The mix was placed on ice for 5 min, then applied to glass fibre filters prewetted with 5% TCA under vacuum. The mix was drawn through the filters, washed three times with 1ml of 5% (w/v) TCA and rinsed once with 3ml of 100% (v/v) ethanol. The filters were then dried under vacuum. To calculate the specific activity of the probe 1µl of eluted probe was applied to duplicate glass fibre filters and allowed to air dry. The six dry filters were then placed into scintillation vials, 2ml of scintillation fluid (Optiphase HiSafe 3, Fisher Scientific UK Ltd) added and the samples counted in a β-counter.

Percentage incorporation was calculated as TCA precipitated cpm/ total cpm x 100.

Specific activity of the probe is expressed as the total incorporated cpm/ total µg RNA synthesised.

2.11 *In situ* hybridisation

2.11.1 Prehybridisation

2.11.1.1 Cryostat sections

Sections of ovarian tissue (14µm) were cut at -20°C using a cryostat (Shandon OT, Shandon Scientific Ltd), and mounted onto prechilled microscope slides (Superfrost-Plus, Merck Ltd). Sections were stored in desiccated air-tight boxes at -80°C until required. For each hybridisation, two sections per animal were hybridised with the antisense probe and one section was hybridised with the sense probe.

Slides were removed from -80°C and allowed to warm to room temperature. All pre-hybridisation steps were performed to minimise RNase contamination, using DEPC treated solutions and baked glassware. Sections were fixed in 4% (v/v) formaldehyde in 1x PBS for 5 min, and then hydrated in 2x SSC for 2 min at room temperature. Sections were acetylated in 0.25% (v/v) acetic anhydride in 0.1M triethanolamine HCl, 0.154 M NaCl (pH8) for 10 min with shaking. Sections were then rinsed in 2x SCC and dehydrated in 60%, 80%, 95% and 100% (v/v) ethanol each for 2 min. Sections were then delipidated in chloroform for 5 min, and returned to 100% and then 95% (v/v) ethanol each for 2 min, before being left to air dry.

2.11.1.2 Paraffin embedded sections.

Sections of ovarian tissue fixed in 4% (w/v) paraformaldehyde and embedded in paraffin were cut at 8 micron on a microtome and floated onto pre-coated microscope slides (Super-Frost, Merck Ltd). Sections were twice placed in Histoclear (National Diagnostics) for 10 min, followed by 100% ethanol (2x 2 min) and 95%, 80%, 60%, and 30% (v/v) ethanol each for 2 min. Sections were then washed in 0.85% (w/v) NaCl and 1x PBS each for 5 min prior to fixation in 4% (w/v) paraformaldehyde in 1x PBS for 20 min. Tissue was then treated with proteinase K (1mg/ml) in 0.01M EDTA, 0.1M Tris for 7.5 min, and twice washed in 1x PBS for 5 min. Slides were then placed into 4% (w/v) paraformaldehyde in 1x PBS for a further 5 min, briefly rinsed in dH_2O and then twice washed in 2x SSC each for 2 min. Slides were subsequently treated as detailed above for frozen sections from the acetylation stage onwards.

2.11.2 Hybridisation

Hybridisation was performed using 50µl of ^{35}S-labelled riboprobe diluted to 1 x 10^7cpm/ml in hybridisation buffer containing 50% (v/v) formamide, 1x Denhardt's solution, 0.05M DTT, 500µg/ml yeast tRNA and 10% (w/v) dextran sulphate. Slides were placed in airtight chambers humidified with 50% (v/v) formamide in 1x SSC, diluted probe applied, and covered with a piece of parafilm. Hybridisation was performed overnight at 55°C.

2.11.3 Post hybridisation

Following hybridisation, the parafilm was removed by repeatedly dipping the slides in 2x SSC pre-warmed to 55°C. Excess probe was removed by washing the slides twice in pre-warmed 2x SSC each for 15 min, at room temperature with shaking. Sections were then treated with RNase A (20 mg/ml) in 2x SSC, to digest mismatched probe and hence reduce non-specific binding. RNase digestion was performed for 1 hour at 37°C in a slow shaking water bath. Slides were then washed at 55°C in 2x SSC for 15 min, 1x SSC for 15 min, 50% (v/v) formamide in 1x SSC for 30 min and twice in 0.1x SSC each for 15 min, with each wash supplemented with 0.1% (v/v) β-ME. Sections were then dehydrated in 60%, 80%, 95% and 100% (v/v) ethanol each for 2 min at room temperature and air dried.

Slides were dipped in (50% v/v) Ilford K-2 emulsion (HA West) warmed to 42°C, and then left to dry overnight. Slides were then placed in air-tight desiccated boxes and kept at 4°C for a three week exposure period. The slides were then placed in 50% (v/v) Kodak D-19 developer (HA West) for 2.5 min, rinsed in water for 30 sec and fixed in 20% (v/v) Ilford Hypam Fix (HA West) for 3 min. Slides were then

lightly counterstained with haemotoxylin and eosin and mounted for microscopic examination under light and darkfield illumination.

2.12 RNase protection assays

Total RNA (25 μg) from luteal and liver homogenates was incubated simultaneously with low specific activity 18S ribosomal RNA antisense riboprobe (3000 cpm/ sample; 1.5 x 10^6 cpm /ml), synthesised using a T7 RNA 18S template (AMS Biotechnology (UK) Ltd.) and high specific activity riboprobes directed against the target RNA of interest (100,000 cpm/ sample; 5 x 10^7 cpm /ml). Riboprobes were synthesised as described above (see section 2.10) using ^{32}P-UTP. Hybridisation was performed in 1,4-piperazine-diethanesulfonic acid (PIPES) buffer (0.025M, pH 6.8) containing NaCl (0.4M), EDTA (1.0mM, pH 8) and formamide (50% v/v). Samples were incubated for 10-min at 85°C, and then overnight at 55°C. Following overnight incubation 500μl of RNase digestion buffer (0.3M NaCl; 7.5M Tris-HCl buffer, pH 8; 5mM EDTA, pH7.5), containing 2μg/ml RNase T1 and 40μg/ml RNase A (Roche Diagnostics Ltd) were added, and samples incubated for a further 30-min at 37°C. RNase digestion was subsequently terminated by incubation with Proteinase K (200μg 30U/mg; Promega) with 20μl 10% (w/v) sodium dodecyl sulphate (SDS) for 30 min at 37°C. Samples were then precipitated in isopropanol, resuspended in gel loading buffer, heated to 90°C and loaded onto 6% monomer sequencing gels (section 2.8.5) (Sequegel, National Diagnostics). Gels were run in 1x TBE buffer, and were subsequently lifted off onto filter paper, and exposed to X-ray film overnight at -70°C with intensifying screens. Films were developed using an

automated processor (X-OGraph Compact X2, supplied by HA West) and protected RNA bands viewed.

The protection of bovine IGF-I, IGF-II, the type 1 IGF receptor, IGFBP-3, IGFBP-4 and porcine IGF-I riboprobes with total luteal and liver RNA are included in the appendix for further confirmation of probe specificity.

2.13 Progesterone Assay

Plasma progesterone concentrations were analysed by radioimmunoassay as described by Corrie *et al.*, (1981) and modified using a non-extraction assay by Law *et al.*, (1992). All reagents were diluted in phosgel assay buffer. Standard solutions were prepared from P-0130 progesterone (Sigma-Aldrich Company Ltd), to give standards of 7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000 and 2000 pg/tube. Plasma (50µl) from ovariectomised animals was added to all standard tubes, which were assayed in triplicate and made up to 500µl in assay buffer. Two quality controls (one high progesterone and one low progesterone) were assayed with the plasma samples. Plasma samples were dispensed in duplicate and made up to 500µl with assay buffer. [^{125}I]-Labelled progesterone (11α glucuronide hemisuccinate; Amersham Pharmacia Biotech UK Ltd) was reconstituted in assay buffer to give 12-15,000 cpm per 100µl, incorporating 1mg/ml of 8-anilino-1-naphthalene sulfonic acid (ANS). Standards and samples were incubated with 100µl of label and 200µl rabbit antiprogesterone antiserum (SAPU: diluted 1:50,000). Primary antibody cross reactivity is detailed in the appendix. Blanks received 200µl assay buffer. All tubes were vortexed and left to incubate at room temperature for at least 3hr. In order to separate free and antibody-bound progesterone, donkey-anti-rabbit serum (100µl) (SAPU) (1:35 in assay buffer

containing 10% 0.1M EDTA), and normal rabbit serum (SAPU) (1:300) were added to all tubes except totals. The tubes were vortexed and left to incubate at 4°C overnight. All tubes except totals were prewashed by adding 1ml of cold assay buffer and spun at 2000g for 30 min at 4°C. The supernatant was then decanted and the tubes allowed to dry briefly, before counting the activity of the precipitates.

Chapter 3. Expression of mRNA encoding IGF-I, IGF-II and the type 1 IGF receptor in the bovine corpus luteum at defined stages of the oestrous cycle.

3.1 Summary

Previous studies have implicated insulin-like growth factors, IGF-I and -II, in the regulation of ovarian function. We have investigated the localisation of mRNA encoding IGF-I, -II and the type 1 IGF receptor to further determine the roles of the IGFs within the bovine CL at precise stages of the oestrous cycle using *in situ* hybridisation. Luteal expression of IGF-I, -II and the type 1 IGF receptor mRNA was detected throughout the oestrous cycle. IGF-I mRNA expression varied significantly during the cycle. IGF-I mRNA levels were significantly higher on day 15 than day 10, and IGF-I mRNA in the regressing CL 48 hours after exogenous $PGF_{2\alpha}$ was significantly greater than in the early or mid-luteal phase (day 5 and 10). In contrast, there was no significant effect of day of the oestrous cycle on mRNA expression for IGF-II and the type 1 IGF receptor in the CL. IGF-II mRNA expression was localised to a subset of steroidogenic luteal cells and was also associated with cells of the luteal vasculature. Messenger RNA encoding the type 1 IGF receptor was widely expressed, in a pattern suggestive of steroidogenic luteal cell expression. These data clearly demonstrate that the bovine CL is a site of IGF production and reception throughout the luteal phase. Importantly however, this study highlights the potential of IGF-II in addition to IGF-I, in the autocrine/paracrine regulation of luteal function.

3.2 Introduction

Whilst pituitary-derived LH is the primary regulator of CL function, it is becoming apparent that other factors of extra- and intra- ovarian origin have the potential to modulate the luteal response to gonadotrophins. The IGF system plays a central role in these interactions and studies *in vitro* have shown that both IGF-I and IGF-II have wide ranging effects on luteal function (Giudice, 1992; Spicer and Echternkamp, 1995).

The formation of the CL in response to the preovulatory surge of LH involves changes in cellular morphology and ultrastructure, and key alterations in steroid hormone synthesis (Smith *et al.*, 1994c). In sheep and cattle following the LH surge, production by the ovulatory follicle of oestradiol and androstenedione ceases and progesterone synthesis increases. This dramatic shift in steroidogenesis is the product of changes in the tissue content of steroidogenic enzymes and factors involved in steroid precursor provision (Couet *et al.*, 1990; Rodgers *et al.*, 1986b, 1987; Voss and Fortune, 1993).

IGF-I enhances LH receptor binding capacity in rat granulosa cells luteinised in culture, acting in synergy with FSH (Adashi *et al.*, 1985c). The increase in receptor numbers is accompanied by increased sensitivity to LH and potentiation of its luteotrophic effects. In addition, IGF-I has direct stimulatory effects on key components of the steroidogenic pathway, and increased progesterone secretion has been demonstrated in rat (Talavera and Menon, 1991), ovine (Khan-Dawood *et al.*, 1994), porcine (Yuan and Lucy, 1996a), rabbit (Constantino *et al.*, 1991), human (Devoto *et al.*, 1995) and bovine (McArdle and Holtorf, 1989; Sauerwein *et al.*, 1992) luteal tissue. IGF-I has potent actions on sterol metabolism that include

amplification of StAR (Balasubramanian *et al.*, 1997) and the delivery and utilisation of steroid precursors (Veldhuis, 1989). The key steroidogenic enzymes P450scc and 3β-HSD are also targets of IGF action, demonstrated by both increased gene expression and enzyme activity in response to IGF-I stimulation in the rat (deMoura *et al.*, 1997; Magoffin *et al.*, 1990; Magoffin and Weitsman, 1993).

Many studies have focused on the potential roles of IGF-I in ovarian function. However, the regulatory effects of IGF-II have not been studied to the same extent, possibly because IGF-II has been considered to exert its effects predominantly prenatally (Hossner *et al.*, 1997). Our recent results have indicated that IGF-II is the major ligand of the IGF system in the bovine follicle (Armstrong *et al.*, 2000a). Moreover, IGF-II has been shown to increase progesterone production by luteal cells *in vitro* (Sauerwein *et al.*, 1992) and detailed studies in porcine granulosa cells have demonstrated potent stimulatory effects of IGF-II on progesterone biosynthesis, largely facilitated by enhanced sterol delivery via increased lipoprotein binding, internalisation and utilisation, and P450scc enzyme activity (Garmey *et al.*, 1993).

Studies *in vitro* strongly suggest a role for IGF-I and/or IGF-II in regulating the acquisition and maintenance of differentiated function in the CL. However, the IGFs may also have additional actions within the ovary, particularly within the CL, that extend beyond the regulation of steroidogenesis to include angiogenesis and apoptosis. The vascularisation of the developing CL increases significantly to support tissue growth and steroid production, in addition to cellular differentiation of luteal cells. The primary regulators of ovarian angiogenesis appear to belong to the VEGF and FGF families (Reynolds and Redmer, 1998), though IGF-I has also been implicated in neovascularisation occurring in response to injury (Hansson *et al.*,

1989), in some disease states (Vialettes *et al.*, 1994) and in the classical angiogenic model of the rabbit cornea (Grant *et al.*, 1993). Immunohistochemical studies also suggest a role for IGF-II in the regulation of luteal vasculature (Amselgruber *et al.*, 1994).

The CL is a transient endocrine gland, which must undergo functional and structural regression in the absence of pregnancy. Morphological and biochemical events suggestive of apoptotic cell death have been associated with this controlled demise (Juengel *et al.*, 1993; Sawyer *et al.*, 1990; Zeleznik *et al.*, 1989). The interaction of the IGF receptor with IGF-I or IGF-II has been shown to protect a range of cell types, including ovarian cells (Chun *et al.*, 1994), from apoptosis (Harrington *et al.*, 1994; Parrizas and LeRoith, 1997; Robinson Singleton *et al.*, 1996).

The aims were to understand the role of the IGFs in the growth and regression of the bovine CL, by describing the temporal and spatial changes in the expression of the mRNAs encoding IGF-I, IGF-II and the type 1 IGF receptor throughout the luteal lifespan.

3.3 Materials and Methods

3.3.1 Animals

The oestrous cycles of 9 Holstein/Friesian cows were synchronised with intravaginal progesterone release (PRID; 1.55g P4, 10mg oestradiol benzoate; Sanofi Animal Health Ltd) maintained for 12 days, and $PGF_{2\alpha}$ analogue (Estrumate; 500 μg; Coopers Animal Health Ltd) administered intramuscularly (i.m) on the day before PRID removal to induce luteolysis. Ovaries were collected on days 5, 10 or 15 after the onset of oestrus (day 0) in the subsequent cycle (n=3 per group). An additional three animals were treated with $PGF_{2\alpha}$ analogue and the ovaries collected 48 hours later to study the regressing CL. Following ovariectomy, all corpora lutea were excised and divided into blocks. Luteal pieces were either, 1) frozen in liquid nitrogen and stored at -80°C prior to cryostat sectioning for *in situ* hybridisation or RNA extraction, or 2) fixed in 4% (w/v) paraformaldehyde and processed for paraffin embedding. Frozen sections from ovaries collected for follicular studies (Armstrong *et al.*, 1998) were also subjected to *in situ* hybridisation.

Blood samples (10ml) were collected on alternate days during the synchronised cycle by jugular or coccygeal venepuncture into tubes containing 100μl of sodium citrate (0.35 g/ml), from haltered and restrained animals. All blood samples were centrifuged (1000g), and plasma separated and frozen at -20°C until assayed for progesterone (see section 5.3.2).

3.3.2 RNA probes

Homologous bovine IGF-II and type 1 IGF receptor probes were prepared using the primer pairs detailed in section 2.4. A plasmid containing a porcine IGF-I probe was donated by Dr M Lucy (University of Missouri). Riboprobe specificity was tested by RNase protection assay (see section 2.12). The protection of [^{32}P]-labelled (Amersham Pharmacia Biotech) bovine IGF-II, bovine type 1 IGF receptor and porcine IGF-I riboprobes with total luteal and liver RNA (25μg) is shown in the appendix.

Isotopic antisense and sense RNA probes for *in situ* hybridisation were transcribed from linearised cDNA templates following a standard transcription protocol (see section 2.10.1) using [^{35}S]-UTP (Amersham Pharmacia Biotech).

3.3.3 *In situ* hybridisation

Frozen sections (14 μm) of ovarian tissue were subjected to *in situ* hybridisation (see section 2.11). Antisense RNA probes for IGF-I, IGF-II and the type 1 receptor were each hybridised to two serial sections per CL or ovary. The sense probe for each mRNA species was applied to a further serial section. The intensity of the *in situ* hybridisation signal was quantified using a NIH-Image analysis system (NIH, Bethesda, MD). Four fields of view were chosen within each slide and the number of pixels occupied by silver grains counted. In order to minimise operator bias the chosen fields of view corresponded to the points of the compass, North, South, East and West on all sections. The position of each field was also selected to minimise possible artefacts, for example, bright spots from scratches in the emulsion, holes or tears in the tissue, and edge effects. The full extent of each field, viewed under the

10x objective, was then selected on the video screen and the signal quantified. This was then presented as a percentage of the total number of pixels within the defined area and antisense signals were then compared to their respective sense (background) signals. Defined areas of a control slide were routinely selected and analysed to ensure that alterations in microscope settings did not create between-session variation.

3.3.4 Statistical Analysis

In situ hybridisation data was analysed using a split-plot analysis of variance (ANOVA) (Genstat 5; Payne *et al.*, 1993) to determine the effect of stage of the oestrous cycle (treatment factor) on mRNA expression. Measurements made within slides were nested within cows for use as the blocking factor. Significant differences between timepoints were tested using least significant differences with a pooled standard error of the difference (SED) determined by ANOVA. Differences between stages were considered significant when $P < 0.05$.

3.4 Results

3.4.1 Expression of IGF-I mRNA

Messenger RNA encoding IGF-I was expressed in CL at all timepoints studied. Expression was low and widespread, with areas of higher abundance towards the periphery and in lines radiating through the CL (Figure 3.1). The exact identity of these cells could not be determined. Concentrations of IGF-I mRNA varied significantly with the day of the cycle ($P = 0.027$) (Figure 3.4a). Steady-state IGF-I mRNA levels were significantly higher on day 15 than day 10, and the expression observed in the regressing CL 48-hours after prostaglandin administration was

significantly greater than IGF-I levels detected in the early and mid-luteal phase (day 5 and 10).

3.4.2 Expression of IGF-II mRNA

Messenger RNA encoding IGF-II was present in CL at all timepoints studied. Expression was localised to a subset of steroidogenic luteal cells and was also present in cells associated with blood vessels of the CL (Figure 3.2). Quantitative analysis showed no significant effect ($P > 0.05$) of day of the oestrous cycle on IGF-II mRNA expression (Figure 3.4b).

3.4.3 Expression of type 1 IGF receptor mRNA

At all timepoints studied, CL expressed mRNA encoding the type 1 IGF receptor. The hybridisation signal was widespread and the pattern of hybridisation suggested that expression was localised to steroidogenic luteal cells (Figure 3.3). Expression was absent from peripheral (stromal/capsular) regions and some large blood vessels. Statistical analysis revealed no significant changes ($P > 0.05$) in expression of mRNA encoding type 1 IGF receptor throughout the luteal phase (Figure 3.4c).

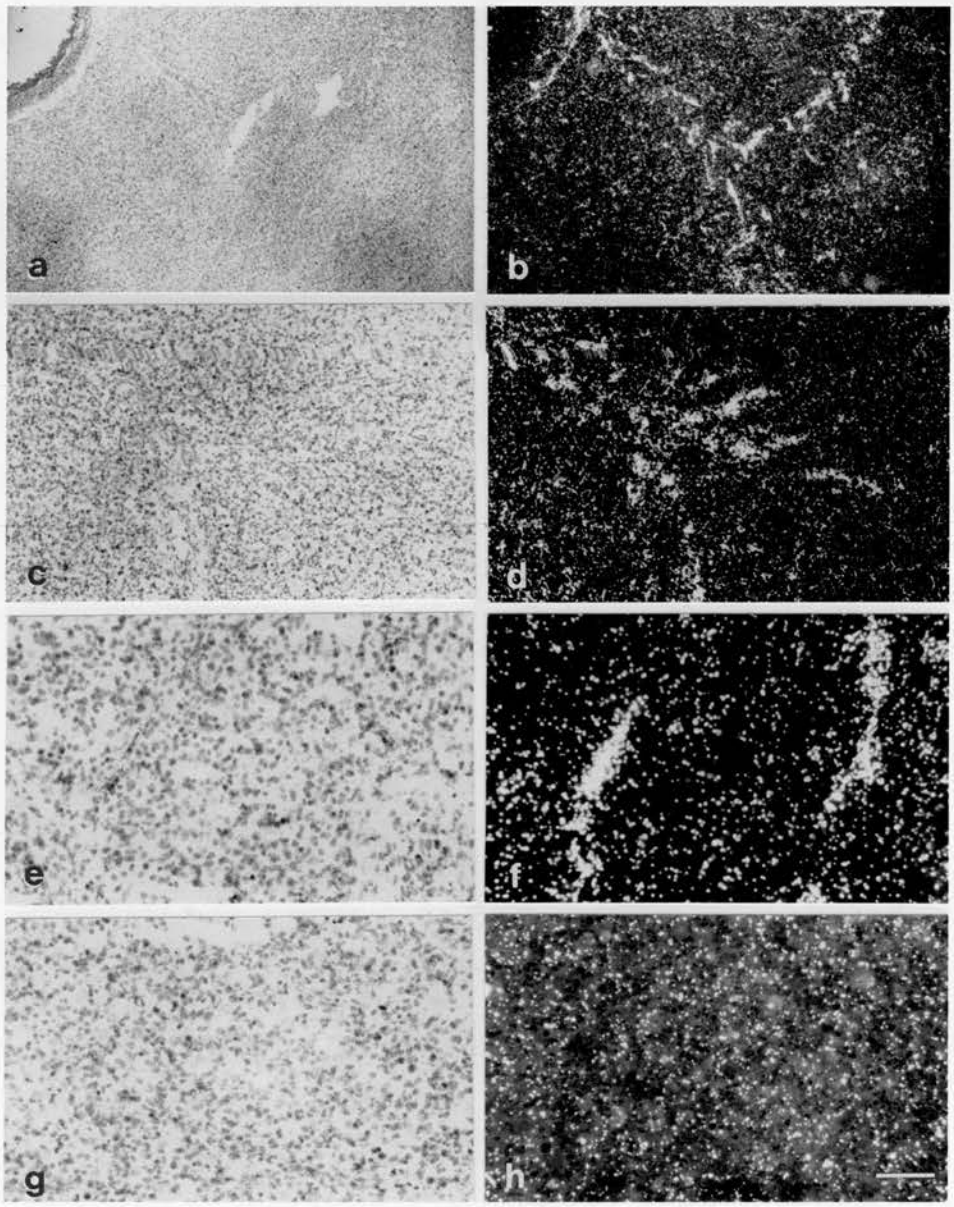


Figure 3.1. Bovine ovarian sections (14 μm) were probed with an IGF-I antisense (a-f) or sense (g and h) riboprobe. The same fields of view are shown under lightfield (a, c, e and g) and darkfield (b, d, f and h) illumination. Localisation of mRNA encoding IGF-I is shown in luteal tissue adjacent to a small follicle, at two different magnifications (a-d), and in a further corpus luteum at higher magnification (e and f). Scale bar represents 450 μm (a and b), 180 μm (c and d) and 90 μm (e-h).

