Analysis of the murine granulosa cell transcriptome during luteinisation

A thesis presented for the degree of Doctor of Philosophy in the Faculty of Veterinary Medicine, University of Glasgow

By

Robert Scott McRae

Division of Cell Sciences
University of Glasgow Veterinary School
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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Declaration

I hereby declare that this dissertation, submitted in fulfilment of the requirements for the degree of Doctorate of Philosophy and entitled "Analysis of the murine granulosa cell transcriptome during luteinisation", represents my own work and has not been previously submitted to this or any other institution for any degree, diploma or other qualification.

Robert Scott McRae

January 2006

Acknowledgements

This thesis is by far the most significant scientific accomplishment in my life. It far surpasses my childhood scientific studies into life expectancy following limb detachment in multileged arthropods, and even ranks above my more recent studies into the change in rate of return of edible delicacies following differing approaches to pet euthanasia.

This work would have been impossible without people who supported me and believed in me. It is the result of several years of work throughout which I have been accompanied and supported by many people.

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For me personally this study has provided an invaluable escape from a lifetime of working with sick animals, except of course those I am now proud to call my friends and colleagues.

Finally in the absence of a beloved wife, darling children, recently deceased relatives, or indeed any surviving pets I dedicate this thesis to the local Chinese takeaway whose culinary support was invaluable during the many late nights I spent completing work I could not be bothered doing during the day.

Abstract

The granulosa cells of the ovarian follicle surround the oocyte and support it during follicle development. Once exposed to the LH surge, the granulosa cells are characterised by the induction of genes necessary for cellular differentiation. The extensive morphological and functional changes which characterise luteinisation involve the regulation of gene and protein expression responsible for the cessation of proliferation and the induction of differentiation in the individual granulosa cells. The differentiating granulosa cell also functions in both an endocrine and paracrine manner mediating follicle and oocyte maturation and subsequent corpus luteum remodelling. The formation of the functional corpus luteum and secretion of progesterone is essential for the establishment of pregnancy following ovulation. Although much is known about the molecular mechanisms responsible for follicular development comparatively little study has been carried out to analyse the control of, and events which occur during, luteinisation. It is therefore pertinent to study gene expression changes to try to clarify and understand mechanisms which regulate and underpin ovarian granulosa cell luteinisation.

In order to investigate the mechanisms underlying these processes we embarked on a time- and cell-specific analysis of gene expression in the granulosa cell during late follicle development and early luteinisation. Changes in gene expression during granulosa cell luteinisation were measured using serial analysis of gene expression (SAGE). Immature normal mice were treated with gonadotrophin to induce formation and luteinisation of ovarian follicles. SAGE libraries were generated from mRNA isolated from granulosa cells collected before and after induction of luteinisation. The combined libraries contained 105,224 tags representing 40,248 unique transcripts. Overall, 715 transcripts showed a significant difference in abundance between the two libraries of which 216 were significantly down-regulated by luteinisation and 499 were significantly up-regulated. Among transcripts differentially regulated, there were clear and expected changes in genes involved in modelling of the extracellular matrix, regulation of the cytoskeleton and intra and intercellular signalling. Also identified were transcripts relating to genes and cellular signalling pathways novel to the granulosa cell, including members of the E2F family of cell cycle regulators and the Notch signalling pathway as well as genes implicated in angiogenesis and cellular metabolism not previously associated with the granulosa cell.

Further studies into an unmatched SAGE tag which was highly differentially expressed revealed that it represented a variable length non-coding transcript which showed a tissue- and temporal-specific expression pattern within granulosa tissue. This transcript is highly conserved across species and lies distal to the 3' end of the inhibin βA subunit. Highest levels of expression were found within the gonadotrophin-stimulated, mature antral follicle prior to the LH surge where it was the 6th most highly expressed transcript. After luteinisation there was a rapid downregulation of expression. It is suggested that this transcript may have involvement in regulating the transcription and/or

translation of the inhibin βA subunit during follicle development and luteinisation.

In conclusion, this thesis provides insight into some of the important mechanisms involved in the regulation of luteinisation, namely angiogenesis, differentiation, cell cycle control and the metabolic machinery within the granulosa cell. We have isolated a large number of candidate genes related to the cellular differentiation processes occurring within the granulosa cell during luteinisation. The data generated and presented here constitutes a new base for the testing of hypotheses in the field of follicle development and luteinisation.

Publications

McRae RS, Johnston HM, Mihm M, and PJ O'Shaughnessy, (2005) Changes in mouse granulosa cell gene expression during early luteinisation. Endocrinology 146(1):309-317.

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Introduction

Mammalian folliculogenesis and luteinisation

1. The Mammalian ovary

The major functions of the female gonad are endocrine support of sexual development and reproductive function, prenatal multiplication of the germ cell line and germ cell storage and release during the postnatal reproductive lifespan.