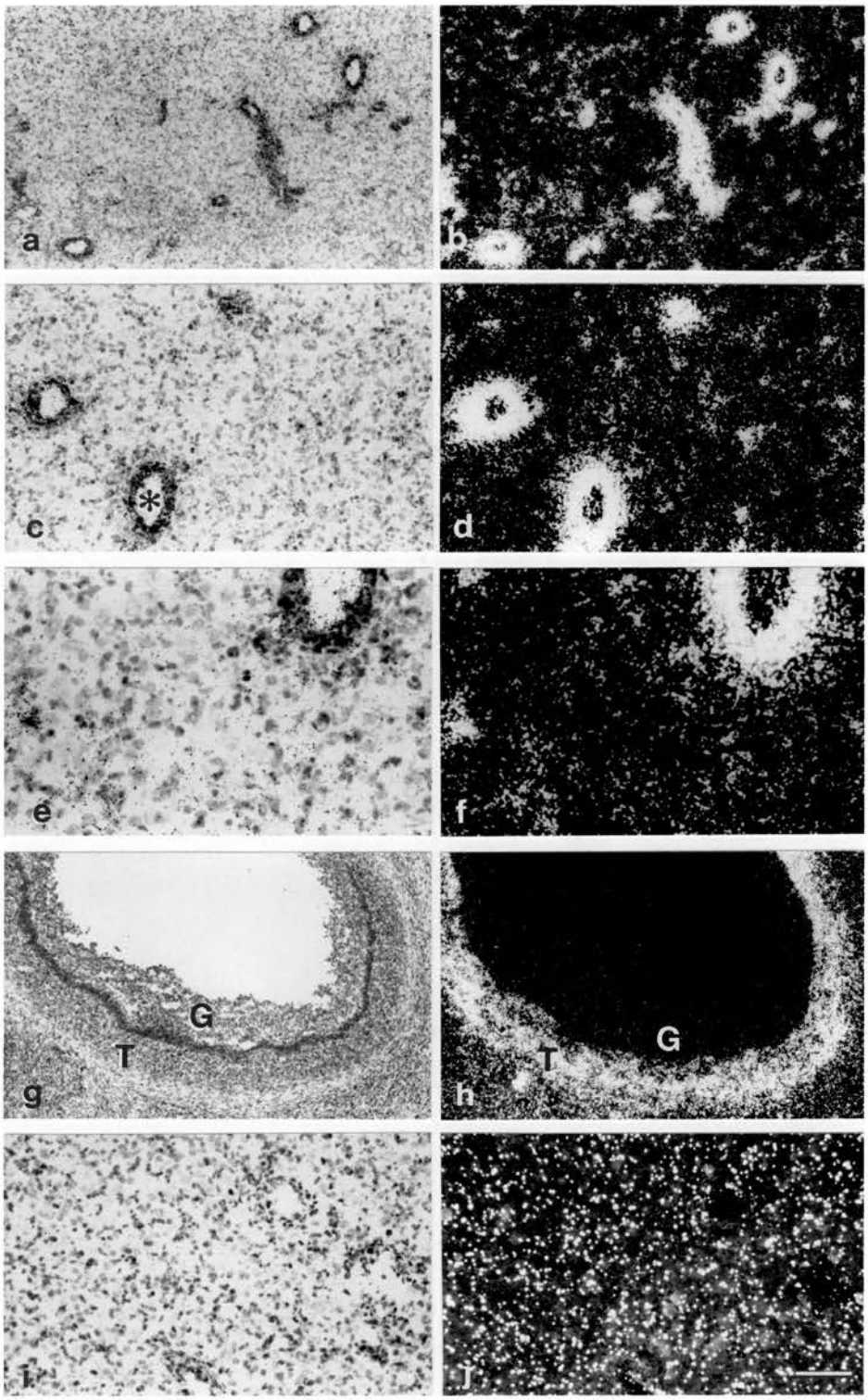


Figure 3.2. Bovine ovarian sections (14 μm) were probed with an IGF-II antisense (a-h) or sense (i and j) riboprobe. The same fields of view are shown under lightfield (a, c, e, g, and i) and darkfield (b, d, f, h, and j) illumination. Localisation of mRNA encoding IGF-II is shown in a typical corpus luteum at three different magnifications (a-f). Follicular expression is shown in theca tissue of a small follicle (g and h). Scale bar represents 180 μm (a, b, g, and h), 90 μm (c, d, i, and j) and 45 μm (e and f). *, G, and T represent a blood vessel, granulosa cells, and theca cells respectively.

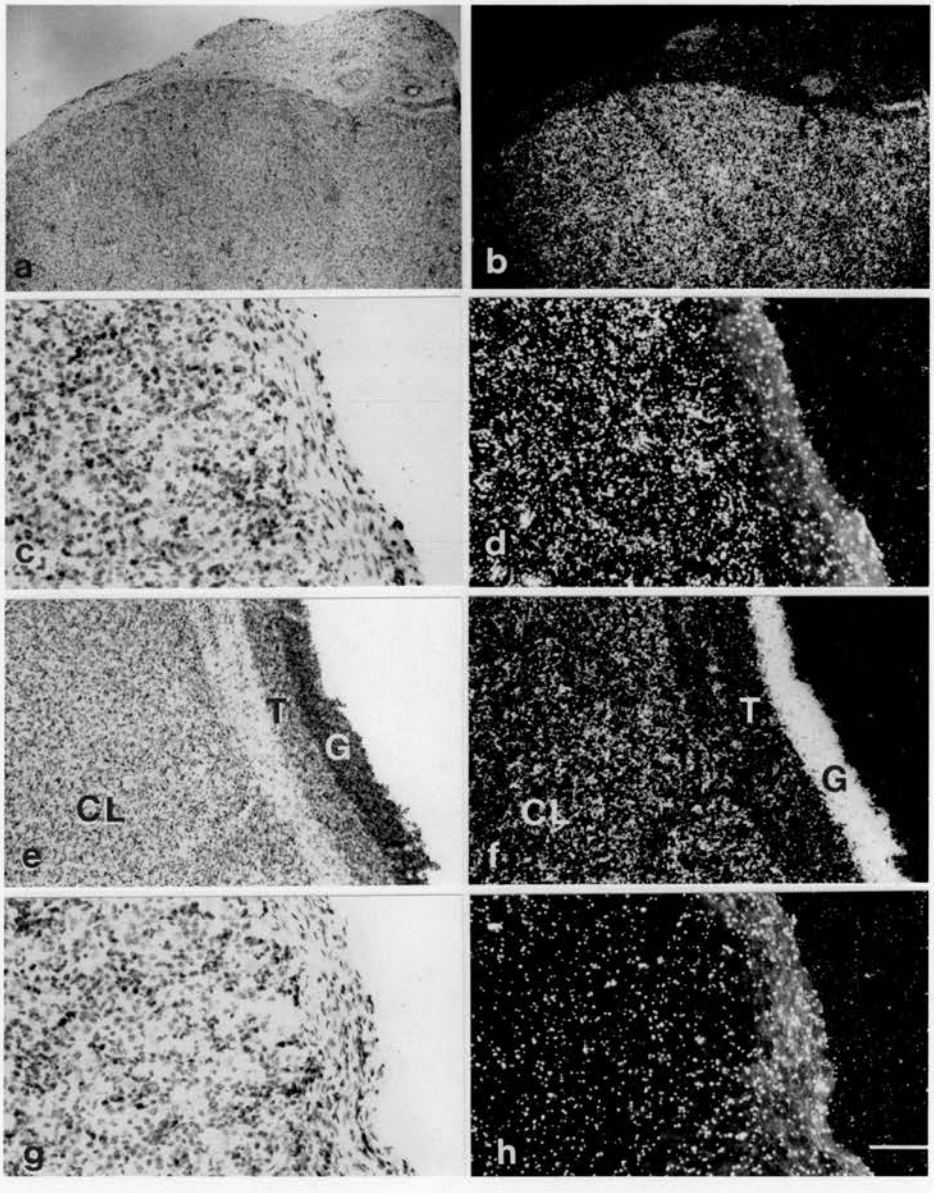
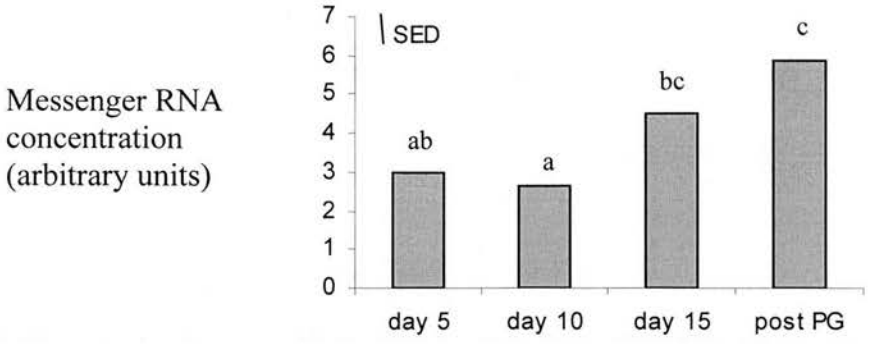
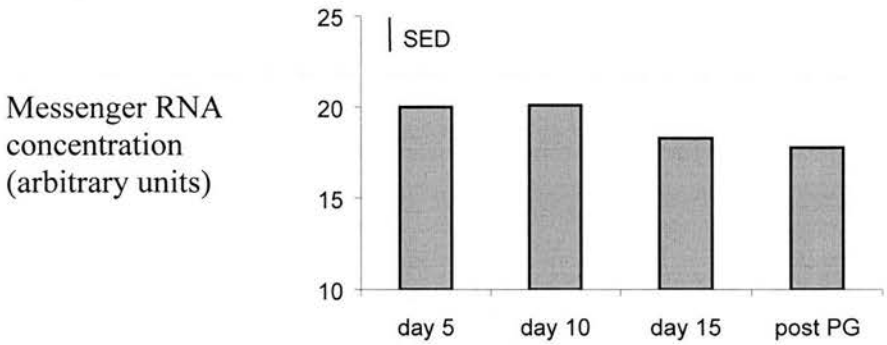


Figure 3.3. Bovine ovarian sections (14 μm) were probed with a type 1 IGF receptor antisense (a-f) or sense (g and h) riboprobe, and the same fields of view are shown under lightfield (a, c, e, and g) and darkfield (b, d, f, and h) illumination. Localisation of mRNA encoding the type 1 IGF receptor is shown in the same CL at two magnifications (a-d) and in luteal tissue adjacent to a small follicle (e and f). The scale bar represents 450 μm (a and b), 180 μm (e and f), and 90 μm (c, d, g and h). G, T, and CL represent granulosa cells, theca cells, and corpus luteum respectively

a) IGF-I



b) IGF-II



c) Type 1 IGF receptor

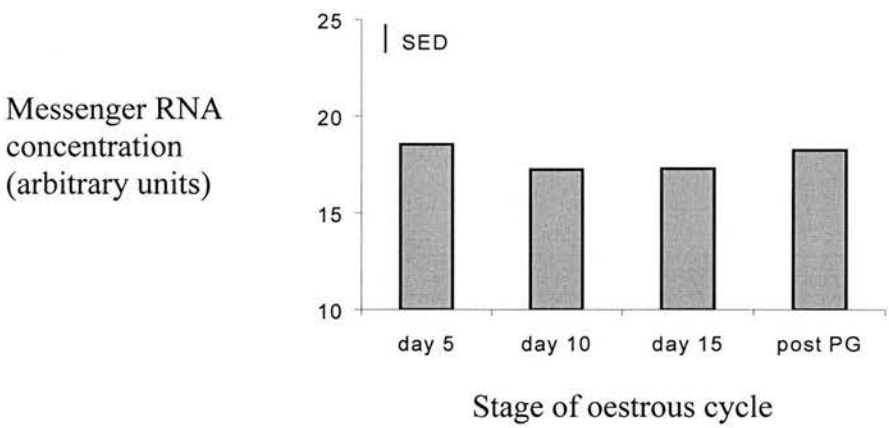


Figure 3.4.

Quantitative analysis of IGF-I (a), IGF-II (b) and the type 1 IGF receptor (c) mRNA concentrations in bovine luteal tissue on days 5, 10 and 15 of the oestrous cycle, and following exogenous prostaglandin (post PG). Luteal timepoints with different superscipts are significantly different ($P < 0.05$). Bars represent pooled standard error of the difference (SED). Messenger RNA concentration is expressed as the percentage of the total number of pixels occupied by silver grains within a defined area. Hybridisation with sense probes gave average background values of 2, 4 and 6 units for IGF-I, IGF-II and the type 1 IGF receptor respectively.

3.5 Discussion

The present study describes the temporal and spatial changes in mRNAs encoding IGF-I and –II, and the type 1 IGF receptor during growth and regression of the bovine CL. It extends the current understanding of the IGF system in the CL of the cow, being to our knowledge, the first detailed report of the localisation of mRNAs encoding IGF-I, IGF-II and the type 1 IGF receptor throughout the luteal phase.

Messenger RNA encoding IGF-I, IGF-II and type 1 IGF receptor showed distinct spatial patterns of expression within the bovine CL. IGF-I mRNA expression was low throughout the CL, with areas of higher abundance towards the periphery and in lines radiating through the CL. In contrast, mRNA encoding IGF-II was found in a subset of steroidogenic luteal cells and also in association with luteal blood vessels. Messenger RNA encoding type 1 IGF receptor was widely expressed throughout the CL in a pattern suggestive of steroidogenic luteal cell expression, but was absent from peripheral regions of the CL and some large blood vessels.

Whilst mRNA encoding IGF-I, IGF-II and type 1 IGF receptor differ spatially within the bovine CL, only IGF-I mRNA showed significant temporal changes in expression. In addition, follicular patterns of expression of mRNA encoding IGF-I, IGF-II and the type 1 IGF receptor were in agreement with our previous observations (Armstrong *et al.*, 2000a).

Previous studies have reported the ability of IGF-I and IGF-II to regulate ovarian function, and the expression of mRNA and protein components of the IGF system within the ovaries of a range of species has led to the hypothesis that the IGF system can exert its influence at the autocrine/paracrine level. Although studies of the local

production of components of the IGF system is less complete for the CL than the follicle, the bovine CL has been shown to express IGF-I mRNA (Einspanier *et al.*, 1990; Kirby *et al.*, 1996; Vandehaar *et al.*, 1995), and IGF-I and -II proteins have also been localised in the bovine CL (Amselgruber *et al.*, 1994). Moreover, binding characteristic of the type 1 IGF receptor has been demonstrated in CL throughout the luteal phase (Sauerwein *et al.*, 1992). Messenger RNA encoding IGF-I, IGF-II and the type 1 IGF receptor has recently been localised in bovine CL at uncharacterised stages of the cycle (Perks *et al.*, 1999). The present study further extends our understanding of the role of the IGF system in bovine luteal function by the use of precisely timed corpora lutea collected throughout the luteal phase.

In the present study, mRNA encoding IGF-I was demonstrated in the CL throughout the oestrous cycle. However, the temporal changes in expression of IGF-I mRNA in the ruminant CL remain controversial. An earlier study of the presence of IGF-I mRNA in the bovine CL by Northern hybridisation demonstrated low expression in the early luteal phase (days 1-5), which increased from days 6-11 to reach maximal on days 12-17, before rapidly decreasing around the time of natural luteolysis (days 18-21) (Einspanier *et al.*, 1990). In contrast, in the present study, IGF-I mRNA levels were low in the early and mid luteal phase (day 5 and 10), increased significantly between day 10 and 15, and were maximal following prostaglandin-induced luteal regression. The increase in IGF-I mRNA between early and mid luteal groups observed by Einspanier *et al.* (1990) is not supported by the results of the present study. Differences in methodology may partly explain differences in IGF-I mRNA expression patterns, since analysis of IGF-I mRNA expression by Northern hybridisation is complicated by the similarity in size of major luteal IGF-I mRNA

transcript(s) (3 to 5kb) and the 28S ribosomal RNA (4.7kb). The differences in IGF-I mRNA expression observed between the present study and that of Einspanier *et al.* (1990) might also be due in part to differences in tissue collection. The early luteal phase group of Einspanier *et al.* comprises corpora lutea judged by macroscopic observations to be 1-5 days post-ovulation, and is therefore likely to include younger CL than the present study where an oestrus synchronisation regime was used to collect luteal tissue of a known age. If luteal IGF-I mRNA levels are lower prior to day 5 this would not be reflected by the present study's first timepoint, but would result in an increase between the early and mid luteal groups of Einspanier *et al.* (1990). Indeed, luteal IGF-I mRNA concentrations were shown to increase between day 3 and day 6 of the ovine oestrous cycle (Juengel *et al.*, 1997), although others had previously observed limited variation throughout the oestrous cycle of the ewe (Perks *et al.*, 1995).

An important regulatory role for IGF-I in the CL is suggested by IGF-I-stimulated progesterone release *in vitro*, and further supported by positive immunostaining for IGF-I in both large and small steroidogenic luteal cells (Amselgruber *et al.*, 1994). Expression of IGF-I mRNA in the bovine CL is confirmed by the present study, and indicates that IGF-I may act as an autocrine/paracrine regulator of luteal function. The correlation between increasing IGF-I mRNA and peak progesterone production (Fields and Fields, 1996; Ireland *et al.*,1980) further implies a physiological significance for locally produced IGF-I within the bovine CL. Increased IGF-I expression during periods of high metabolic activity may reflect an important role for IGF-I in stimulating key components of the steroidogenic pathway. It is interesting to note that whilst IGF-I has been shown to influence the acquisition of differentiated

function, that local expression is low during luteinisation.

It has also been suggested that IGF-I derived from the peripheral circulation might act in an endocrine manner to regulate luteal function. Support for this theory is provided by an apparent heterogeneity of immunostaining for IGF-I in the CL, which may be dependent on the proximity of luteal cells to blood vessels, implying luteal uptake of IGF-I (Amselgruber *et al.*, 1994). In addition, there is evidence in extra-ovarian tissues of IGF transport, whereby IGF peptides have been localised to cellular sites within human foetal tissues which are distinct from the site of mRNA expression (Hill *et al.*, 1988), such that immunostaining reflects site of IGF binding rather than production. Hence, although the IGF-I peptide is associated with luteal cells (Amselgruber *et al.*, 1994), these cells may not be the primary source of IGF-I within the bovine CL. The relative importance of endocrine versus autocrine/paracrine IGF-I stimulation in the CL remains to be determined. Furthermore, the significance of high expression of IGF-I mRNA seen as radiating lines within the CL remains unclear, though a similar pattern was described by Perks *et al.* (1995) in the ovine CL. These cells may be endothelial in nature, although the function and importance of IGF-I mRNA expression by cells lining a small number of blood vessels in such a restricted pattern is unknown. Alternatively, the cells may be steroidogenic luteal cells, and the expression pattern may be related to the morphogenesis of the luteal lobules. Immune cells are also suggested sites of luteal IGF-I expression (Perks *et al.*, 1995).

The potential role of IGF-I during luteal regression also remains unclear. Einspanier *et al.* (1990) demonstrated a reduction in IGF-I mRNA expression around the time of natural luteolysis in the bovine CL, whilst induced luteolysis in the ewe (Juengel *et*

al., 1997) was not associated with any significant alteration in the expression of mRNA encoding IGF-I up to 24 hours following the administration of $PGF_{2\alpha}$. Furthermore, the expression of IGF-I mRNA was shown to be higher in the corpus albicans than the CL of the ewe (Perks *et al.*, 1995) and to double following prostaglandin-induced luteal regression in the pregnant rat (Tamada *et al.*, 1995). The demonstration of a rapid decrease in IGF-I expression and content on days 18-21 of the cycle (Einspanier *et al.*, 1990) suggests that bovine luteolysis is associated with reduced IGF-I action. Since numerous stimulatory effects of IGF-I have been shown in ovarian cells *in vitro* (Giudice, 1992; Spicer and Echternkamp, 1995), this reduction of IGF-I expression *in vivo* might be expected to influence luteal function both directly, and indirectly by modulating the trophic support of LH, so contributing to the demise of the CL. In addition, decreased IGF-I and the subsequent abrogation of type 1 IGF receptor activation may also influence apoptotic cascades. However, studies to date have not addressed the potential of reduced IGF-I stimulation as a proximal cause of luteal regression and cell death.

In the present study we observed maximal IGF-I mRNA expression 48 hours after exogenous prostaglandin, at which time both functional and structural regressive changes will have occurred (Juengel *et al.*, 1993). As suggested in the ewe (Perks *et al.*, 1995) the high expression may be due to immune cell activity during the cellular destruction and phagocytocis associated with structural luteal regression (Pate and Townson, 1994; Penny *et al.*, 1998). IGF-I is produced by immune cells (Baxter *et al.*, 1991; Rappolee *et al.*, 1988), affects immunoregulation extensively (Renier *et al.*, 1996) and stimulates wound healing (Suh *et al.*, 1992). Identification of the

cellular source of IGF-I mRNA expression in the regressing CL will further our understanding of the role of IGF-I in tissue remodelling.

The demonstration of local ovarian IGF-I mRNA production in previous studies has favoured IGF-I rather than IGF-II as an autocrine/paracrine intra-luteal regulator. We report here that the bovine CL is a site of high IGF-II mRNA expression, in agreement with recent results from unstaged large bovine corpora lutea (Perks *et al.*, 1999). Although we were unable to detect any change in the level of IGF-II mRNA expression throughout the luteal phase, the results highlight the importance of locally produced IGF-II and suggest a major role in luteal function. It remains unclear whether the balance of IGF-I to IGF-II has an important influence on luteal function or if the growth factors serve different but crucial functions in different cell types.

The intense hybridisation for IGF-II mRNA in the luteal vasculature agrees spatially with immunohistochemical observations (Amselgruber *et al.*, 1994) which localised IGF-II protein to perivascular cells. The site of IGF-II mRNA expression was not fully determined in the present study. However, the hybridisation signal was not limited to endothelial cells lining the vessels, and pericytes, fibroblasts or smooth muscle cells may contribute to IGF-II mRNA expression. Whether the association of IGF-II with blood vessels reflects a role for IGF-II in controlling angiogenesis, luteal blood flow or interactions between vascular and steroidogenic cells requires further investigation. Prior to this study, the interest in the potential role of IGFs in the CL was based primarily on the ability of IGFs to stimulate luteal steroid biosynthesis. However, for IGF-II in particular, additional functions and targets should now be considered.

The IGFs exert their effects by interacting with cell surface receptors. The type 1 IGF receptor is activated by both IGF-I and IGF-II binding. The presence of the type 1 IGF receptor within the bovine CL has been indicated by competitive binding characteristics, as well as by the ability of IGF-I (and to a lesser degree IGF-II) to modulate luteal function. The expression of mRNA encoding the type 1 IGF receptor throughout the lifespan of the CL therefore confirms and extends previous reports. The spatial pattern of expression appears similar to that described in the ovine CL (Perks *et al.*, 1995). The lack of a temporal change in the pattern of expression is also in agreement with the absence of alterations in receptor number observed previously in the bovine CL (Sauerwein *et al.*, 1992).

In addition to regulation at the level of gene expression, the IGFs are subject to modulation by the specific binding of IGFBPs. IGFBPs control the bioactivity of IGFs in a complex and incompletely understood manner, and can regulate the bioavailability of IGFs and hence their interactions with IGF receptors. Future studies of the complex interactions of IGFBPs with the IGFs and IGF receptors will be necessary to further our understanding of the roles of IGF-I and IGF-II in regulating bovine CL function.

In conclusion, the present study (i) describes the temporal and spatial patterns of IGF-I, IGF-II and type 1 IGF receptor mRNA expression in the bovine CL throughout the luteal phase, (ii) demonstrates that the bovine CL is a site of IGF production and reception, and (iii) highlights the potential importance of IGF-II, in addition to IGF-I, in bovine luteal function.

Chapter 4. Expression of mRNA encoding insulin-like growth factor binding protein -2, -3 and -4 in the bovine corpus luteum at defined stages of the oestrous cycle

4.1 Summary

The insulin-like growth factors, IGF-I and -II, have important effects on luteal cells *in vitro*, and the demonstration of luteal expression of mRNA encoding IGF-I, IGF-II and the type 1 IGF receptor in Chapter 3 is suggestive of paracrine/autocrine roles for both IGF-I and IGF-II in the regulation of bovine luteal function. The actions of IGF-I and IGF-II are modulated by association with members of a family of high affinity IGFBPs, and to date the interaction of IGFs with IGFBPs has been shown to attenuate the actions of IGFs on ovarian cells *in vitro*.

We have investigated the localisation of mRNA encoding IGFBP-2, -3 and -4 using *in situ* hybridisation to further determine the regulation of the IGFs within the bovine CL at precise stages of the oestrous cycle. Luteal expression of mRNA encoding IGFBP-2, -3 and –4 was detected throughout the oestrous cycle, and showed limited temporal variation. Luteal expression of mRNA encoding IGFBP-2 and –4 was predominantly low. In addition, a number of large blood vessels showed moderate to intense hybridisation for IGFBP-2 mRNA. IGFBP-3 mRNA expression was also associated with the luteal vasculature, and hybridisation was localised to cells lining microvessels, suggestive of endothelial cell expression.

These data clearly demonstrate that the bovine CL is a site of IGFBP production and hence IGF regulation throughout the luteal phase. In addition, this study highlights the contribution of vascular expression of IGFBPs to bovine luteal function.

4.2 Introduction

The actions of IGFs are modulated by binding to up to six high affinity IGFBPs (Rechler and Clemmons, 1998). IGFBPs bind IGF-I and IGF-II with high affinity and so regulate their access to IGF receptors. IGFBPs have been shown to consistently block IGF-stimulation of ovarian cells. IGFBPs inhibit steroid production (Mason *et al.*, 1998; Spicer *et al.*, 1997; Ui *et al.*, 1989), and cell proliferation (Bicsak *et al.*, 1990), reduce ovulation rate and oocyte maturation (Yoshimura *et al.*, 1996), and induce apoptotic cell death (Chun *et al.*, 1994). However, in certain cases IGFBPs can potentiate IGF action (Conover *et al.,* 1996).

IGFBP-2, -3 ,–4 and –5 have been detected in the follicular fluid of cattle, and IGFBP concentrations vary with the stage of follicular development (Echternkamp *et al.*, 1994; Stewart *et al.*, 1996). These changes are thought to regulate follicle selection and dominance by altering IGF availability and follicular sensitivity to gonadotrophins. Follicular fluid IGFBP concentrations are thought to be modulated via changes in local production (Armstrong *et al.*, 1998; Perks and Wathes, 1996), changes in uptake from the plasma (Armstrong *et al.*, 1996) or variable proteolysis (Besnard *et al.*, 1996, 1997).

IGFBPs have also been detected in both the CL and luteinised granulosa cells of human and non-human primates (Fraser *et al.*, 1998; Giudice *et al*, 1991), pig (Gadsby *et al.*, 1996; Samaras *et al.*, 1992; Zhou *et al.*, 1996,), rat (Erickson *et al.*, 1993, 1994; Nakatani *et al.*, 1991) and sheep (Armstrong *et al.*, 1996; Perks and Wathes, 1996). In the cow, granulosa and theca cells in culture secrete IGFBPs following luteinising stimuli (Sakal *et al.*, 1992) and mRNA encoding IGFBP-2 and -3 has been detected in luteal tissue (Kirby *et al.*, 1996). Whilst marked species

variations have been shown to exist with regard to the expression of components of the IGF system, evidence suggests that the IGFBPs could play important regulatory roles in luteal function.

The work in this chapter aims to describe the temporal and spatial changes in expression of mRNA encoding IGFBP-2, -3 and -4 within the bovine CL throughout the oestrous cycle.

4.3 Materials and Methods

4.3.1 Animals

The expression of mRNA encoding IGFBP-2, -3 and –4 was examined in the same CL as described in section 3.3.1. In brief, CL were collected on day 5, 10 or 15 following oestrus synchronisation (n=3 per group), and 48 hours following $PGF_{2\alpha}$ administration (n=3).

4.3.2 RNA probes

A plasmid containing the bovine IGFBP-2 probe was a gift from Dr. M Lucy (University of Missouri), and corresponds to positions 1084-1192 of a bovine IGFBP-2 cDNA (Bourner *et al.*, 1992). Homologous bovine IGFBP-3 and -4 probes were prepared after reverse transcriptase PCR from total theca cell RNA (gifted by Dr. DG Armstrong).

IGFBP-3 mRNA transcripts were amplified using a 21-mer upstream primer (5'-ATTCCACCCCATCCACACCAA-3') and a 21-mer downstream primer (5'-CACGTCCCCTTTCCCCTTCAC-3'). The amplified product (358 bp) corresponds to position 611 to 968 of a bovine IGFBP-3 cDNA (Spratt *et al.*, 1991). IGFBP-4 mRNA transcripts were amplified using a 21-mer upstream primer (5'-

ATCGAGGCCATCCAGGAAAGC-3') and a 21-mer downstream primer (5'-CGCCCGGTGCAGCTCACTCTG-3'). The amplified product (228 bp) corresponds to position 523 to 750 of a bovine IGFBP-4 cDNA (Moser *et al.*, 1992).

Riboprobe specificity was tested by RNase protection assay (see section 2.12). The protection of [^{32}P]-labelled (Amersham Pharmacia Biotech) bovine IGFBP-3 and IGFBP-4 riboprobes with total luteal and liver RNA (25μg) is shown in the appendix. A very weak protection of bovine IGFBP-2 was also detected in bovine luteal RNA extracts (results not shown).

Isotopic antisense and sense RNA probes for *in situ* hybridisation were transcribed from linearised cDNA templates using [^{35}S]-UTP according to the protocol detailed in section 2.10.

4.3.3 *In situ* hybridisation

Frozen sections (14 μm) of ovarian tissue were subjected to *in situ* hybridisation for IGFBP-3 and IGFBP-4 as detailed in section 2.11. Additional paraffin sections were utilised for IGFBP-2 (see section 2.11.1.2). Antisense RNA probes for IGFBP-2, -3 and -4 were each hybridised to two serial sections per corpus luteum or ovary. The sense probe for each mRNA species was applied to a further serial section.

The intensity of the *in situ* hybridisation signal was quantified (see section 3.3.3), and antisense signals were then compared to their respective sense (background) signals.

4.3.4 Statistical Analysis

The effect of stage of the oestrous cycle on mRNA concentration was determined by a split-plot ANOVA (Genstat 5; Payne *et al.*, 1993) using the hierarchical structure previously described (see section 3.3.4).

4.4 Results

4.4.1 Expression of IGFBP-2 mRNA

Messenger RNA encoding IGFBP-2 was expressed in CL at all timepoints studied. Luteal expression of IGFBP-2 was predominantly low. In several animals a more intense punctate expression pattern was observed for IGFBP-2 mRNA (Figure 4.1a-b). In addition, moderate to intense expression of mRNA encoding IGFBP-2 was detected in large blood vessels within both the luteal and stromal tissue, and may be localised to smooth muscle cells (Figure 4.1d-f). Vascular expression was observed to be heterogeneous, with some vessels appearing negative for IGFBP-2 adjacent to those expressing IGFBP-2 mRNA. IGFBP-2 mRNA concentrations were not significantly affected by day of the oestrous cycle ($P > 0.05$).

4.4.2 Expression of IGFBP-3 mRNA

Messenger RNA encoding IGFBP-3 (Figure 4.2) was present in the CL at all timepoints studied. Expression was predominantly localised to the vascular system, and the strong expression by cells lining microvessels suggests that endothelial cells are a probable site of expression. Quantitative analysis showed no significant effect ($P > 0.05$) of day of the oestrous cycle on IGFBP-3 mRNA concentrations (Figure 4.4).

4.4.3 Expression of IGFBP-4 mRNA

At all timepoints studied, CL expressed mRNA encoding IGFBP-4. The hybridisation signal was diffuse and detected at moderate level. The pattern of hybridisation is suggestive of steroidogenic cell expression (Figure 4.3). IGFBP-4 mRNA concentrations were similar throughout the oestrous cycle ($P > 0.05$).

4.5 Discussion

The present study describes the expression of mRNA encoding IGFBP-2, -3 and -4 during growth and regression of the bovine CL. Previous reports have demonstrated that bovine theca and granulosa cells stimulated in culture to differentiate into luteal-like cells secrete IGFBPs (Sakal *et al.*, 1992) and bovine corpora lutea of the late luteal phase (day 17) express mRNA encoding IGFBP-2 and -3 (Kirby *et al.*, 1996). The results presented here confirm that the CL of the cow is a local site of expression of mRNA encoding IGFBP-2, -3 and -4.

Messenger RNA encoding IGFBP-2 was detected at low levels within the CL throughout the oestrous cycle. During the development of follicular dominance IGFBP-2 concentrations in follicular fluid (Stewart *et al.*, 1996) and IGFBP-2 mRNA expression were decreased (Armstrong *et al.*, 1998). The low level of expression demonstrated during the final stages of bovine follicular development was maintained in the CL. IGFBP-2 mRNA expression has also been shown to persist at low levels in the CL of the sheep (Perks and Wathes, 1996), pig (Gadsby *et al.*, 1996; Zhou *et al.*, 1996) and rat (Erickson *et al.*, 1994). In addition, the synthesis and release of IGFBP-2 by human luteinising granulosa cells is inhibited by hCG

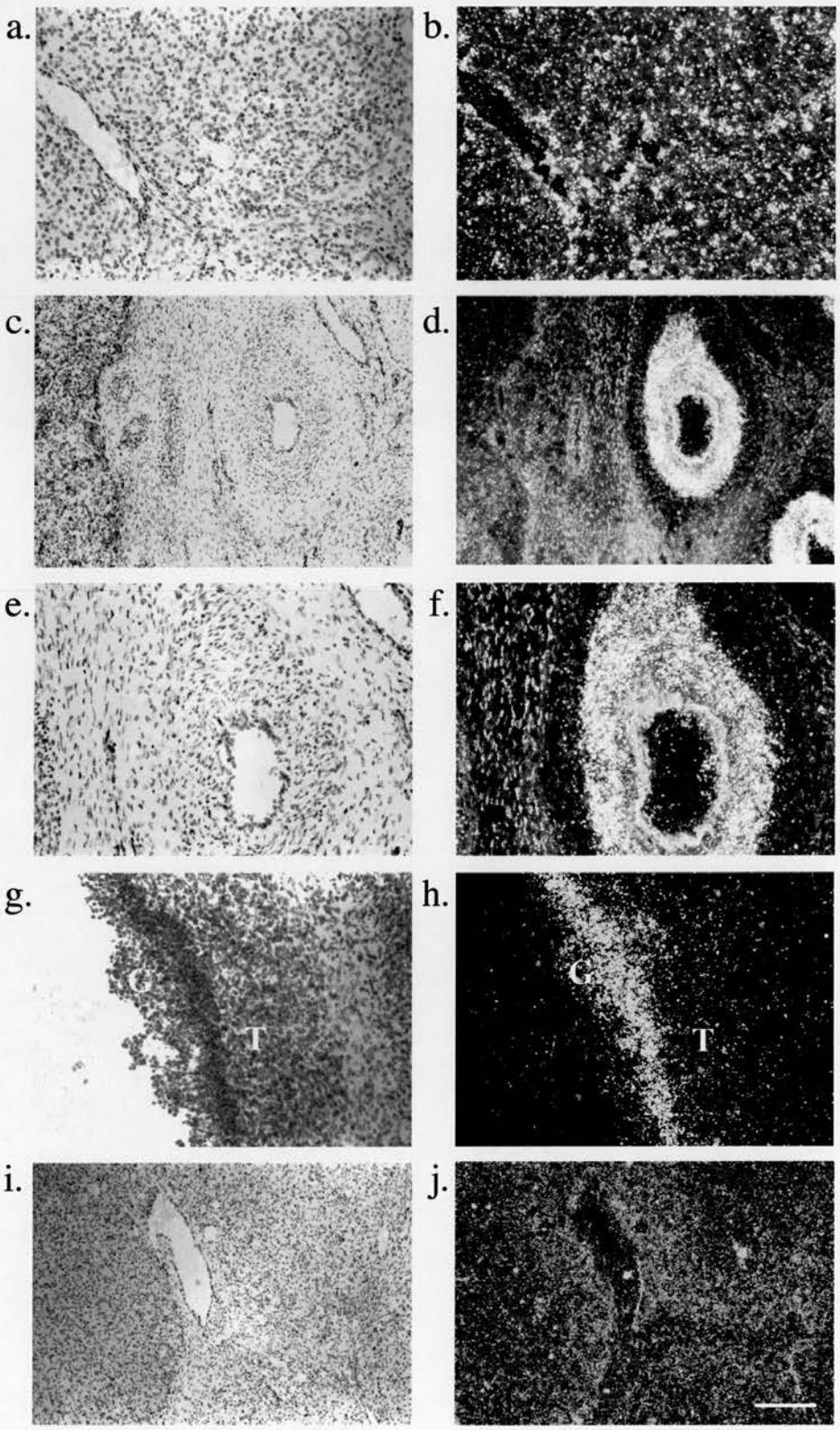


Figure 4.1. Bovine ovarian sections (14 μm) were probed with an IGFBP-2 antisense (a-h) or sense (i and j) riboprobe, and the same fields of view are shown under lightfield (a, c, e, g and i) and darkfield (b, d, f, h and j) illumination. Localisation of mRNA encoding IGFBP-2 is shown in luteal tissue at low magnification (a and b), and in a further corpus luteum at two different magnifications (c-f). Follicular expression is shown in the granulosa cell layer of a small follicle (g and h). The scale bar represents 100 μm (a, b, e, f, g, and h), and 200 μm (c, d, i and j).G and T represent granulosa cells and theca cells respectively.

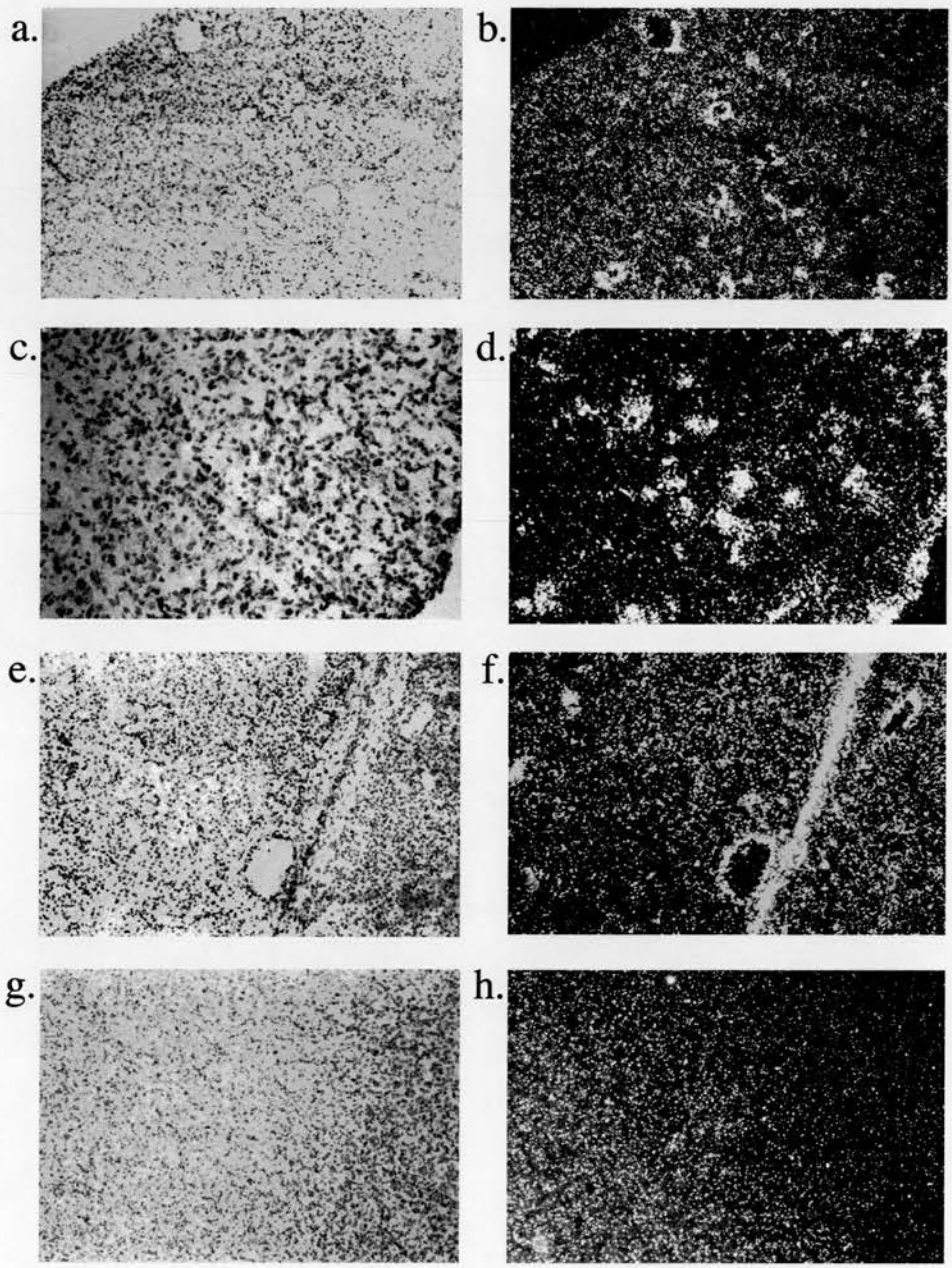


Figure 4.2. Bovine ovarian sections (14 μm) were probed with an IGFBP-3 antisense (a-f) or sense (g and h) riboprobe, and the same fields of view are shown under lightfield (a, c, e, and g) and darkfield (b, d, f, and h) illumination. Localisation of mRNA encoding IGFBP-3 is shown in typical corpora lutea at three different magnifications (a-f). The scale bar represents 100 μm (c and d) and 200 μm (a, b, e, f, g, and h).

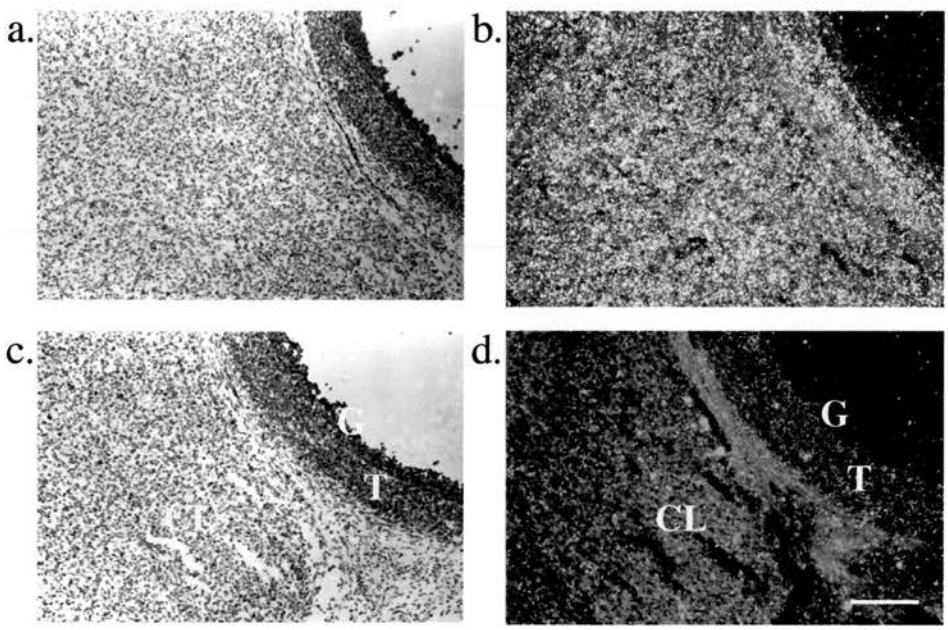


Figure 4.3. Bovine ovarian sections (14 μm) were probed with an IGFBP-4 antisense (a and b) or sense (c and d) riboprobe, and the same fields of view are shown under lightfield (a and c) and darkfield (b and d) illumination. Localisation of mRNA encoding the IGFBP-4 is shown in luteal tissue adjacent to a small follicle (a and b). The scale bar represents 200 μm (a - d). G, T, and CL represent granulosa cells, theca cells, and corpus luteum respectively.

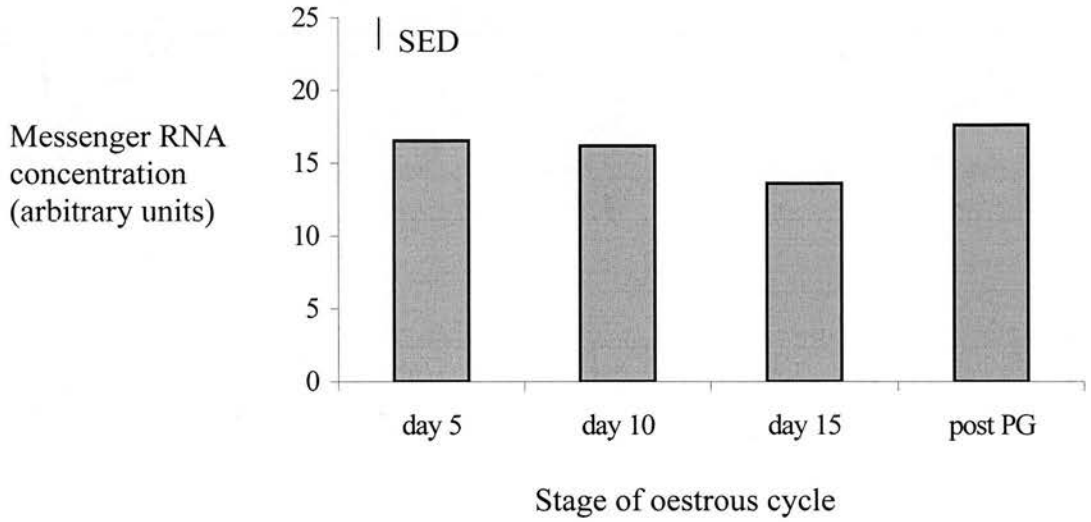


Figure 4.4

Quantitative analysis of IGFBP-3 mRNA concentrations in bovine luteal tissue on days 5, 10 and 15 of the oestrous cycle, and 48 hours after $PGF_{2\alpha}$ treatment (post PG). There was no effect of stage of cycle on the concentration of mRNA encoding IGFBP-3 ($P > 0.05$). Bar represents pooled standard error of the difference (SED). Messenger RNA concentration is expressed as the percentage of the total number of pixels occupied by silver grains within a defined area. Hybridisation with an IGFBP-3 sense probe gave an average background value of 4 units.