1.1 Historical perspective

The recognition of a role for
the ovary as the major organ for reproductive function began in the 3rd century BC with the hypothesis put forward by Herophilus that the ovary was the source of female semen. It took until the work of Galen, in the second century AD, to recognise the transmission of this 'semen' through the fallopian tubes to the uterus. Fifteen hundred years later came the first proposition of the role of the oocyte was made by William Harvey with his treatise, *Exercitationes de Generatione Animalium* (1651) proposing the concept *ex ovo omnia*, that all living things come from eggs, a deduction inspired largely by his work in the avian field. The first recording of the ovarian follicle by van Horne, and further identification and investigation of the tertiary follicle by Regnier de Graaf in the late 17th century led to the proposition that the follicle itself was the mammalian egg, although this was never reconciled with the problem of transmission of this egg through the much narrower fallopian tube to the uterus. The corpus luteum was first accurately depicted in de Graaf's publication "De Mulierum Organis Generationi Inservientibus Tractatus Novus" of 1672 in which he proposed that the corpora lutea are transient and provide an estimation of the number of embryos. The discovery of the oocyte within the fallopian tube was made in 1797 by William Cruickshank, and in 1827 the origin of the oocyte was identified by von Baer. This can be regarded as the start of the accurate elucidation of the cellular structure of the Graafian follicle.
Although the follicular lineage of luteal tissue was not conclusively determined until the latter half the 20th century its function was unravelled much earlier. In 1897 John Beard proposed that the corpus luteum inhibits ovulation during

gestation and degenerates before parturition (as cited by Amoroso 1968). Although corpora lutea were referred to as glands by Malpighi, de Graaf, and Fabricius, Prenant (1898) was the first to suggest that the corpus luteum was an endocrine gland. He wrote "As for the physiological role of the corpus luteum there can be no doubt from a study of its histological appearance that it acts as a gland of internal secretion, releasing one or more products into the bloodstream" (as cited by Short, 1977). In 1903 Frankel found that removing the ovaries of pregnant rabbits terminated pregnancy and thus provided evidence for Gustav Born's hypothesis that corpora lutea are required for implantation (as cited by Amoroso, 1968). Further investigation into the internal secretion of the corpus luteum culminated in the purification of progesterone by several research groups (Allen and Wintersteiner, 1934; Butenandt and Westphal,1934; Hartman and Wettstien, 1934; Slotta et al, 1934).

1.2 Development, anatomy and physiology of the ovary

1.2.1 Gonadal development and sexual differentiation

The undifferentiated gonad develops during early foetal life on the cranioventral mesonephros. This early gonad has the ability to differentiate into either testes or ovary as determined by the genetic sex of the individual. By embryonic day 10 in mice it is recognisable as the gonadal ridge on the ventral mesonephros. Sexual differentiation is initiated by embryonic day 11.5 (Viger et al 2005).

The germ cells themselves arise extra-gonadally and migrate to the developing gonad. Different origins have been suggested for the primordial germ cells but a recent study suggests the posterior primitive streak as the likely source (Anderson et al 2000). In mice, germ cell migration is complete by embryonic day 10.5 (McLaren, 2003).

The developing gonad is covered by coelomic epithelium. The granulosa cells of the ovary arise from cell populations within the region of the developing gonad, which in the male, become the Sertoli cells of the seminiferous tubules. In both sexes the cellular function is homologous: to nurture and aid maturation of the

germ cells and to mechanically discharge them into the genital duct system. The precise origin of the somatic cells is controversial, the coelomic epithelium, local mesenchymal cells and mesonephros have all been proposed as potential sources. It has been suggested, on the basis of immunohistochemical detection of steroidogenic factor 1, SF-1 (Hatano et al.,1996) and ultrastructural stereological studies of calf embryos (Wrobel and Süβ, 1999) that the steroid producing cells of the gonads and adrenal gland develop from the same cell population, an adreno-genital primordium that originates in the mesonephros. This is supported by the observation that when sexually undifferentiated female gonads were inoculated into nude mice, only those with attached mesonephros developed normal follicles, indicating the importance of the mesonephros in follicle development (Byskov,1974; Byskov et al., 1977). Various cellular markers have been used to trace the origin of granulosa cells including the ligand receptor pair SCF-c-kit and cytokeratin (Tisdall et al 1999, Löffler et al 2000). Both these studies support the notion that the first generation of granulosa cells develop from the mesonephros, but do not exclude the possibility of an origin in the surface epithelium, which is supported by the work of Sawyer et al (2002). In the male, the Sertoli cell is known to develop from the coelomic epithelium (Karl and Capel 1998) but an analogous situation in the female has yet to be proven. The importance of the origin of the granulosa cell is debatable since it has been shown that somatic cells from newborn rat ovaries mixed with oocytes from newborn mouse ovaries and implanted underneath the kidney capsule develop into hybrid follicles of rat granulosa cells and mouse oocytes (Eppig and Wigglesworth 2000) which suggests that the oocyte may have the ability to exert influence on sufficiently undifferentiated somatic cells to develop into granulosa cells.