(Cataldo *et al.*, 1993). This low expression of IGFBP-2 mRNA is suggestive of a limited role for this binding protein in luteal function.

IGFBP-2 expression was predominantly low throughout the whole CL, although a more punctate pattern was observed in several animals. However, additional intense hybridisation was observed associated with a small number of large blood vessels, probably arterioles. Since this high concentration of IGFBP-2 mRNA was heterogeneous, and not observed in all sections, it remains unclear whether such expression is restricted to a certain size or type of vessel, or alternatively whether it reflects a difference in expression between animals, or between stages of luteal development. The association of IGFBP-2 with blood vessels has been shown previously in other tissues. IGFBP-2 mRNA has been detected in bovine aortic vascular smooth muscle cells, although at comparatively low levels (Boes *et al.*, 1996). Since IGF-I has been implicated in the regulation of vascular tone (Walsh *et al.*, 1996) the intense hybridisation suggests a role for IGFBP-2 in modulating regional blood flow.

Bovine granulosa and theca cells in culture have previously been shown to secrete IGFBPs, and luteinising stimuli caused a shift in this secretion to high molecular weight binding proteins, putatively identified as IGFBP-3 (Sakal *et al.*, 1992). The present data confirm the local expression of IGFBP-3 mRNA within the bovine CL, and demonstrate that IGFBP-3 mRNA is readily detectable within the corpus luteum throughout the oestrous cycle.

IGFBP-3 mRNA was also detected in the ovine CL throughout the cycle. However, in contrast to the present study, luteal IGFBP-3 mRNA concentrations were very low

(Perks and Wathes, 1996). Studies in the pig have shown that IGFBP-3 mRNA was abundantly expressed by the CL and was maintained at levels that display no stage-dependant changes (Gadsby *et al.*, 1996) until the onset of luteolysis when expression was significantly decreased (Samaras *et al.*, 1992). Similarly high concentrations of IGFBP-3 mRNA were observed in the marmoset CL until the late follicular phase when levels showed a marked decline (Fraser *et al.*, 1998). Additionally, mRNA encoding IGFBP-3 was detected at high levels during the early luteal phase in the human CL, and was significantly reduced during the mid- and late-luteal phase (Fraser *et al.*, 2000). The rat exhibited a different temporal pattern of luteal IGFBP-3 expression. *In situ* hybridisation has shown that IGFBP-3 mRNA was absent from newly formed CL, became detectable with continuing luteal development and was most abundant during luteolysis (Erickson *et al.*, 1993). Whilst luteolysis was associated with an upregulation of IGFBP-3 mRNA concentrations in the rat, the absence of any increase of IGFBP-3 mRNA following prostaglandin-induced luteolysis suggests this is not the case in the cow CL. Indeed the presence of IGFBP-3 mRNA from early in the luteal phase is more suggestive of a role in CL formation and function rather than regression.

IGFBP-3 mRNA was distributed throughout the entire structure of the bovine CL. The hybridisation signal was particularly intense in cells associated with blood vessels, and based on the intensity of hybridisation signal lining microvessels, endothelial cells are a probable site of IGFBP-3 mRNA expression. Where cellular identification has been possible in other studies this spatial pattern of expression seems well conserved between species, despite marked temporal differences in the expression of IGFBP-3 mRNA. In the rat IGFBP-3 mRNA was detected throughout

the CL (Nakatani *et al.*, 1991) predominantly localised to endothelial cells, some perivascular cells and for a short period during luteolysis granulosa and theca lutein cells (Erickson *et al.*, 1993). In the porcine CL following dissociation and elutriation, IGFBP-3 mRNA was enriched in the small-cell fraction which contains endothelial cells (Gadsby *et al.*, 1996). Vascular cells of the human and non-human primate CL also showed the most intense localisation of IGFBP-3 mRNA (Fraser *et al.*, 1998, 2000). Whilst the temporal pattern of expression of IGFBP-3 in the rat suggests a role for vascular IGFBP-3 in luteal regression, the presence of IGFBP-3 mRNA from early in luteinization through the functional lifespan of the CL in the cow, pig, human and monkey is more suggestive of actions in angiogenesis. An important role for vascular IGFBP-3 in other reproductive tissues has also been suggested. In the bovine uterus during pregnancy IGFBP-3 mRNA was detected in vascular endothelial cells of the myometrium (Keller *et al.*, 1998).

Whilst IGFBP-3 is the most abundant IGFBP in serum (Clemmons, 1997) and the predominant IGFBP in the follicular fluid of pre-ovulatory follicles (Funston *et al.*, 1996), mRNA encoding IGFBP-3 has only been detected at low levels and does not increase with follicular development (Yuan *et al.*, 1998). This lack of follicular IGFBP-3 expression is in sharp contrast to the detection of luteal IGFBP-3 expression. Since IGFBP-3 mRNA was detected in the bovine CL early in the luteal phase we hypothesise that it plays an important role in luteinisation. The earliest timepoint in the present study was day 5 of the luteal phase. It remains to be established how early in luteal development IGFBP-3 mRNA expression was upregulated.

Messenger RNA encoding IGFBP-4 was detected within the bovine CL throughout the oestrous cycle. This is the first report of IGFBP-4 expression within the luteal tissue of the cow to our knowledge. IGFBP-4 mRNA has been detected in the CL of the pig (Zhou *et al.*, 1996), where it was determined to be the most abundant IGFBP (Gadsby *et al.*, 1996) and the sheep (Perks and Wathes, 1996), where expression levels were low by contrast. An IGFBP putatively identified as IGFBP-4 was also present in culture medium conditioned by human luteinising granulosa cells (Cataldo *et al.*, 1993). An association of IGFBP-4 with luteal formation and function was also suggested by increased IGFBP-4 production and mRNA expression following the addition of a luteinizing dose of LH to ovine and bovine theca cell cultures (Armstrong *et al.*, 1996, 1998).

IGFBP-4 mRNA was detected throughout the bovine CL, and did not appear to be associated with major structural components such as blood vessels. Whilst the identification of the cellular site of expression was limited by low resolution, the diffuse pattern is suggestive of steroidogenic cell expression of IGFBP-4 mRNA. In the bovine follicle mRNA encoding IGFBP-4 was localised in the theca cell layer (Armstrong *et al.*, 1998). However, it remains to be established whether luteal IGFBP-4 expression is predominantly localised to large or small luteal cells.

Whilst IGFBPs have been shown to potentiate IGF-stimulation in certain cases (Conover *et al.,* 1996), IGFBPs inhibit the actions of IGFs on ovarian cells in culture. Therefore it is unclear why mRNA encoding any IGFBP should be synthesised throughout the functional lifespan of the corpus luteum. However, the present study details changes in local IGFBP mRNA concentration and additional

post-transcriptional events may serve to vary the final IGFBP protein concentration and hence the regulation of IGFs during the luteal phase.

The actions of IGFs are regulated in part by the relative abundance of IGFBPs, since the high affinity binding of IGFBPs for IGF-I and IGF-II prevents ligand-receptor interactions. In addition to regulation of the IGFs:IGFBPs ratio, IGFBPs can undergo post-synthetic modification. The affinities of IGFBPs for IGFs can be significantly lowered by variable proteolytic cleavage, phosphorylation and binding to cell surfaces or extracellular matrix in ways that may lead to the potentiation of IGF actions (Clemmons, 1997). It is unknown whether such changes modulate luteal IGFBP function.

IGFBP proteases have been identified in a variety of physiological and pathological conditions (Fowlkes, 1997; Rajaram *et al.*, 1997). IGFBP-degrading activity has been identified in human (Chandrasekhar *et al.*, 1995), porcine (Besnard *et al.*, 1997), ovine (Besnard *et al.*, 1996) and bovine (Chandrasekhar *et al.*, 1996) follicular fluid. IGFBP-specific proteolytic activity has also been detected in human and rat granulosa cell conditioned media (Iwashita *et al.*, 1998; Liu *et al*, 1993), and bovine theca cell conditioned media (Nicholas *et al.,* 2000). Whilst protease activity has been correlated with follicular growth and development, the possible role of IGFBP proteases in regulating luteal function has not been investigated. Our understanding of the roles of IGFBPs in luteal function is further complicated by the possibility that IGFBPs can exert IGF-independent inhibitory effects on target cell growth and DNA synthesis. However, it remains to be determined how widespread these effects may be (Rechler, 1997).

In conclusion, the present study (i) describes the temporal and spatial patterns of IGFBP-2, -3 and -4 mRNA expression in the bovine corpus luteum throughout the luteal phase, (ii) demonstrates that the bovine CL is a site of IGF regulation and (iii) highlights the potential importance of vascular expression of IGFBPs in bovine luteal function.

Chapter 5. Follicular response to an ovulatory challenge of GnRH in the early luteal phase.

5.1 Summary

The CL is essential for the establishment and maintenance of pregnancy, and luteal insufficiency is therefore a major contributor to infertility. Measures to reduce reproductive wastage have included supplemental progesterone regimes to augment inadequate luteal function. One strategy to improve endogenous progesterone concentrations is via the induction of accessory corpora lutea.

The oestrous cycles of mature dairy cows were synchronised and follicular dynamics observed by ultrasonography. Administration of GnRH on day 6 caused ovulation of the dominant follicle of the first wave and formation of a secondary luteinized structure. There was a trend for plasma progesterone concentrations to be increased in animals treated with GnRH when compared to control animals, although no significant effect was detected on day 14. The GnRH-induced CL has been shown previously to have limited progesterone secreting capacity. The luteal insufficiency observed following GnRH administration may be the result of sub-optimal follicular development, inadequate luteotrophic support or premature luteolytic stimuli.

5.2 Introduction

The CL is essential for the establishment and maintenance of pregnancy. Inadequate or abnormal luteal function therefore has significant implications for fertility. Indeed, insufficient progesterone levels have been associated with early embryo loss (Mann *et al.*, 1999), which accounts for the majority of reproductive wastage, with the greatest losses occurring within 20 days of fertilisation (Sreenan and Diskin, 1983). Cows in which pregnancy failed had significantly lower milk progesterone concentrations on day 6 and between days 12 and 15 after mating, when compared with pregnant cows (Mann *et al.*, 1999). In addition, pregnancy failure has been associated with delayed increases in post-ovulatory concentrations of progesterone (Lamming and Darwash, 1995). These results suggest that progesterone concentrations critically regulate early embryo growth and survival. In contrast, others have failed to demonstrate a positive relationship between pregnancy outcome and peripheral progesterone concentrations (Hasler *et al.*, 1980).

Measures to improve reproductive efficiency have included the use of exogenous progesterone supplementation (Robinson *et al.*, 1989), and the administration of human chorionic gonadotropin (hCG) (Breuel *et al.*, 1990) or GnRH (Lewis *et al.*, 1990), the rationale being that increased peripheral progesterone would support embryo survival, until such time as the corpus luteum could be maintained through the embryo's own luteotrophic and/ or anti-luteolytic effects (Sreenan and Diskin, 1983). This assumes that supplemental therapy is additive to endogenous progesterone, although there is evidence to suggest that whilst supplemental progesterone increases plasma progesterone concentrations it also decreases endogenous luteal progesterone production (Robinson *et al.*, 1989). However, these

methods have had some success, although they have not consistently improved pregnancy rates (Robinson *et al.*, 1989; Sreenan and Diskin, 1983).

It has been postulated that the most efficient means to augment endogenous progesterone production is via the induction of accessory corpora lutea (Rajamahendran and Siangama, 1992). The induction of a second CL is expected to increase plasma progesterone levels, despite the lack of significant difference in progesterone between animals displaying single or twin ovulations (Morris *et al.*, 1987). The induction of accessory CL formation should coincide ideally with the presence of large follicles on the ovary. The bovine oestrous cycle is characterised by waves of follicular growth and development, as demonstrated by peaks of oestradiol secretion and daily ultrasound observations. Large luteal-phase follicles, whilst destined not to spontaneously ovulate are responsive to gonadotrophin stimulation and oestrogen-active (Ireland and Roche, 1983a). Large follicles respond to hCG and GnRH treatment with ovulation, and produce a secondary luteinized structure (Fricke *et al.*, 1993; Price and Webb, 1989; Rajamahenran and Sianangama, 1992; Schmitt *et al.*, 1996a, 1996b). Large follicles were most responsive to exogenous hCG during the early luteal phase (days 4-7) (Price and Webb, 1989), at which time the large follicle of the first wave displays increased LH-binding (Ireland and Roche, 1983b).

The luteinized structure formed in response to a second gonadotrophin surge has been characterised and treatment with either hCG or GnRH induces a functional CL. However, peripheral progesterone concentrations were markedly higher in hCG treated animals, when compared to those induced to ovulate with GnRH-agonist (Schmitt *et al.*, 1996a, 1996b). In addition, GnRH agonist-induced CL displayed

reduced LH-stimulated progesterone secretion *in vitro* (Schmitt *et al.*, 1996a). The formation of a CL with reduced steroidogenic capacity following the administration of GnRH was demonstrated both *in vivo* and *in vitro* (Rusbridge, 1993). Serum progesterone levels produced by the GnRH-induced CL following removal of the original CL failed to reach those following spontaneous ovulation, whilst in culture, cells of the induced CL displayed reduced cAMP-stimulated progesterone secretion (Rusbridge, 1993).

IGF-I and -II have been shown to have numerous stimulatory effects on ovarian steroidogenesis. We hypothesised, therefore, that the reduction in steroidogenic capacity shown by the GnRH-induced CL could be due to a disruption of the expression of the local IGF system in the CL. This chapter describes the production of accessory CL in response to GnRH treatment in the early luteal phase (day six), and the following chapter describes the effect of GnRH-administration on the luteal IGF system.

5.3 Materials and Methods

5.3.1 Animals

All animals were Friesian/Holstein crossbred cows, aged between 3 and 8 years, housed at the Roslin Institute Farm at Blythbank. Cows were housed in covered courts with straw bedding. Water was available ad libitum and cows were fed a maintenance diet. All experiments were carried out in accordance with the 1986 UK Animals (Scientific Procedures) Act.

5.3.2 Procedures

The experimental protocol is illustrated in Figure 5.1. The oestrous cycles of fourteen mature dairy cows were synchronised by intravaginal progesterone release (PRID; 1.55g P4, 10mg oestradiol benzoate; Sanofi Animal Health Ltd) over 9 days. Luteolysis was induced by $PGF_{2\alpha}$ analogue (Estrumate; 500 μg; Coopers Animal Health Ltd) administered i.m on the day of PRID removal. Oestrous behaviour was monitored by daily observation, together with the use of heat detection devices (Heatmount Detectors, Kamar Inc). Oestrus was expected to have occurred within 48 hours of progesterone withdrawal, and ovulation was confirmed by ultrasound scanning. Ovarian dynamics were monitored by daily rectal ultrasound scanning using a real-time B-mode ultrasound scanner (Echo Camera SSD-220 DX II, Aloka Company Ltd) equipped with a 7.5 MHz intrarectal probe. Scans were recorded on videotape, and ovarian structures mapped. Six days after the synchronised oestrus (oestrus = Day 0) cows received 0.5 mg synthetic GnRH (Fertagyl, Intervet Laboratories Ltd) by i.m injection, and daily ultrasonography was continued until slaughter 5 or 10 days later. Blood samples (10ml) were taken by jugular or coccygeal venepuncture into tubes containing 100μl of sodium citrate (0.35 g/ml), from haltered and restrained animals at the time of scanning. All blood samples were centrifuged (1000g), and plasma separated and frozen at -20°C until assayed for progesterone (see section 2.13).

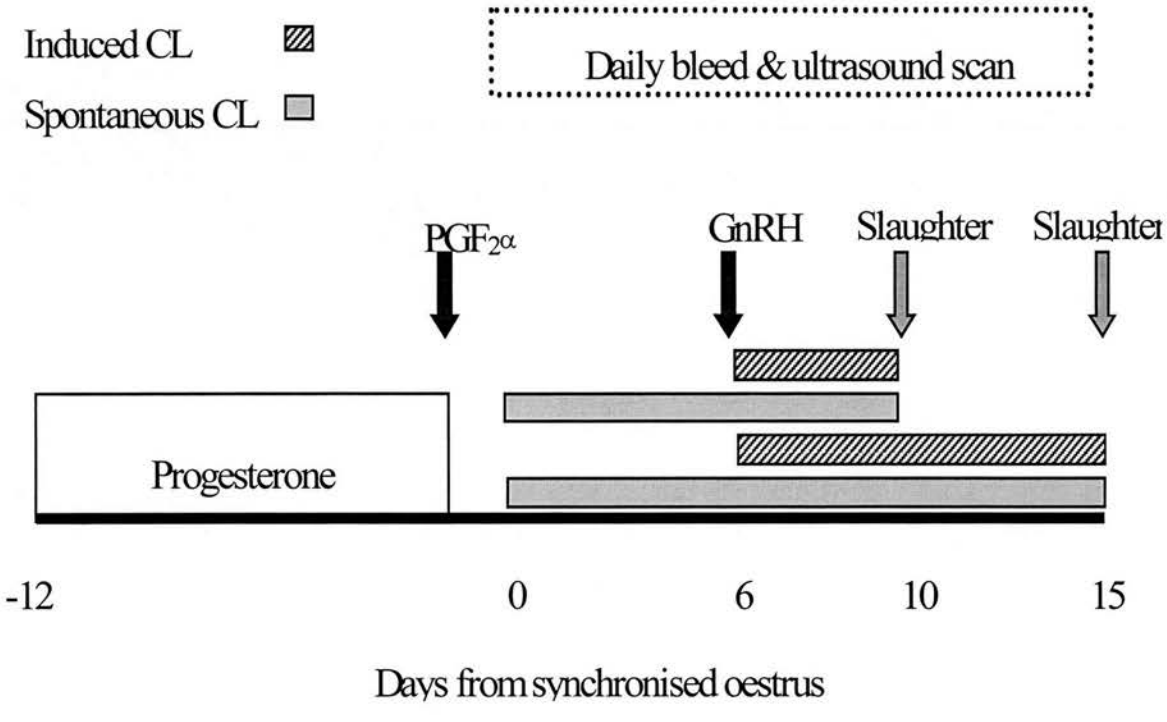


Figure 5.1

Oestrous cycles were synchronised by intravaginal progesterone release and i.m $PGF_{2\alpha}$. Ultrasonography and blood sampling were performed daily from the expected day of oestrus (Day 0). GnRH analogue was administered on day 6 to cause ovulation of the first wave dominant follicle and the formation of a GnRH-induced CL. Cows were slaughtered on day 10 or day 15 and the spontaneous CL and GnRH-induced CL from each pair of ovaries collected.

Peripheral progesterone concentrations were compared between control animals following oestrous synchronisation (see section 3.3.1) and animals induced to ovulate in response to GnRH administration and form a second CL. Samples were assayed in two assays. The mean sensitivity was 0.26 ng/ml. The intra- and inter-assay coefficients of variation were 8.1% and 9.1% respectively. The effect of GnRH treatment on progesterone concentrations on day 14 of the control and GnRH-treated cycles (9 days post-GnRH) was analysed using an unpaired Student's t-test.

5.3.3 Tissue collection

Ovaries were collected at slaughter, and photographed in pairs from each cow (Figure 5.2). Corpora lutea were excised, divided and frozen in liquid nitrogen within 20mins of slaughter, and stored at -80°C prior to cryostat sectioning or RNA extraction.

5.3.4 *In situ* hybridisation

In situ hybridisation was performed to detect the expression of mRNA encoding the LH receptor and cytochrome P450arom in CL from GnRH-treated animals, and similar-aged control CL from the synchronised cycle (see section 3.3.1). Ovarian tissue collected for follicular expression studies (Armstrong *et al.*, 1998) was also subjected to *in situ* hybridisation.

[^{35}S]-UTP-labelled riboprobes were generated from templates kindly gifted by Professor A. Garverick (University of Missouri) (Xu *et al.*, 1995b, 1995c) using the transcription protocol described previously (see section 2.10). The 730-bp LH receptor riboprobe encodes a portion of the extracellular domain of the ovine LH receptor, and corresponds to positions 193-922 of porcine (p) and human (h) LH

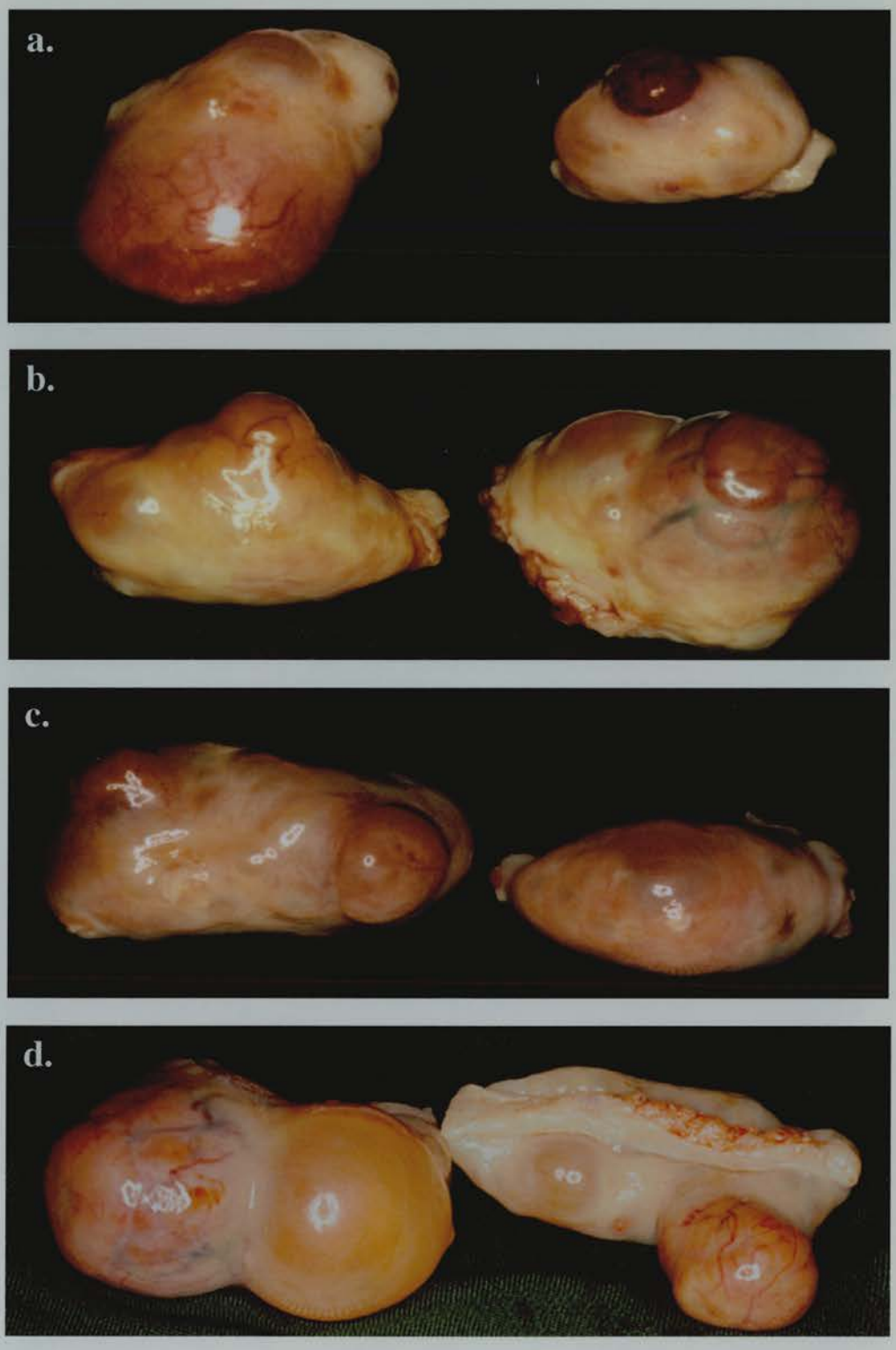


Figure 5.2 Pairs of ovaries were collected 4 days (a) and 9 days (b-d) after GnRH administration. Ovulation was induced both on the ovary ipsilateral (c) and contralateral (a, b, d) to the ovary bearing the spontaneous CL. In one animal (041) ovaries were found to bear a spontaneous CL, a GnRH-induced CL and a large follicular cyst (d).

receptor (LHr) cds (EMBL id: pLHr - SSLHHCGA; hLHr – HSLHHCGR) (Loosfelt *et al.*, 1989; Minegish *et al*, 1990). The 579-bp aromatase riboprobe corresponds to positions 139-718 of human P450arom cDNA (EMBL id: HSARMA) (Harada, 1988).

Antisense RNA probes for LH receptor and cytochrome P450arom were each hybridised to two serial sections per corpus luteum or ovary. The sense probe for each mRNA species was applied to a further serial section. The intensity of the *in situ* hybridisation signal was quantified (see section 3.3.3), and antisense signals were then compared to their respective sense (background) signals.

The effect of GnRH administration on LH receptor mRNA concentration was determined by a split-plot ANOVA (Genstat 5; Payne *et al.*, 1993), using the blocking factor cow/ slide/ replicate as previously described (see section 3.3.4).

5.4 Results

5.4.1 Ovarian observations

One animal had an ossification of the left ovary following oestrous synchronisation and was removed from the experiment. The remaining 13 animals used for this study all displayed three waves of follicular growth and development. Ultrasound observations confirmed the presence of a large preovulatory follicle following PRID withdrawal, which was subsequently replaced by a "spontaneous" CL. The emergence of a large follicle from the first wave of follicle growth was observed following synchronised oestrus. Ultrasonography confirmed ovulation and the presence of an "induced" accessory CL in the GnRH responders following GnRH treatment (Figure 5.3a). All 13 animals formed an additional CL. However, in two

animals the second ovulation was not due to ovulation of the largest follicle present at GnRH injection. The time from GnRH injection to the appearance of a newly formed CL was markedly extended (5 days and 4 days) in these animals, and there was no clear ovulation of a dominant follicle. In one of these late-responding animals, the large follicle present at GnRH administration failed to ovulate, and became cystic reaching a maximum diameter of 25mm: this animal was not included in further experimental analysis (Figure 5.4). Ovulation occurred in the remaining 11 animals within 48 hours. It is of note that the largest follicles present in the ovaries of both late responding animals were under 9mm in diameter at the time of GnRH administration. Among all 13 cows, the large follicle at the time of GnRH administration ranged from 8-19mm, with a mean diameter of 13.7mm. A bolus of GnRH induced single ovulations in all animals, and led to the formation of accessory CL contralateral (n=6) or ipsilateral (n=6) to the spontaneous CL. The dynamics of medium-sized follicle development was also recorded by ultrasonography, and a reduction in medium follicle number was observed which was associated with the acquisition of dominance of the large follicle in each wave (Figure 5.3b).

5.4.2 Plasma progesterone concentrations

No difference in plasma progesterone concentration was observed between control animals and animals treated with GnRH in the period prior to GnRH administration. Profiles of progesterone concentrations were unaffected by GnRH treatment until day 14, when there was a tendency for progesterone concentrations in the GnRH treated animals to be higher than control levels (Figure 5.5). However, no significant effect of treatment on day 14 was detected by the Student's t-test ($P = 0.12$).

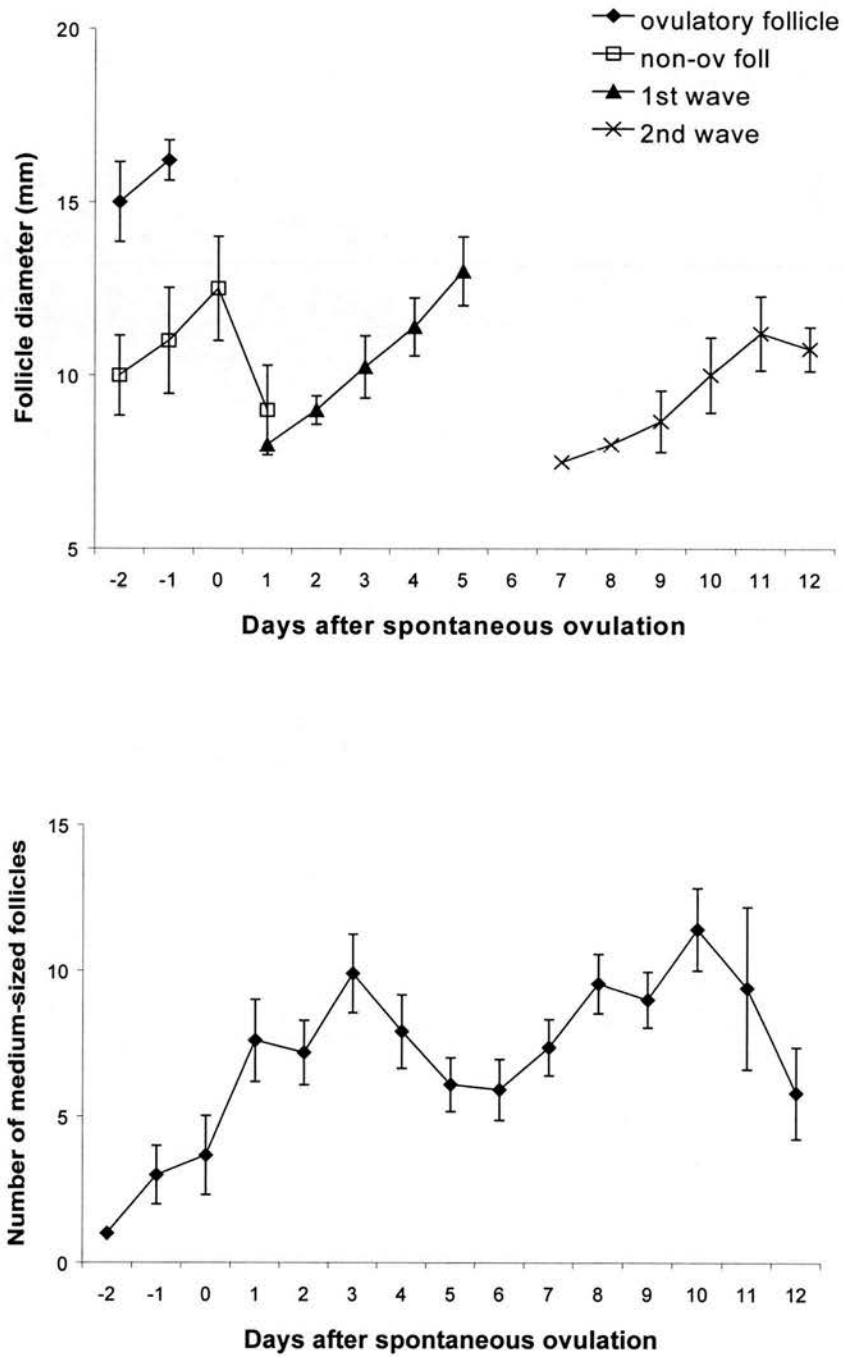


Figure 5.3

Follicle dynamics of GnRH responders as assessed by ultrasonography. Growth profile of the largest follicle (a) and changes in the number of medium-sized follicles (b). Mean values ± SEM.

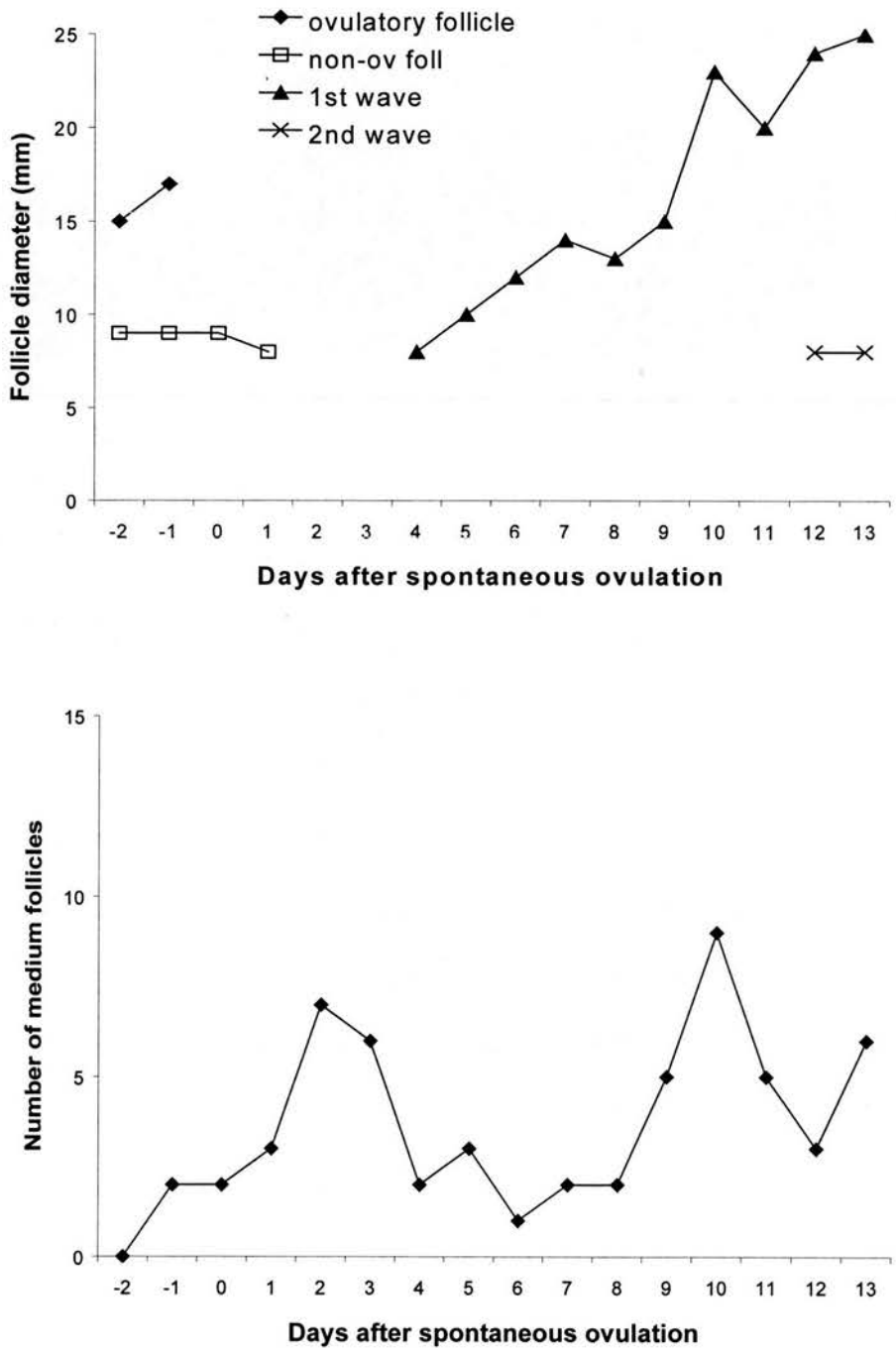


Figure 5.4

Ultrasound assessment of follicle dynamics in animal 041, which developed a follicular cyst. Growth profile of largest follicle (a) and changes in the number of medium-sized follicles (b).

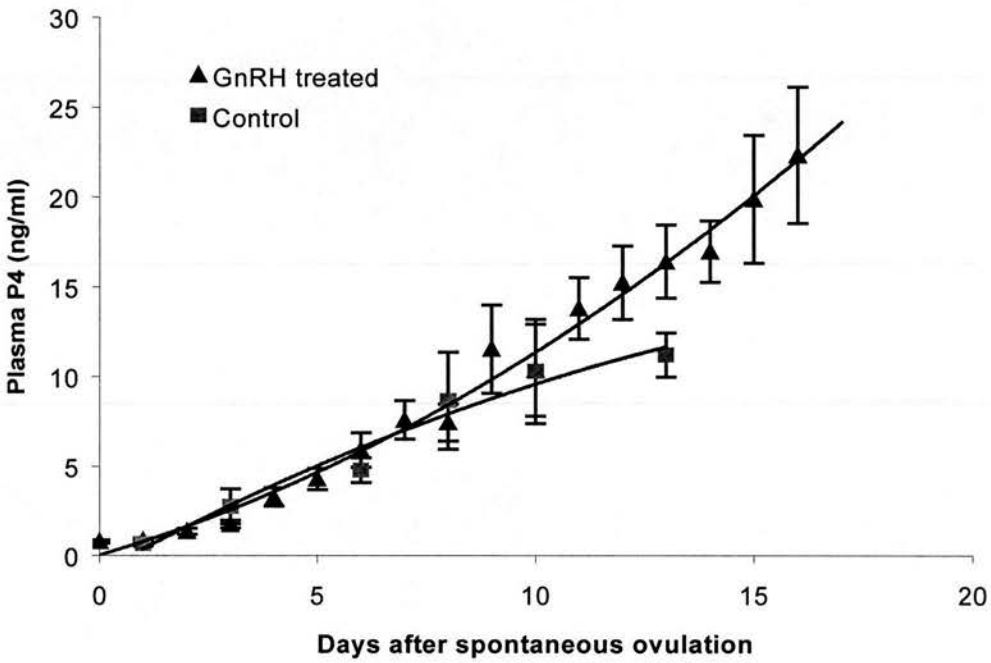


Figure 5.5

Plasma progesterone concentrations before and after administration of GnRH (day 6). Mean values ± SEM.

5.4.3 *In situ* hybridisation

Messenger RNA encoding P450arom was detected in sections of bovine follicular tissue, and was localised to granulosa cells. Expression of mRNA encoding P450arom was absent from CL of the control cycle and CL induced by GnRH treatment.

Messenger RNA encoding the LH receptor was detected in the GnRH-induced corpora lutea. LH receptor mRNA levels in GnRH-induced corpora lutea were not significantly reduced ($P > 0.05$) when compared to spontaneous corpora lutea of similar age.

5.5 Discussion

This study has confirmed that the administration of GnRH on day 6 of the oestrous cycle results in ovulation of the dominant follicle of the first wave, and the subsequent formation of a secondary luteinized structure in >80% of animals. Previous studies in our laboratory have demonstrated that following the removal of the spontaneous CL, the luteinized structure formed in response to a second surge release of LH begins to secrete progesterone *in vivo*. However, its steroidogenic activity falls markedly below that of a spontaneously formed CL of a similar age. In addition, the induced CL has a shortened lifespan, with progesterone concentrations falling below 1 ng/ml within 10 days of GnRH administration (Rusbridge, 1993).

Abnormal luteal function may be the result of one or more of the following generalised effects: 1) sub-optimal follicular development, 2) inadequate luteotrophic support or 3) premature luteolytic stimuli.

5.5.1 Sub-optimal follicular development

Whilst the early stages of folliculogenesis are gonadotrophin-independent, FSH and LH are the primary regulators of the final maturation and development of antral follicles. FSH is required for the growth of follicles up to 9mm, whilst pulsatile LH is essential for continued growth (Gong *et al.*, 1996a, 1996c). The period of ovulatory follicle selection is characterised by low progesterone concentrations, decreasing FSH support and high frequency-low amplitude LH pulses prior to the LH surge (Campbell *et al.*, 1999; Scaramuzzi *et al.*, 1993). Following ovulation, the dominant follicle of the first wave develops in a very different hormonal environment, which may not provide the follicle with sufficient stimulation in preparation for subsequent luteal function. Indeed in the ewe, premature induction of ovulation resulted in luteal phase insufficiency, suggestive of a lack of suitable follicular maturation (Murdoch *et al.*, 1983). The first wave dominant follicle is exposed to increasing progesterone concentrations produced by the developing spontaneous CL, and studies using exogenous progesterone supplementation in the early luteal phase significantly reduced the growth rate and size of the first dominant follicle in cattle (Adams *et al.*, 1992; Burke *et al.*, 1994), demonstrating the potential for progesterone to be inhibitory to follicle development. This suppressive effect may be the result of the negative feedback of progesterone on the frequency of release of LH pulses by the pituitary gland (Bergfeld *et al.*, 1995), as the abolition of preovulatory LH pulses has been shown to limit CL development (Quintal-Franco *et al.*, 1999).

Short-lived CL are often observed following reproductive quiescence in cows and sheep (Braden *et al.*, 1989a; Keisler *et al.*, 1983; Knight *et al.*, 1981; Lamming *et al.*,

1981) and it has been suggested that this naturally occuring reduction in luteal lifespan may also be partly the result of altered follicular development (Garverick *et al.*, 1992; Hunter, 1991). Indeed, developing follicles expected to form short-lived CL post-partum were shown to have fewer gonadotrophin receptors and lower follicular fluid oestradiol concentrations than preovulatory follicles of normally cycling cows (Braden *et al.*, 1989b).

No comparison of the steroidogenic capacity of the ovulatory and anovulatory follicles was made in the present study. However, the mean diameter of the largest follicle present at GnRH administration was lower than that preceding spontaneous ovulation, suggesting that progesterone production by the spontaneous CL may have had an inhibitory effect on subsequent follicle waves.

5.5.2 Inadequate gonadotrophic support

Corpus luteum formation is a complex process involving tightly co-ordinated changes in cellular form and function (Smith *et al.*, 1994c). The primary drive for luteinization is the preovulatory LH surge, and sustained luteotrophic support in the form of LH pulses post-ovulation are required for both structural and functional luteal development (Quintal-Franco *et al.*, 1999). It is clear therefore that alterations in LH stimulation have the potential to disrupt CL function greatly. However, despite the requirement of LH for normal luteal development and function, there is little evidence to suggest that naturally occurring short-lived CL are the result of reduced luteotrophic support (Garverick *et al.*, 1992; Hunter, 1991) Indeed, premature luteolysis is thought to be the primary cause of an inadequate luteal phase (Hunter, 1991). Gonadotropic stimulation was not significantly different between post-partum

cows having short or normal oestrous cycles (Garverick *et al.*, 1988), and repeated injections of hCG failed to decrease the incidence of short cycles in cows following prolonged postpartum anoestrous (Carruthers *et al.*, 1986).

Whilst luteotrophic support was not reduced in short-lived CL, abnormal luteal function might result from a lack of sensitivity to LH. However, besides receiving the same LH stimulation, short-lived and normal CL in the early luteal phase had similar numbers of LH receptors (Rutter *et al.*, 1985), and adenylate cyclase and phosphodiesterase activities were not different (Smith *et al.*, 1986). However, day 7 corpora lutea expected to have a short lifespan were less responsive to LH *in vitro* than cyclic CL (Kesler *et al.*, 1981) and short-lived CL had significantly reduced LH receptor mRNA on day 8 (Smith *et al.*, 1996).

In the present study induced CL did not exhibit significantly reduced LH receptor mRNA levels when compared to spontaneous CL of similar age. Indeed, Rusbridge (1993) demonstrated that the GnRH-induced CL had increased LH receptor numbers, suggesting that the limited function of the GnRH-induced CL was not caused by a lack of LH reception. However, GnRH-induced CL had a reduced response to cAMP-stimulation *in vitro,* suggesting that alterations downstream of the LH receptor may serve to limit the response to LH stimulation.

The transition from follicle to CL following the LH surge is characterised by increased progesterone production. In the bovine CL this is also accompanied by a decrease in oestradiol secretion, resulting from decreased cytochrome P450arom and P450c17 mRNA levels and enzyme content (Rodgers *et al.*, 1986b, 1987; Voss and Fortune, 1993). Whilst it has been suggested that GnRH-induced ovulation of the

first-wave dominant follicle might not allow sufficient follicle maturation, and that the normal transition from the follicular to the luteal phase might be disrupted, the results of the present study indicate that cytochrome P450arom mRNA expression was similar in the GnRH-induced CL and the spontaneous CL.

5.5.3 Premature luteolytic stimuli

There is considerable evidence to suggest that naturally occurring CL displaying a short lifespan are not pre-programmed to be short-lived, but regress prematurely in response to an early luteolytic stimuli. Prevention of the luteolytic signal by hysterectomy, indomethacin infusion and immunisation against $PGF_{2\alpha}$ extended the lifespan of CL anticipated to be short-lived (Copelin *et al.*, 1987, 1989; Troxel and Kesler, 1984). In further support of advanced secretion of $PGF_{2\alpha}$, short luteal phases have been correlated with increased PGFM and endometrial oxytocin binding sites when compared to normal corpora lutea (Hunter, 1991; Hunter *et al.*, 1989).

In the studies by Rusbridge which established the GnRH-induced CL model (Rusbridge, 1993), prostaglandin was administered on day 9 after oestrus (day 3 of the induced cycle) to cause regression of the spontaneous CL. At this time the spontaneous CL was sensitive to the luteolytic signal, whilst the GnRH-induced CL did not undergo luteolysis. The lack of responsiveness of the 3 day old induced CL to prostaglandin treatment is in agreement with data from the natural cycle, where corpora lutea acquire sensitivity to $PGF_{2\alpha}$ after day 5 of the oestrous cycle. This suggests therefore that the GnRH-induced CL does not develop sensitivity to prostaglandin in advance of normal CL. In contrast, others have shown that CL

induced with hCG on day 10 undergo luteolysis following exogenous $PGF_{2\alpha}$ administered within 2 days of ovulation (Howard and Britt, 1990).

To further examine the development of the luteolytic response in animals with induced CL, heifers were subjected to an oxytocin challenge 7 days post-GnRH. Administration of oxytocin resulted in a PGFM response which was of similar magnitude to that of control animals, and it was concluded that there was no alteration in uterine secretory responsiveness that might account for the short luteal phase observed following GnRH-induced ovulation (Rusbridge, 1993). Prostaglandin was released in response to oxytocin despite the low levels of progesterone produced by the GnRH-induced CL. However, the uterus had previously been exposed to progesterone produced by the spontaneous CL, which would be expected to have contributed to uterine priming.

The results of Rusbridge demonstrated that animals bearing a GnRH-induced CL were able to respond to an oxytocin challenge with a PGFM response within the normal range. Whilst this suggests that there was no increase in uterine sensitivity to oxytocin, it does not preclude the possibility of raised endogenous $PGF_{2\alpha}$ concentrations. Intrauterine indomethacin infusion was used to determine the effect of prostaglandin-suppression on the development and function of hCG-induced CL (Sianangama and Rajamahendran, 1996). Indomethacin treatment prevented pulsatile PGFM secretion, but did not enhance the growth of the induced CL. Progesterone production was significantly increased during the infusion period, but PG-suppression was unable to elevate progesterone concentrations beyond levels observed in control cows during the early luteal phase. The authors concluded that whilst endogenous prostaglandin may have contributed to the limited secretory

function of the hCG-induced CL, the luteal inadequacy could not be wholly attributed to the luteolysin $PGF_{2\alpha}$ (Sianangama and Rajamahendran, 1996). However, whilst indomethacin reduces luteolytic prostaglandins, it is not specific for $PGF_{2\alpha}$ and also reduces luteotrophic prostaglandins.

In summary, early embryonic death accounts for a major proportion of reproductive wastage, and evidence suggests that luteal dysfunction contributes significantly to infertility. Progesterone supplementation has been proposed as a possible corrective measure and one possible route is via the induction of accessory corpora lutea. The present study confirms that administration of GnRH to cows in the early luteal phase induces ovulation of the first wave dominant follicle and formation of an induced CL, and plasma concentrations of progesterone were increased in the presence of a GnRH-induced CL. However, previous studies have demonstrated that the contribution of the induced CL to progesterone production is limited. The inadequate function of the GnRH-induced CL may be the result of a number of factors including sub-optimal follicular development, inadequate luteotrophic support or premature luteolytic stimuli. Messenger RNA encoding LH receptor was not reduced in the present study, suggesting that the induced CL did not lack luteotrophic support. However, LH responsiveness may be altered downstream of the LH receptor as suggested by the reduced response of cells from the induced CL to cAMP stimulation *in vitro* (Rusbridge, 1993). Previous suggestion of aberrant luteal oestradiol production are not supported by the present study, since mRNA encoding P450arom was undetectable in the GnRH-induced CL. There is substantial evidence to suggest that inadequate luteal function occurring naturally often results from premature induction of luteolysis, although others have suggested that luteal

inadequacy is not wholly attributable to the actions of $PGF_{2\alpha}$. Whilst the development of the CL induced in response to GnRH is likely to be markedly different from that of naturally occurring inadequate corpora lutea, the possible causes of its limited function warrant future study.

Chapter 6. Effect of GnRH administration in the early luteal phase on the IGF system in the bovine corpus luteum

6.1 Summary

The administration of GnRH to cattle on day 6 causes ovulation of the first wave dominant follicle and formation of an additional corpus luteum. This GnRH-induced CL has been characterized and displays reduced steroidogenic capacity. Studies *in vitro* have demonstrated that IGF-I and –II regulate many aspects of ovarian function, including steroidogenesis. We have tested the hypothesis that diminished IGF stimulation may contribute to the limited function of the GnRH-induced CL.

In situ hybridization has demonstrated that mRNA encoding IGF-I, IGF-II, the type 1 IGF receptor and IGFBP-2, -3 and –4 were expressed within the GnRH-induced CL. The spatial patterns of mRNA expression were not different between GnRH-induced and control corpora lutea. In contrast however, significant differences were detected in mRNA concentrations following GnRH-administration in both the induced CL and the original CL present on the ovary at the time of treatment compared to control CL levels. The concentration of type 1 IGF receptor mRNA was significantly decreased in the GnRH-induced CL when compared to early luteal phase control, and there was a trend for increased IGFBP-3 mRNA concentrations in the GnRH-induced CL when compared to day 10 control CL. These changes are expected to reduce both the bioavailability and bioactivity of IGF-I and IGF-II in the GnRH-induced CL, thus compromising the response to luteotrophic support. This may in part explain the limited steroidogenic function of CL induced by the administration of GnRH in the early luteal phase.

6.2 Introduction

IGF-I and IGF-II are important regulators of ovarian steroidogenesis. IGF-stimulation of luteal cells *in vitro* increases progesterone release in a number of species (Constantino *et al.*, 1991; Devoto *et al.*, 1995; Khan-Dawood *et al.*, 1994; McArdle and Holtorf, 1989; Talavera and Menon, 1991; Yuan and Lucy, 1996a), resulting from multiple effects on the steroidogenic pathway. IGFs augment sterol uptake and utilisation (Veldhuis, 1989), transport of steroid precursors to mitochondria (Balasubramanian *et al.*, 1997), and the subsequent enzymatic conversion of cholesterol to pregnenolone, and of pregnenolone to progesterone (deMoura *et al.*, 1997). The *in vitro* results suggest therefore that the IGFs have great potential to regulate luteal progesterone production *in vivo*.

The first-wave dominant follicle can be induced to ovulate in response to a second surge release of LH and subsequently form a secondary luteinized structure (Fricke *et al.*, 1993; Price and Webb, 1989; Rajamahenran and Sianangama, 1992; Rusbridge, 1993; Schmitt *et al.*, 1996a, 1996b; see chapter 5). However, whilst corpora lutea formed following GnRH administration in the early luteal phase (day 6) begin to secrete progesterone, peripheral progesterone concentrations fall below those produced by spontaneously formed CL of a similar age (Rusbridge, 1993).

We hypothesise that the subnormal steroidogenic function of the GnRH-induced CL is the result of changes in the luteal IGF system, resulting in reduced IGF bioavailability and /or bioactivity and hence diminished local IGF-stimulation of luteal function.

6.3 Materials and Methods

6.3.1 Tissue collection

6.3.1.1 GnRH-treated animals

Secondary corpora lutea were induced by early luteal phase GnRH administration, as detailed in section 5.3.2. In brief, the oestrous cycles of mature dairy cows were synchronised by intravaginal progesterone release (PRIDs) and $PGF_{2\alpha}$ analogue (Estrumate) injection. On day 6 after oestrus cows received 0.5mg synthetic GnRH (Fertagyl) to induce ovulation of the first-wave dominant follicle and formation of a second corpus luteum. Pairs of ovaries were collected on either day 10 or day 15 of the oestrous cycle (Figure 5.1), and both the GnRH-induced and spontaneously formed CL within each pair were processed for *in situ* hybridisation using frozen sections.

6.3.1.2 Control animals

The oestrous cycles of mature dairy cows were synchronised using PRIDs and $PGF_{2\alpha}$ analogue injection (see section 3.3.1). Corpora lutea collected on day 5, 10 and 15 after oestrus were studied.

6.3.2 *In situ* hybridisation

In situ hybridisation (see section 2.11) was performed to detect the expression of mRNA encoding IGF-I, IGF-II, the type 1 IGF receptor and IGFBP-2, -3 and –4 in frozen sections of luteal tissue (14μm). Antisense and sense riboprobes were generated using a standard transcription protocol and labelled with [^{35}S]-UTP (see section 2.10.1). Target sequences are detailed in sections 2.4 and 4.3.2. The intensity of the *in situ* hybridisation signal was quantified by image analysis (see section 3.3.3)

and mRNA concentrations were compared between similar aged corpora lutea of the control cycle, GnRH-induced CL and spontaneous CL present at the time of GnRH administration.

6.3.3 Statistical Analysis

The effect of GnRH administration on mRNA concentration was determined by a split-plot ANOVA (Genstat 5; Payne *et al.*, 1993), using the blocking factor cow/ slide/ replicate as previously described (see section 3.3.4).

6.4 Results

In situ hybridisation detected mRNA encoding IGF-I, IGF-II, the type 1 IGF receptor and IGFBP-2, -3 and –4 within GnRH-induced CL at both timepoints studied (4 and 9 days post GnRH). The spatial pattern of mRNA localisation was similar for GnRH-induced and spontaneously-formed CL, and is detailed in chapter 3 and 4. Analysis of the intensity of the *in situ* hybridisation signal demonstrated a significant difference in the concentration of mRNA encoding the type 1 IGF receptor between GnRH-induced and control CL of a similar age. Differences in expression of IGFBP-3 mRNA approached significance. There was no significant effect of treatment on the concentrations of mRNA encoding IGF-I, IGF-II, IGFBP-2 or –4.

IGFBP-3 mRNA levels were not significantly different ($P>0.05$) between control CL of the early luteal phase and GnRH-induced CL of a similar age (Figure 6.1a). Differences in IGFBP-3 mRNA expression between control CL, GnRH-induced CL and the spontaneous CL present at GnRH administration of around 10 days old approached significance ($P=0.052$) (Figure 6.1b). IGFBP-3 mRNA levels in control CL were below those in both GnRH-induced and spontaneous CL.

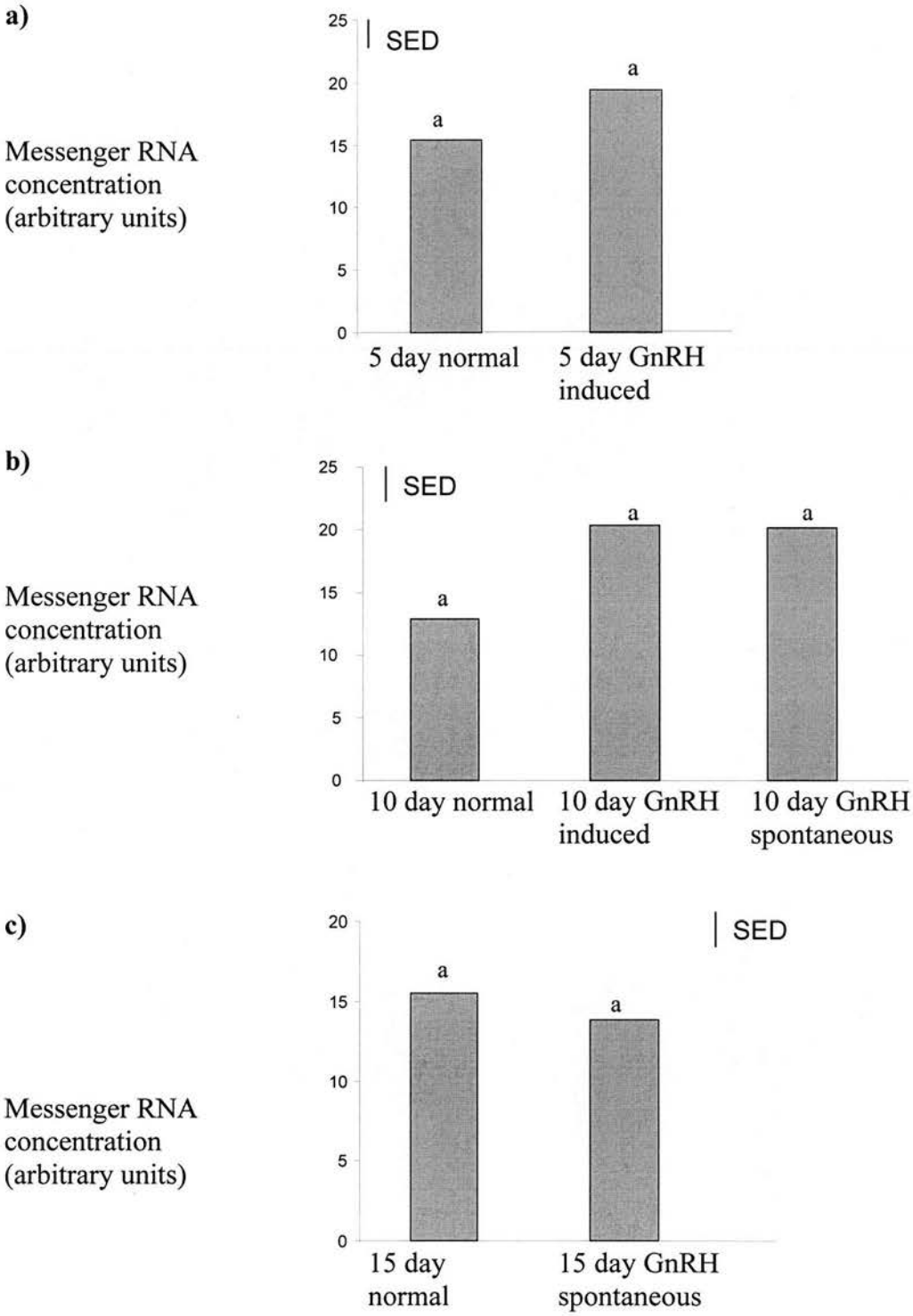


Figure 6.1

Quantitative analysis of IGFBP-3 mRNA concentrations in bovine luteal tissue. Concentrations were compared between 5 day old (a), 10 day old (b) and 15 day old (c) corpora lutea from control animals, and animals treated with GnRH bearing both a spontaneous and GnRH-induced CL. Luteal timepoints with different superscipts are significantly different ($P < 0.05$). Bars represent pooled standard error of the difference (SED). Concentration of mRNA is expressed as the percentage of the total number of pixels occupied by silver grains within a defined area. Hybridisation with an IGFBP-3 sense probe gave an average background value of 5 units.

Messenger RNA encoding IGFBP-3 was not significantly different (P>0.05) between control CL of the late luteal phase and age-matched spontaneous CL (Figure 6.1c).

Messenger RNA encoding type 1 IGF receptor was observed at significantly higher levels (P =0.045) in CL of control animals on day 5 than similar aged GnRH-induced CL (Figure 6.2a). A significant difference (P=0.05) in type 1 IGF receptor mRNA concentration was detected amongst control CL, GnRH-induced CL and spontaneous CL around day 10; concentrations of type 1 IGF receptor mRNA were highest in control CL and lowest in the spontaneous CL (Figure 6.2b). Type 1 IGF receptor mRNA levels were not significantly different (P>0.05) between day 15 CL of control animals and age-matched spontaneous CL (Figure 6.2c).

6.5 Discussion

GnRH-treatment in the early luteal phase causes ovulation and formation of a secondary luteal structure (Rusbridge, 1993). However, the GnRH-induced CL has reduced secretory function (Rusbridge, 1993) that is correlated with significant alterations in the local IGF system.

This study demonstrates that the GnRH-induced corpora lutea expresses mRNA encoding IGF-I, IGF-II, the type 1 IGF receptor and IGFBP-2, -3 and –4 at both timepoints studied. The spatial patterns of expression were not different between CL induced by a second surge release of LH and those formed during the natural cycle. In addition, there was no significant effect of treatment on the concentrations of mRNA encoding IGF-I, IGF-II, IGFBP-2 or –4. However, there were significant differences in the levels of mRNA encoding the type 1 IGF receptor and a trend for alterations in IGFBP-3 mRNA levels following GnRH administration.

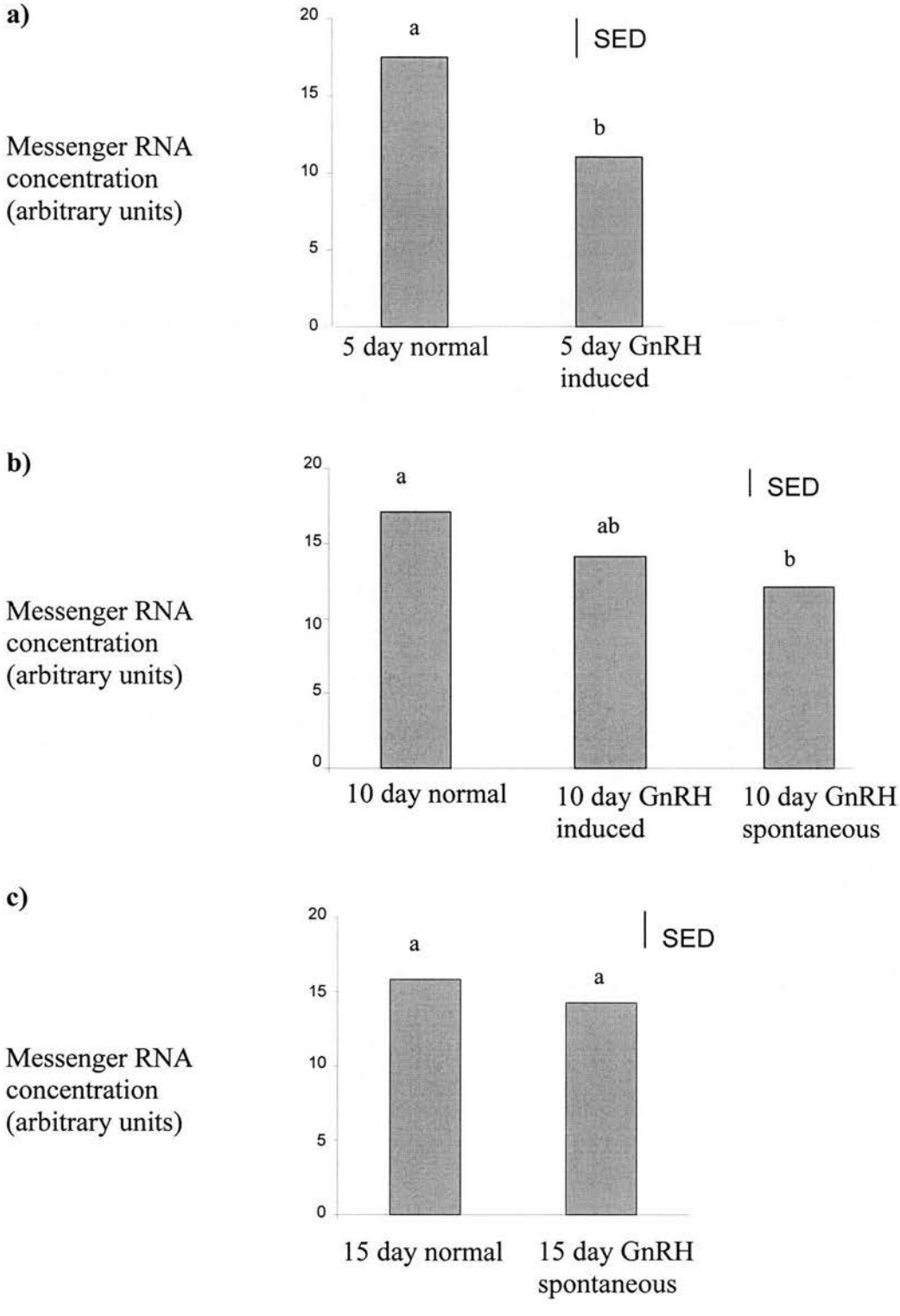


Figure 6.2

Quantitative analysis of type 1 IGF receptor mRNA concentrations in bovine luteal tissue. Concentrations were compared between 5 day old (a), 10 day old (b) and 15 day old (c) corpora lutea from control animals, and animals treated with GnRH bearing both a spontaneous and GnRH-induced CL. Luteal timepoints with different superscipts are significantly different ($P < 0.05$). Bars represent pooled standard error of the difference (SED). Concentration of mRNA is expressed as the percentage of the total number of pixels occupied by silver grains within a defined area. Hybridisation with a type 1 IGF receptor sense probe gave an average background value of 6 units.

Concentrations of mRNA encoding the type 1 IGF receptor were significantly reduced in GnRH-induced CL compared to similar-aged CL from the early luteal phase of the control cycle. The reduction in mRNA is assumed to result in reduced receptor numbers, and subsequently reduced IGF-stimulation in GnRH-induced CL. Since progesterone production by luteal cells is stimulated *in vitro* by IGF-I and IGF-II a reduction in IGF-reception in the GnRH-induced CL may contribute to the diminished steroidogenic output observed experimentally. However, induced corpora lutea of around 10 days old did not exhibit significantly different type 1 IGF receptor concentrations compared to control levels.

There was a trend for increased concentrations of mRNA encoding IGFBP-3 in GnRH-induced corpora lutea compared to age-matched CL of the mid-luteal but not early-luteal phase. In its IGF-dependant role, IGFBP-3 binds both IGF-I and –II with high affinity, preventing ligand-receptor interaction IGFBP-3 inhibits IGF-I-stimulated progesterone production by bovine granulosa and theca cells in culture, and this inhibitory effect is thought to result from IGFBP-3 sequestering IGF-I (Spicer and Chamberlain, 1999; Spicer *et al.*, 1997). Therefore, increased IGFBP-3 would be expected to further reduce IGF-stimulation of luteal cell steroidogenesis.

Failure of the IGF system to develop to normal levels in the GnRH-induced CL may be the result of disruption to both follicular and luteal events. The hormonal environment of the first-wave dominant follicle differs markedly from that of the ovulatory follicle. In particular, the dominant follicle of the first wave is exposed to increasing progesterone produced by the spontaneous CL and subsequently reduced LH-pulse frequency (Bergfeld *et al*, 1995; Rahe *et al.*, 1980).

Bovine dominant follicles undergo atresia in response to exogenous progesterone treatment. The concentrations of low molecular weight IGFBPs were increased in follicular fluid during progesterone-induced atresia, with a profile similar to natural atresia. In addition, atresia was associated with reduced follicular fluid IGF-I and –II concentrations (Manikkam and Rajamahendran, 1997). In the present study progesterone produced by the original CL was not sufficient to cause atresia of the first wave follicle, since GnRH administration resulted in ovulation. However, progesterone concentrations may regulate components of the IGF system in the first wave dominant follicle and subsequently the GnRH-induced CL.

In the follicle, the IGFs stimulate cellular proliferation and steroidogenesis, and in synergy with gonadotrophins, stimulate follicular growth and differentiation. The actions of both IGF-I and –II are mediated via the type 1 IGF receptor and a reduction in IGF-reception and hence IGF-stimulation would be expected to have major implications for both the follicle and CL. IGF-receptor binding and mRNA encoding the type 1 IGF receptor have been detected within bovine follicles. However, it remains unclear whether receptor numbers alter with follicular development or hormonal regulation (Spicer and Echternkamp, 1995). *In vitro*, granulosa cells from large bovine follicles had greater IGF-I binding than those of small follicles (Spicer *et al.*, 1994). In contrast, expression of mRNA encoding the type 1 IGF receptor did not vary with follicle size *in vivo*, although this study did not examine changes in expression at different points during follicle growth (Armstrong *et al.*, 2000a).

Type 1 IGF receptor levels are regulated in response to a number of physiological and pathological stimuli (LeRoith *et al.*, 1995; Werner *et al.*, 1995). The binding of

IGF-I to cultured rat granulosa cells was increased by FSH and LH treatment (Adashi *et al.*, 1988). However, neither LH nor progesterone influenced IGF-I binding in cultured bovine granulosa cells (Spicer *et al.*, 1994). This lack of regulatory action suggests that differences in LH and progesterone secretion between the growth of the ovulatory follicle and the dominant follicle of the first wave may not be the cause of reduced type 1 IGF receptor mRNA levels in the induced CL. A significant difference in type 1 IGF receptor mRNA concentrations was observed between early luteal CL from control animals and similar aged GnRH-induced CL. This result suggests that the type 1 IGF receptor is sensitive to changes in the hormonal environment, however it remains unclear whether this reflects a response to changes in late follicular or early luteal development. Whilst progesterone did not influence IGF-I-binding to cultured bovine granulosa cells, steroid hormones have been shown to regulate type 1 IGF receptor gene expression in reproductive tissues. Oestradiol caused a two- to three-fold increase in type 1 IGF receptor mRNA levels in normal human breast xenografts, whilst progesterone treatment down-regulated type 1 IGF receptor mRNA concentrations to half that of controls (Clarke *et* al., 1997). Progestins also reduced mRNA encoding the type 1 IGF receptor in cultured T-47D human breast-cancer cells (Goldfine *et* al., 1992), an effect that may be mediated by enhanced IGF-II secretion (Papa *et al.*, 1991). It remains to be determined whether type 1 IGF receptor mRNA concentrations are similarly reduced in the GnRH-induced CL in response to the high levels of progesterone produced by the CL present at GnRH administration. Type 1 IGF receptor gene expression is also regulated by local and circulating levels of IGF-I. Patients with low circulating IGF-I levels as a result of GH deficiency or Laron-type dwarfism display raised

erythrocytic IGF-I binding sites and increased lymphocytic type 1 IGF receptor mRNA levels (Eshet *et al.*, 1993). An inverse correlation between IGF-I concentration and receptor number has also been demonstrated in cell culture. Increasing concentrations of IGF-I cause a reduction in IGF receptor number *in vitro* in a number of different cell types (Hernandez-Sanchez *et al.*, 1997; Rosenfeld and Dollar, 1980; Rosenfeld and Hintz, 1980). However, it is not clear whether a similar relationship operates in the ovary.

A number of other growth factors regulate type 1 IGF receptor abundance. Basic FGF, for example, stimulates IGF-I binding and type 1 IGF receptor mRNA levels in muscle cells (Pfeifle *et al.*, 1987; Rosenthal *et al.*, 1991). In contrast, in bovine granulosa cells bFGF decreased IGF-I receptor numbers (Spicer *et al.*, 1994). PDGF also increases the number of IGF-I binding sites and the expression of the IGF-I receptor gene, an effect that is due at least in part to PGDF-stimulation of IGF-receptor promoter activity (Rubini *et al.*, 1994). The physiological role of these factors in regulating luteal type 1 IGF receptor numbers remains uncertain.

IGFBP-3 is the most abundant IGFBP in serum (Rajaram *et al.*, 1997) and the predominant IGFBP in bovine preovulatory follicular fluid (Funston *et al.*, 1996). However, mRNA encoding IGFBP-3 is expressed at low levels in the follicle, and does not vary with follicle size (Yuan *et al.*, 1998), and neither ovine granulosa or theca cell cultures produced significant quantities of IGFBP-3 (Armstrong *et al.*, 1996). It has been suggested therefore that follicular fluid IGFBP-3 is primarily the product of transudation from serum. There is limited information on the regulation of IGFBP-3 expression in the ovary. Messenger RNA encoding IGFBP-3 is abundant in the bovine CL by day 5 (Chapter 4). However, it remains to be established when in

early luteal development IGFBP-3 mRNA expression is upregulated, and whether this occurs as a direct response to the LH surge. An increase in IGFBP-3 mRNA concentration was observed between day 10 control CL and similar aged GnRH induced CL, whilst no effect was detected in the early luteal phase. The lag between GnRH treatment and significant alterations in IGFBP-3 concentrations may suggest that IGFBP-3 mRNA is more sensitive to hormonal changes during luteal growth and development than during follicular growth and development. In addition, it suggests that the signal which upregulates IGFBP-3 mRNA levels from the relatively low follicular levels is still in place following GnRH treatment.

A number of IGFBPs are expressed at any one time within the ovaries of many species. The IGF system may have in-built redundancy, such that an increase in the concentration of one IGFBP can be accompanied by a compensatory decrease in the concentration of another. However, the addition of IGFBP-3 to ovarian cells in culture or its administration to the whole ovary *in vitro* has had dramatic results, suggesting a lack of compensation. IGFBP-3 profoundly inhibited IGF-stimulated cell proliferation and steroidogenesis in bovine theca and granulosa cells in culture by sequestering IGF-I and –II (Spicer and Chamberlain, 1999; Spicer *et al.*, 1997). The addition of IGFBP-3 to perfused rabbit ovaries *in vitro* inhibited hCG-induced ovulation, oocyte development and oestradiol production (Yoshimura *et al.*, 1996). In human luteinising granulosa cells IGFBP-3 reduced the stimulatory effect of IGF-I on oestradiol production (Barreca *et al.*, 1996).

The primary regulator of plasma IGFBP-3 concentrations is GH, produced by the pituitary somatotrophs. Analysis of the rat and cow IGFBP-3 gene promoters localized consensus sequences for a number of putative response elements including

GH (Albiston *et al.*, 1995; Erondu *et al.*, 1997). The secretion of IGFBP-3 by human luteinising granulosa cells in culture was stimulated by GH (Barreca *et al.*, 1996), supporting a role for GH in ovarian IGFBP-3 regulation. However, in the reproductive tissues of cattle, concentrations of mRNA encoding IGFBP-3 were unaffected by exogenous GH treatment, suggesting that GH does not directly affect gene expression in this species (Kirby *et al.*, 1996). Hence, with the limited information available it remains unclear to what extent GH may regulate IGFBP-3 expression in the cow CL. Furthermore we have no clear understanding of how GH-stimulated IGFBP-3 mRNA expression might be altered in the GnRH-induced CL to account for increased IGFBP-3 concentrations.

However, regulatory actions of GH within the ovary have been demonstrated (Lucy *et al.*, 1999). Treatment of mature ewes with GH resulted in enhanced follicle development and increased follicular secretion of IGF-I in culture, in addition to increased peripheral concentrations of IGF-I (Gong *et al.*, 1996b). Many of the actions of GH are mediated by IGF-I, which is released from the liver in response to GH-stimulation. However, GH may also act directly on the ovary, since the GH receptor has been detected in the bovine ovary and GH receptor concentrations were 20-fold higher in the CL than in follicles (Lucy *et al.*, 1993). Abnormalities of GH receptor expression (either occurring naturally or following gene knockout experiments) have demonstrated that GH is not essential for reproduction, but it appears to have an important facilitatory role (Chase *et al.*, 1998; Zhou *et al.*, 1997). In CL, GH influences luteal growth and development (Juengel *et al.*, 1997) and increases progesterone secretion *in vitro* (Liebermann and Schamms, 1994).

A number of other factors have been shown to modulate the production of IGFBP-3 by a range of cell types in culture. IGFBP-3 protein and mRNA levels are increased by treatment with factors including IGF-I, the tumour suppressor p53, retinoic acid, PDGF and vasopressin (Buckbinder *et al.*, 1995; Corps and Brown, 1991; Gucev *et al.*, 1996). In addition, porcine granulosa cells respond to oestradiol and EGF treatment with increased IGFBP-3 production, whilst the addition of FSH and TGF-β inhibited IGFBP-3 secretion (Mondschein *et al.*, 1990). Under luteinising conditions porcine granulosa cell IGFBP-3 production is stimulated by $PGF_{2\alpha}$ and attenuated by PGE_2 (Grimes *et al.*, 1993). It is unknown whether any of these factors regulate IGFBP-3 mRNA concentrations in the bovine corpus luteum during the oestrous cycle, or whether these factors are influential in creating the changes in IGFBP-3 mRNA concentrations observed in the GnRH-induced CL.

The inhibitory effects of IGFBP-3 on ovarian function (Barreca *et al.*, 1996; Spicer and Chamberlain, 1999; Spicer *et al.*, 1997; Yoshimura *et al.*, 1996) have been largely attributed to the sequestering of IGFs and subsequent prevention of IGF receptor activation. However, IGFBPs can exert additional IGF-independent actions that do not involve IGF binding or influence IGF reception. The growth inhibitory actions may be mediated by binding of IGFBP-3 to cell surface proteins (Oh *et al.*, 1993b, 1993c). In addition, the type V TGF-β receptor has been suggested to be a putative IGFBP-3 receptor, although the nature of any signalling response to IGFBP-3 is unknown (Leal *et al.*, 1997). IGF-independent actions of IGFBP-3 have also been proposed to occur intracellularly (Li *et al.*, 1997), although the extent and mechanism by which IGFBP-3 interacts with the nuclear machinery remain to be determined. The growth inhibitory actions of IGFBP-3 may also involve the

induction or accentuation of apoptotic cell death (Gill *et al.*, 1997; Rajah *et al.*, 1997), via an IGF-receptor independent pathway and possible interaction with other growth inhibitors. It remains to be established whether IGFBP-3 influences follicular and luteal function via IGF-independent mechanisms, in addition to its ability to bind IGF.

Whether IGFBP-3 exerts IGF-independent luteal growth inhibition or sequesters stimulatory IGFs, it is unclear why the CL should express IGFBP-3 from the early luteal phase in the natural cycle and at times of high steroidogenesis. Despite this paradox, increased expression of IGFBP-3 mRNA in the induced CL would be expected to be detrimental to both luteal lifespan and function.

Our understanding of the role of IGFBP-3 in ovarian function is further complicated by its ability to potentiate the effects of IGF *in vitro*. Potentiation is thought to involve IGF-binding to cell surface-associated IGFBP-3, and may enhance delivery of IGF to its receptor (Conover *et al.*, 1990). Cell associated IGFBP-3 has a 10-fold lower binding affinity for IGF-I than IGFBP-3 in solution, and a 2-fold lower affinity for IGF-I than the type 1 IGF receptor (Conover, 1991). Additionally proteolysis of IGFBP-3 may potentiate IGF action, since cleavage of IGFBP-3 produces a fragment with reduced IGF-binding affinity (Schmid *et al.*, 1991). Reduced affinity of binding proteins for IGF would then be expected to increase the IGF available for ligand-receptor interactions.

GnRH treatment in the early luteal phase causes ovulation and subsequent formation of a luteinised structure. In addition, the administration of GnRH and/or the presence of the induced CL also influence the spontaneous corpus luteum present on the ovary at the time of treatment. Concentrations of mRNA encoding both IGFBP-3 and the

type 1 IGF receptor were altered in the spontaneous CL when compared to control corpora lutea of a similar age. IGFBP-3 mRNA levels were raised above control levels in the mid-luteal spontaneous CL, but were not different in the late luteal phase. In contrast, type 1 IGF receptor mRNA concentrations in GnRH-treated spontaneous CL were significantly below the levels in control CL of the mid-luteal phase, whilst no difference was observed in the late luteal phase.

Exposure of the developing corpus luteum to an LH surge following GnRH administration has been shown to impair the function of the original CL (Martin *et al.*, 1990). This effect may be the result of LH receptor down-regulation (Rodger and Stormshak, 1986) or post LH-receptor disruptions (Conti *et al.*, 1977) leading to desensitisation to gonadotrophin stimulation. In contrast, others have suggested that increases in plasma progesterone observed two days post-hCG could not be accounted for by the induced CL and must therefore result from a stimulatory effect of hCG on the original CL (Fricke *et al.*, 1993).

In the present study the relative contributions of the original and GnRH-induced CL to plasma progesterone production were not determined, therefore it remains unclear whether GnRH had a stimulatory or supressive effect on the progesterone secretion of the original CL. However, the observed increase in IGFBP-3 and decrease in the type 1 IGF receptor mRNA levels would not be expected to be consistent with increased progesterone production, therefore, the functional capacity of the original CL may also have been compromised. Interestingly, whilst the GnRH-induced CL exhibited alterations in mRNA concentrations at both timepoints studied, the original CL was only affected when compared to mid-luteal CL of control animals and no differences in mRNA concentrations were detected when compared to late luteal

control CL. The original CL therefore appears to display only a transient variation from control IGFBP-3 and type 1 IGF receptor mRNA levels. It is unclear whether this reflects an intrinsic quality of the original CL or its advanced stage of luteal development to recover from earlier hormonal changes. Alternatively, there may have been a diminution of the signal that caused the initial variations or the sensitivity of the original CL to that signal, although the increase in IGFBP-3 mRNA levels observed in the GnRH-induced CL also present on the ovary suggests the former not to be the case.

In conclusion, this study demonstrates that (1) many components of the IGF system are expressed in the GnRH-induced CL, (2) differences in type 1 IGF receptor and IGFBP-3 mRNA concentrations from control levels may contribute to the reduced functional capacity of the GnRH-induced CL (3) GnRH administration also affects the original CL present on the ovary at the time of treatment.

Chapter 7. General Discussion

The corpus luteum is crucial to reproductive success. Its primary secretory product progesterone is required for the establishment and maintenance of pregnancy, and inadequate progesterone production is a major cause of infertility and embryonic loss. The corpus luteum is also central to the regulation of cyclicity. Whilst LH and $PGF_{2\alpha}$ are the principal luteotrophic and luteolytic hormones in domestic animals, there is increasing interest in the potential of local factors to modulate the response of the CL to hormonal action.

The insulin-like growth factors are putative regulators of ovarian function, and both IGF-I and IGF-II have direct effects on ovarian cells in culture. Whilst there is considerable evidence to support a role for the IGFs in regulating normal follicular development, there is a relative paucity of information regarding the actions of the IGFs in the CL. However, an important role for the IGFs in regulating luteal function is suggested both by the multiple stimulatory effects of the IGFs on follicular steroidogenesis, and by the direct stimulation of progesterone production by luteal cells *in vitro*.

Whilst the *in vitro* evidence suggests that the IGFs are important regulators of both follicular and luteal function, the local expression of mRNAs encoding the IGFs in the bovine ovary remains controversial.

This series of studies has examined the temporal and spatial pattern of expression of mRNA encoding IGF-I, IGF-II, the type 1 IGF receptor and IGFBP-2, -3 and –4 during the growth and regression of the bovine corpus luteum. Corpora lutea were collected at defined stages of the oestrous cycle after synchronised oestrus (see

Chapter 3), and following GnRH-induced ovulation of the first wave dominant follicle (see Chapter 5).

The expression of IGF-I mRNA in the ruminant ovary has been the subject of much debate. This study confirms that the bovine corpus luteum is a site of IGF-I production (see Chapter 3), and suggests an important regulatory role for locally produced IGF-I. Expression of IGF-I mRNA was low and widespread, but increased towards the periphery and in lines radiating through the corpus luteum. The exact identity of these cells could not be determined, however, endothelial cells, steroidogenic cells and immune cells are all suggested sites of IGF-I mRNA expression. It is clear that accurate identification of the cellular sites of IGF-I mRNA expression is essential to a greater understanding of the role of locally produced IGF-I in luteal function.

The results of follicular cell culture suggest that IGF-I acts with the gonadotrophins to regulate the acquisition of differentiated function. However, the results of the present study do not support a similar role in the bovine corpus luteum for endogenously produced IGF-I, since the expression of mRNA encoding IGF-I was low during luteinisation. However, the correlation between increasing luteal IGF-I mRNA concentrations and peak progesterone production observed in the present study, in addition to enhanced progesterone biosynthesis in response to IGF-I *in vitro*, suggests that local IGF-I might act as a stimulator of luteal steroidogenesis.

The variation in IGF-I mRNA concentrations during the oestrous cycle suggests that regulated local expression is important for luteal function. However, it is unclear how critical a role is played by the low levels of locally produced IGF-I, since there are high levels of IGF-I present in the circulation. It is not known whether the CL

receives significant IGF-I stimulation from peripheral sources, and if so, whether locally produced IGF-I supplements the actions of endocrine IGF-I or serves an entirely different role in CL regulation. Liver-specific inactivation of the IGF-I gene caused a large decrease in blood IGF-I concentrations (Sjogren *et al.*, 1999; Yakar *et al.*, 1999). However, mutant mice with liver IGF-I gene deletion were fertile and had litters of normal size (Yakar *et al.*, 1999), suggesting that systemic IGF-I may not be crucial to the stimulation of reproduction in the mouse. There is little known about the contribution of circulating IGF-I to luteal function. An endocrine effect of IGF-I on luteal growth was suggested in nutrient-restricted heifers, where reduced CL size was correlated with low blood IGF-I, whilst luteal IGF-I mRNA expression was unaffected (Vandehaar *et al.*, 1995). However, whilst the treatment of cows with GH increases peripheral IGF-I concentrations, subsequent progesterone secretion has been unaffected (Gong *et al.*, 1991), increased (Lucy *et al.*, 1994) or decreased (Jimenez-Krassel *et al.*, 1999).

Maximal expression of mRNA encoding IGF-I was detected in the regressing CL, 48 hours after exogenous prostaglandin. The role of IGF-I in luteolysis is unclear, given the potential of IGF-I to stimulate luteal function. However, the high expression may reflect the intense immune cell activity associated with luteal regression. The role of IGF-I in the regulation of immune cell function during luteal regression could be further examined by the collection of CL at additional timepoints following natural or induced luteolysis, and the subsequent identification of the sites of IGF-I mRNA expression.

Previous studies have concentrated on the potential of IGF-I rather than IGF-II to regulate ovarian function. The present study reports that the bovine corpus luteum is

a site of IGF-II mRNA expression (see Chapter 3). Despite the limited number of studies that have previously examined the potential role of IGF-II in the ovary, IGF-II has been shown to enhance steroidogenesis (Garmey *et al.*, 1993; Kamada *et al.*, 1992; Mason *et al.*, 1994), including the stimulation of progesterone production by bovine luteal cells in culture (Sauerwein *et al.*, 1992). The local production of IGF-II by the bovine corpus luteum suggested by the detection of IGF-II mRNA therefore allows for local regulation of steroidogenesis. However, the expression of mRNA encoding IGF-II was shown to be particularly intense in the luteal vasculature, and suggests that IGF-II may regulate aspects of luteal function in addition to the stimulation of steroid biosynthesis. Hybridisation was not limited to endothelial cells, and pericytes, fibroblasts or smooth muscle cells may also be sites of IGF-II mRNA expression. Indeed, previous immunohistochemical observations localised IGF-II protein to perivascular cells (Amselgruber *et al.*, 1994). It remains unclear whether this reflects a role for locally produced IGF-II in the control of vascular growth and function, or the regulation of vascular and steroidogenic cell interactions, and warrants further examination.

IGF-I and IGF-II interact with the same receptor and binding proteins, albeit with different affinities. It is unclear how the IGFs might co-operate to support luteal growth and development. However, the distinct cellular sites of expression of mRNA encoding the IGFs suggest that these factors may serve different functions in different cell-types. It remains to be determined whether locally produced IGF-II is sufficient to influence luteal function in the presence of a potential excess of IGF-I from circulatory sources, since IGF-I has a higher affinity for the type 1 IGF receptor than IGF-II.

The detection of mRNA encoding the type 1 IGF receptor within the corpus luteum throughout the oestrous cycle (see Chapter 3) suggests that stimulation by IGF-I and IGF-II (produced locally or derived from the circulation) may be important throughout the lifespan of the corpus luteum. Whether these multifunctional proteins stimulate different aspects of luteal function at different times is unknown.

Type 1 IGF receptor mRNA was widespread, and steroidogenic luteal cells are the suggested site of mRNA expression. These results confirm and extend previous reports of binding characteristic of the type 1 IGF receptor in bovine luteal membrane preparations (Sauerwein *et al.*, 1992). The diffuse pattern of mRNA expression encoding the type 1 IGF receptor suggests that IGFs may influence luteal cells which do not themselves produce IGF-I or –II, given the low levels of locally produced IGF-I, and the predominant association of IGF-II mRNA with the luteal vasculature. This suggests that local IGFs may act as paracrine regulators of non-expressing cells. Additionally it may further support a role for IGFs derived from the circulation in the regulation of luteal function.

The actions of IGF-I and IGF-II are modulated by association with members of a family of high affinity IGFBPs (Clemmons, 1997; Jones and Clemmons, 1995; Rechler and Clemmons, 1998). Current evidence suggests that the interaction of IGFs with IGFBPs serves to attenuate the actions of IGFs on ovarian cells (Bicsak *et al.*, 1990; Chun *et al.*, 1994; Mason *et al.*, 1998; Spicer *et al.*, 1997; Ui *et al.*, 1989; Yoshimura *et al.*, 1996). This study has determined that the bovine CL is a site of IGFBP expression (see Chapter 4) and indicates that there is a requirement for the regulation of IGF-actions in the CL. Messenger RNA encoding IGFBP-2, -3 and –4 was detected throughout the oestrous cycle, and showed limited temporal variation.

IGFBP-4 mRNA was expressed by steroidogenic luteal cells and may act to block IGF-stimulation of steroid production as has been demonstrated *in vitro*. However, mRNA encoding IGFBP-2 and -3 was predominantly localised to non-steroidogenic luteal cells. Indeed, mRNA encoding IGFBP-2 and –3 showed particular association with the luteal vasculature. A number of large blood vessels showed moderate to intense hybridisation for IGFBP-2 mRNA which may be localised to smooth muscle cells. Messenger RNA encoding IGFBP-3 was detected in cells lining microvessels, suggestive of endothelial cell expression. IGFBP-2 and –3 may therefore serve a number of functions. They may 1) regulate the actions of IGFs on vascular function, 2) act as paracrine factors outwith the vasculature in the regulation of IGF-stimulated steroidogenic cell function, or 3) act as a sink for peripheral IGFs, enabling subsequent controlled release of the ligands in response to changes in affinity of the IGFBPs for the IGFs due to proteolysis or ECM-binding.

The IGFBPs exhibit different binding preferences for IGF-I and -II. IGFBP-2, -5 and –6 bind IGF-II with higher affinity than IGF-I (Jones and Clemmons, 1995). It is not known whether luteal IGFBP-2 is therefore primarily involved in regulating the actions of IGF-II within the corpus luteum due to differential binding affinity, whilst other IGFBPs such as IGFBP-3 and -4 may interact predominantly with IGF-I.

These studies have detected limited temporal variation in mRNA levels throughout the oestrous cycle, despite the dramatic changes in luteal growth and function that occur during the oestrous cycle. Evidence from *in vitro* studies support a role for the IGFs in enhancing steroidogenic activity. However, only IGF-I mRNA levels varied significantly with day of the oestrous cycle, whilst concentrations of mRNA encoding IGF-II and the type 1 IGF receptor showed no correlation with changes in

steroidogenic activity. The bioactivity of IGF-I and –II can be further modified by changes in levels of IGFBPs. However, IGFBP mRNA concentrations did not vary with day of the oestrous cycle.

The lack of a correlation between major luteal events and alterations in steady-state mRNA levels might imply a lack of critical function. However, *in vitro* evidence supports an important role for the IGFs *in vivo* (Poretsky *et al.*, 1999; Spicer and Echternkamp, 1995). A reduction in IGF-bioavailability following the addition of exogenous IGFBPs to ovarian cells in culture and the subsequent sequestration of IGFs had dramatic effects on steroid production (Mason *et al.*, 1998; Spicer *et al.*, 1997; Ui *et al.*, 1989), cell proliferation (Bicsak *et al.*, 1990), ovulation rate and oocyte maturation (Yoshimura *et al.*, 1996), and apoptosis (Chun *et al.*, 1994).

GnRH-administration on day 6 of the oestrous cycle results in ovulation of the first wave dominant follicle and subsequent formation of a secondary luteinized structure (see Chapter 5). Removal of the spontaneous CL has previously demonstrated that the GnRH-induced CL does secrete progesterone. However, the steroidogenic activity of the induced CL falls below that of a spontaneously formed CL of a similar age. Ovulation of the first wave dominant follicle and formation of an induced CL has been demonstrated in response to the administration of hCG, synthetic GnRH or GnRH-agonist. However, peripheral progesterone concentrations were greater following hCG treatment than GnRH-agonist treatment (Schmitt *et al.*, 1996a, 1996b). This may be due to differences in LH-like exposure induced by the two treatments. Human chorionic gonadotrophin has a markedly slower clearance rate than LH, and hence an increased plasma half-life. In addition, hCG bound to the LH receptor is internalised much slower than LH bound to its receptor resulting in

extended stimulation. The increase in plasma progesterone concentrations in response to hCG may be the result of increased stimulation of the original CL or greater differentiation of the induced CL.

There may also be differential effects of hCG and GnRH-agonist on luteal morphology. Alterations in the size distribution of steroidogenic luteal cells could potentially affect the luteal response to both LH-like stimulation and $PGF_{2\alpha}$ binding, since large luteal cells contain the majority of $PGF_{2\alpha}$ membrane receptors, whilst only small luteal cells respond to LH stimulation with increased progesterone production. The extent of morphological differences induced by GnRH treatment is unknown and warrants further investigation.

In the present study GnRH-induced CL did not exhibit significantly reduced LH receptor mRNA levels when compared to spontaneous CL of similar age, suggesting that a lack of LH reception was not the cause of the limited function of the induced CL. Indeed, Rusbridge (1993) demonstrated that the GnRH-induced CL had increased LH receptor numbers. The cause and biological significance of increased LH receptor concentration observed by Rusbridge remains unclear (1993), however, it was suggested that progesterone production by the endogenous CL may have been responsible for the premature induction of LH receptors. The reason for the discrepancy in LH receptor concentration assessed by LH-binding (Rusbridge, 1993) or detection of mRNA encoding the LH receptor is unknown.

Evidence from the GnRH-induced CL demonstrates that significant alterations to the luteal IGF system are correlated with reduced functional capacity (see Chapter 6). Increased IGFBP-3 mRNA concentrations and decreased type 1 IGF receptor mRNA concentrations are expected to result in reduced bioavailability and bioactivity of

IGF-I and –II. The resultant reduction in IGF-stimulation may compromise the response of the GnRH-induced CL to luteotrophic support, and thus contribute to its limited steroidogenic function. Abnormal luteal function such as that exhibited by the GnRH-induced CL may also reflect a lack of suitable follicular maturation prior to CL formation. A reduction in progesterone secretion has critical implications for subsequent fertility, since low luteal phase progesterone concentrations are detrimental to the successful maintenance of pregnancy (Mann *et al.*, 1999).

The current studies were designed to determine the local production of components of the IGF system, and were therefore limited to detection at the RNA level. Determination of IGF and IGFBP protein concentrations would reflect changes in both local production and transudation from plasma. Any resultant changes in IGF bioavailability and bioactivity are beyond the scope of this work, but would be a valuable extension of the present studies. Indeed they may even provide insights into the relative importance of endocrine versus autocrine/paracrine IGF stimulation of luteal function.

IGFBPs are subject to proteolytic degradation (Fowlkes, 1997; Maile and Holly, 1999). IGFBP-specific proteases have been identified within follicular fluid, and their ability to alter IGF-bioavailabilty related to follicular growth (Besnard *et al.*, 1996, 1997; Cwyfan Hughes *et al.*, 1997; Grimes and Hammond, 1994; Mason *et al.*, 1996). IGFBP-4 proteolytic activity has also been identified in luteinized human granulosa cells (Iwashita *et al.*, 1996). However, there has been little research thus far on the role of IGFBP-specific proteases in the regulation of the luteal IGF system. IGFBP- proteases are potentially important regulators of the IGF system within the CL and hence luteal function.

Much of this study is based on results generated by *in situ* hybridisation. *In situ* hybridisation reveals the cellular localisation of specific nucleic acids in cells and tissues, and therefore has the potential to greatly increase our understanding of cellular function. However, the technique does have associated difficulties and limitations.

Accurate identification of cell-types was not always possible in this study, thus limiting the power of the analysis. In order to identify the precise positions of gene transcription target nucleic acids must be immobilised. A number of methods are suitable for the preparation of tissue prior to *in situ* hybridisation, and this study primarily utilised tissue snap-frozen in liquid nitrogen and prepared as cryostat sections. The use of frozen tissue allows good penetration of the probe to the *in situ* target, but the preservation of tissue morphology can be poor. In contrast, paraformaldehyde-fixation and paraffin-embedding stabilises the tissue, but the creation of cross-links can cause problems of probe penetration. Paraffin sections were used to detect luteal IGFBP-2 mRNA expression, and tissue integrity was much improved without increased problems of probe accessibility. Future studies are likely therefore to use fixed tissue sections for improved identification of the cellular sites of expression. Further improvement might also be achieved by a reduction in the thickness of tissue sections which may have contributed to limited resolution.

In the follicle, theca and granulosa cells are physically separated into distinct layers by the basement membrane allowing for easy cell identification, in contrast, the corpus luteum is a complex tissue comprised of a number of different cell-types which display a heterogeneous distribution. In order to distinguish more precisely between different cells, specific markers of cell-type can be detected by

immunohistochemistry. Cell-specific markers have previously been used in the corpus luteum to identify steroidogenic cells, endothelial cells and a number of immune cells (Jablonka-Shariff *et al.*, 1993; Penny *et al.*, 1999). Immunolocalisation performed in conjunction with the standard *in situ* hybridisation protocol, either on the same or parallel slides, could be used in subsequent studies to more accurately determine the sites of mRNA expression.

In tissues where the expression of a specific mRNA is restricted to a small sub-population of cells *in situ* hybridisation may prove more sensitive than techniques which rely on the extraction of RNA by tissue disruption. However, quantification of selected fields of view may prove to be unrepresentative if the pattern of hybridisation is non-uniform. In this situation techniques such as RNase protection assays or Northern blotting may provide a more representative approach to quantitative analysis. In addition, these methods have the further advantage that they allow for both relative and absolute quantification of mRNA concentrations. The analysis of mRNA expression levels by *in situ* hybridisation may also be subject to more bias than RNase protection assays or Northern blotting, since the investigator must locate the fields of view to be quantified.

The accuracy of quantification can also be affected by the process of exposure and development. In order for expression levels to be compared it is assumed that a doubling of hybridisation signal reflects a doubling in target mRNA, however, the response of photographic film or emulsion to exposure is not a simple linear one. By exposing slides to emulsion or gels to film for varying times an appropriate length of exposure can be chosen to ensure that readings are taken from the linear part of the

sigmoid curve, and that saturation has not been reached. The linearity of signal is also influenced by the specific activity of the probe hybridised to the RNA target.

In spite of potential limitations, *in situ* hybridisation studies have extended our knowledge of the spatial patterns of mRNA expression for components of the IGF system. Whilst it is clear that there is much still to be learnt about the complex interactions of the IGF system within the CL, the work presented here supports a role for the modulation of luteal function by locally produced IGFs. In general terms, much of our understanding of the importance of IGF-I and –II to luteal function relies heavily on information gained from follicular studies, and IGF-actions in the follicle are extrapolated to the CL, often with little direct evidence. Clearly, the luteal targets of IGF-stimulation should be the subject of further detailed study, and should consider IGF effects on both the steroidogenic and non-steroidogenic components of the CL. However, collectively the data in this thesis have demonstrated that the bovine CL is a site of IGF production, reception and regulation.

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APPENDIX.

Porcine IGF-I and 18S ribosomal RNA.

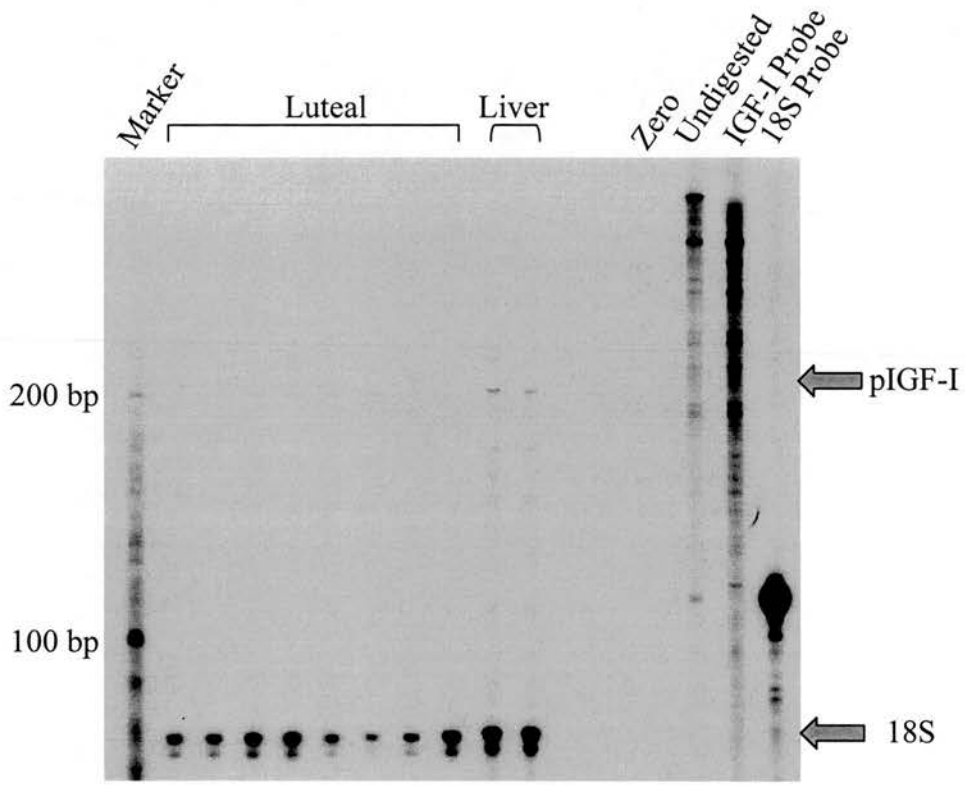

Figure I.
The protection of ^{32}P-labelled porcine IGF-I and 18S ribosomal RNA probes with total RNA (25μg) isolated from bovine luteal and liver tissue.

Protected hybrid of size 80 base pairs was detected with the 18S ribosomal RNA riboprobe. Full length porcine IGF-I probe (472 bp) was not protected in bovine tissue due to mismatches. Undigested probes for 18S and pIGF-I are also shown.

Bovine IGF-I

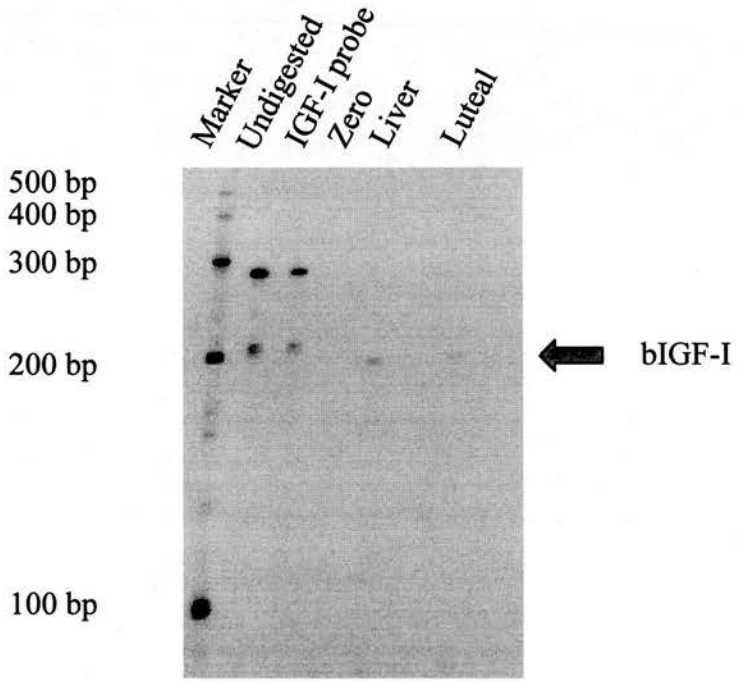

Figure II.
The protection of ^{32}P-labelled bovine IGF-I RNA probe with total RNA (25μg) isolated from bovine luteal and liver tissue.

Protected hybrid of size 196 was detected with a bovine IGF-I probe. Undigested probe of size 285 base pairs is also shown.

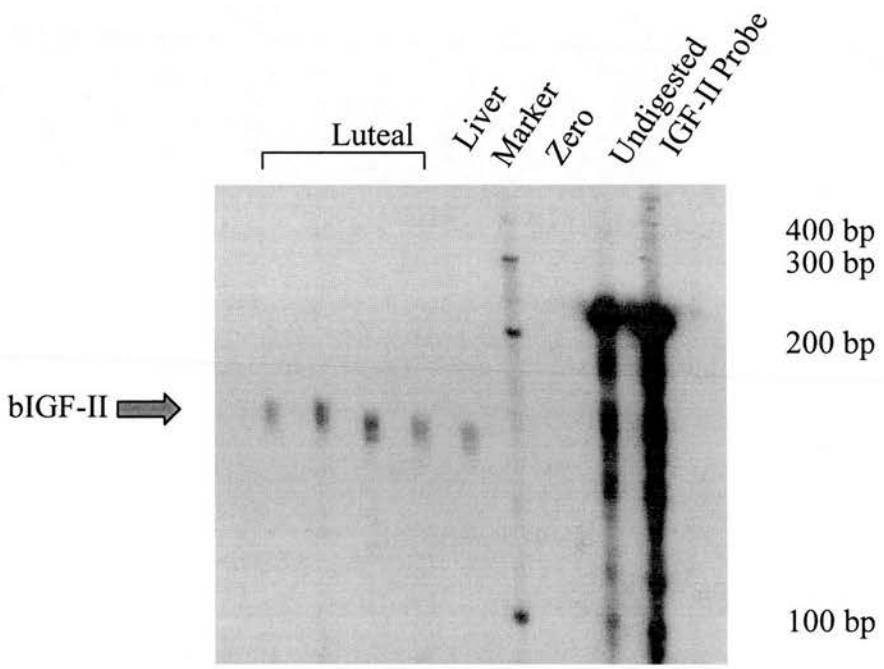

Figure III.
The protection of ^{32}P-labelled bovine IGF-II RNA probe with total RNA (25μg) isolated from bovine luteal and liver tissue.

Protected hybrid of size 154 base pairs was detected with a bovine IGF-II probe. Undigested probe of size 242 base pairs is also shown.

Bovine type 1 IGF receptor, IGFBP-3 and 18S ribosomal RNA.

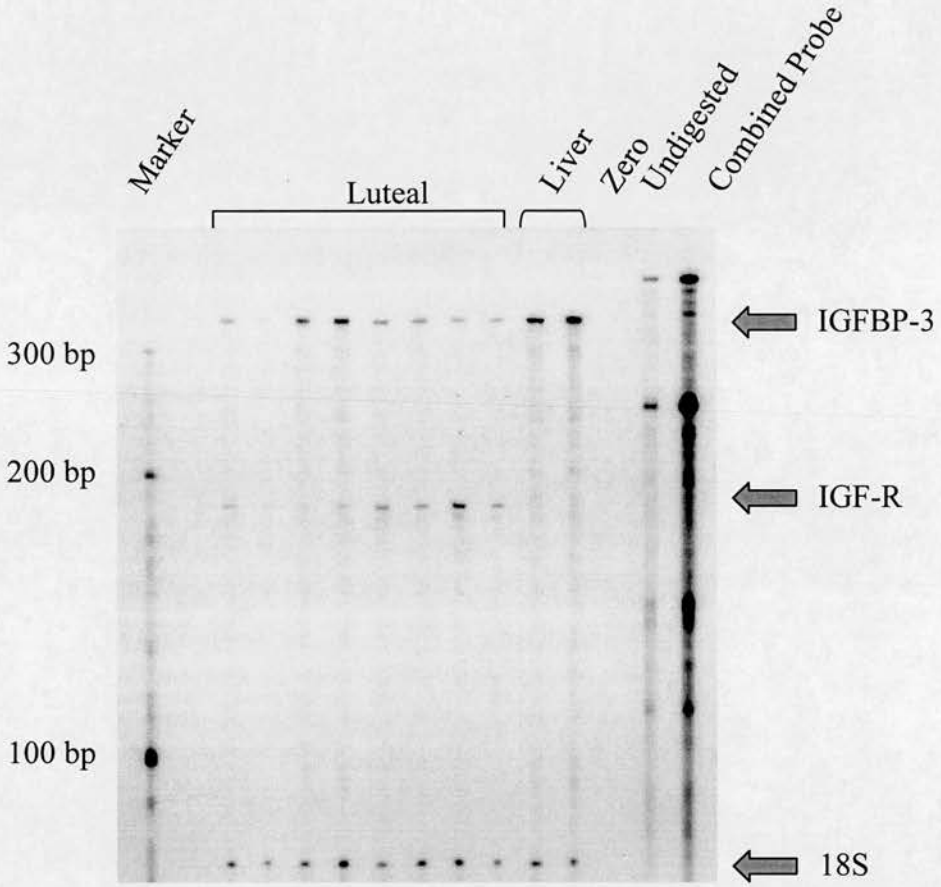

Figure IV.
The protection of ^{32}P-labelled bovine type 1 IGF receptor , IGFBP-3 and 18S ribosomal RNA probes with total RNA (25µg) isolated from bovine luteal and liver tissue.

Protected hybrids of size 189, 358 and 80 base pairs were detected with type 1 IGF receptor (IGFr), IGFBP-3 and 18S ribosomal RNA probes respectively. Undigested probe of sizes 251 and 433 base pairs for the type 1 IGF receptor and IGFBP-3 are also shown.

IGFBP-4 and 18S ribosomal RNA

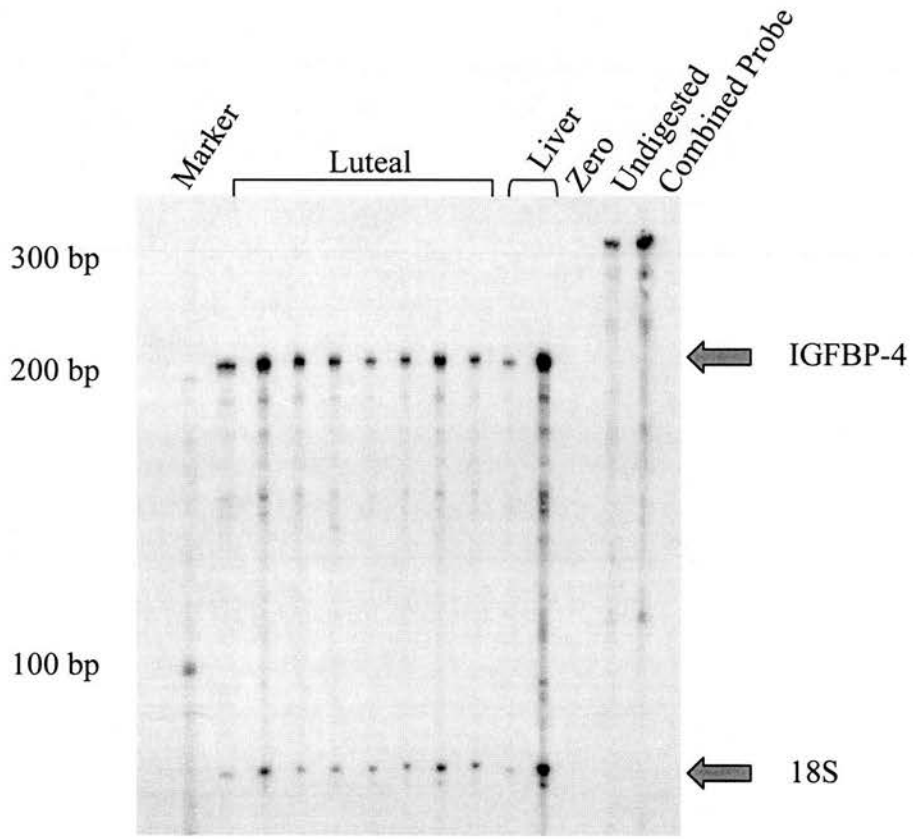

Figure V.
The protection of ^{32}P-labelled bovine IGFBP-4 and 18S ribosomal RNA probes with total RNA (25μg) isolated from bovine luteal and liver tissue.

Protected hybrids of size 227 and 80 base pairs were detected with IGFBP-4 and 18S ribosomal RNA probes respectively. Undigested probe of size 326 base pairs for the IGFBP-3 probe is also shown.

BUFFERS

Denaturing solution (100ml)

47.264g guanadine thiocyanate (4M)

10ml 250mM trisodium citrate (25mM)

Adjust to pH 7 with NaOH (5M), filter through 0.22μm nylon bottle top filter and store in the dark.

Denhardt's solution (1x)

0.02% (w/v) Ficoll

0.02% (v/v) polyvinylpyrolidone

0.02% (w/v) bovine serum albumin (BSA)-Fraction V

L. agar plates with ampicillin (per litre)

10g Bacto-Tryptone

5g Bacto-Yeast extract

5g NaCl

15g agar

Adjust pH to 7.5 with NaOH, and autoclave.

Remelt in microwave when required, and allow to cool to 55°C before adding ampicillin. For liquid media omit the agar.

MOPS (10x)

0.2M 3-[N-Morpholino]propanesulfonic acid (MOPS)

0.05M NaAc pH7

0.01M EDTA

Paraformaldehyde 4% (w/v)

Add 80g paraformaldehyde to 1000ml DEPC H_2O,

heat to 60-70°C until the paraformaldehyde dissolves,

add a few drops of 2M NaOH until the solution clears,

make up to 2000ml with 200mM PBS (pH 7.4) and

adjust to pH 7.4 .

PBS (1x)

0.05M $NaPO_4$ pH7.5

0.9% (w/v) NaCl

Phosgel assay buffer

18g NaCl

2g swine skin gelatin

200mg thimerosal

200ml 0.5M PO_4 buffer (pH7.5)

Proteinase K buffer (10x)

0.1M Tris pH7.8

0.05M EDTA

5% (w/v) SDS (warm to dissolve).

Reverse transcription mix

42µl 50mM $MgCl_2$,

42µl 10x RTase buffer,

42µl dNTP mix (each at 10mM; Promega),

12µl RTase Superscript II (25U/µl; Life Technologies) and

add dH_2O to 200µl.

SSC (20x)

3M NaCl

0.3M sodium citrate

adjust to pH7

SSPE (20x)

3M NaCl

0.2M $NaPO_4$ pH7

0.02M EDTA (disodium salt)

TAE (Tris/acetate/EDTA) electrophoresis buffer (50x)

242g Tris base

57.1 ml glacial acetic acid

100 ml 0.5M EDTA pH8

final working concentration 40mM Tris acetate, 1mM EDTA.

TBE (Tris/borate/EDTA) (10x)

108g Tris base (890mM)

55g boric acid (890mM)

40ml 0.5M EDTA pH8 (20mM)

TE buffer

10mM Tris-HCl pH 7.5

1mM EDTA

TM buffer

50mM Tris-Cl, pH 7.5

10mM $MgSO_4$

ANTIBODY DATA

Anti-progesterone (rabbit) antibody; SAPU product S235-201

Cross reactivities;

11 Hydroxyprogesterone	< 300%
17 Hydroxyprogesterone	< 5%
11 Deoxycorticosterone	< 10%
5-α-Pregnan 3,20 dione	< 20%
Hydrocortisone	< 0.25%

SUPPLIERS

Aloka Company Ltd

Tokyo, Japan.

Amersham Pharmacia Biotech UK Ltd

Little Chalfont, Buckinghamshire, UK.

AMS Biotechnology (UK) Ltd

Abingdon, Oxfordshire, UK.

Appligene Oncor

Watford, Hertfordshire, UK.

Beckman Instruments (UK) Ltd

High Wycombe, Buckinghamshire, UK.

Bibby Sterilin

Stone, Staffordshire, UK.

Biometra Ltd

Maidstone, Kent, UK.

Bio-Rad Laboratories Ltd

Hemel Hempstead, Hertfordshire, UK.

Coopers Animal Health Ltd

Crewe, Cheshire, UK.

CP Laboratories

Bishop's Stortford, Hertfordshire, UK.

Cruachem

Glasgow, UK.

Fisher Scientific UK Ltd

Loughborough, Leicestershire, UK.

Flowgen

Ashby de la Zouch, Leicestershire, UK.

GCG (Genetics Computer Group)

Madison, Wisconsin, USA

Intervet Laboratories Ltd

Cambridge, UK

Kamar Inc

Steamboat Springs, Colorado, USA.

Life Technologies

Paisley, UK.

Merck Ltd

Poole, Dorset, UK

National Diagnostics

Hessle, Hull, UK.

New England BioLabs (UK) Ltd

Hitchin, Hertfordshire, UK.

Promega

Southampton, Hampshire, UK.

Qiagen

Crawley, West Sussex, UK.

Roche Diagnostics Ltd

Lewes, East Sussex, UK.

Sanofi Animal Health Ltd

Watford, Hertfordshire, UK.

SAPU (Scottish Antibody Production Unit)

Law Hospital, Carluke, UK.

Shandon Scientific Ltd

Runcorn, Cheshire, UK.

Sigma –Aldrich Company Ltd

Poole, Dorset, UK.

Whatman International Ltd

Maidstone, Kent, UK.

HA West (X-ray) Ltd

Edinburgh, UK.