Sexual differentiation of the gonad becomes evident in male mice around embryonic day 12.5 with the development of testicular cords (Upadhyay et al., 1981), initiated by sex determining region Y (*Sry*) expression on the Y chromosome (Gubbay et al 1990; Sinclair et al 1990; Koopman et al 1991). Ovarian differentiation follows slightly later and was for a time considered the default pathway of development in the absence of expression of testis

determining genes. Ovary determining genes are however now being identified, among them *Wnt-4* (Vainio et al 1999) and *Dax-1* (Swain et al 1998) which lends weight to the argument for a specific gene pathway that drives ovarian development rather than passive differentiation in the absence of testes.

Following gonadal differentiation the proliferating germ cells become concentrated in the cortex and undergo mitosis to give rise to oogonia, which continue to proliferate. Shortly thereafter meiosis is initiated in the oogonia and they become oocytes, surrounded by granulosa cells and stroma, and as such are regarded as primordial follicles. These primordial follicles were thought to be the source of all developing follicles during the reproductive lifespan. However recent evidence has shown that mammalian ovaries possess persistant large germline stem cells and gives rise to the possibility of follicular renewal in adult females (Johnson et al 2004).

1.2.2 Histological structure of the ovary

The mammalian ovary consists of three distinct regions, the hilum, which contains the entry point for the blood vessels, the medulla, containing a heterogeneous group of cells, and the cortex, the dominant zone, lined by germinal epithelium and containing the oocytes. In the mature cycling, fertile, female follicle development is ongoing and a variety of stages of developing and atretic follicles may be present at any one time. In addition, depending on the species and stage of the ovarian cycle, active or regressing copora lutea may be present. (figure 1.1)

The outermost layer covering the ovary consists of germinal epithelium, directly underneath this there is a dense layer of connective tissue known as the tunica albuginea. The ovarian follicles, in conjunction with surrounding fibroblasts, collagen and elastic fibres, form the ovarian cortex located under the tunica albuginea. The ovarian medulla contains the blood vessels, lymphatic vessels and nerve terminals.

The ovary is an organ of constantly fluctuating populations of cells due to continual growth and regression of follicles and corpora lutea. These

developmental processes within the ovary involve cell proliferation (Reynolds and Redmer 1998) and differentiation (Chang et al 1977, Anderson and Little 1985), angiogenesis (McClellan et al 1975) and cell death (Ingram 1962). Normal ovarian function in mammalian species requires that during every oestrus cycle only a limited number of follicles reach the stage of Graafian follicles and ovulate. This is essential for the prevention of excess embryos during pregnancy. In each stage of the cycle about 50% of the large preantral and antral follicles will be in the process of apoptotic death (Almog et al 2001), the stimuli for apoptosis or survival can be autocrine, paracrine or

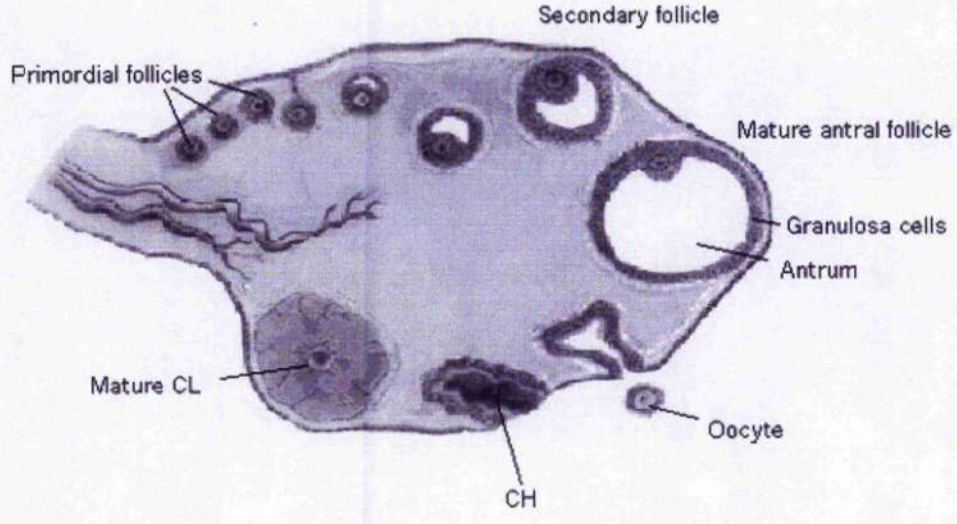


Figure 1.1 Schematic of an ovary depicting the life cycle of a follicle destined to ovulate. The ovum of a mature follicle is situated within the fluid filled antrum on a pedicle of granulosa cells. At ovarian rupture the follicular antrum is filled with blood forming the corpus haemorrhagicum (CH), the clot is resorbed and replaced by lutein cells forming the corpus luteum (CL)

endocrine (Amsterdam et al 1999). The ultimate goal of the successful follicle is a release of the female germ cell, termed ovulation (Hartman 1932, Hisaw 1947, Corner 1963).

1.2.3 Endocrine control of ovarian function

Reproductive function is controlled by a variety of physiological and hormonal factors. The mouse has oestrus cycles of 4-6 days duration (Schwartz 1973), although considerable variation in cycle length has been recorded (Whitten 1958, 1959). Ovulation is spontaneous but in absence of copulation corpora lutea fail to develop (Schwartz 1973, Greenwald and Rothchild 1968).

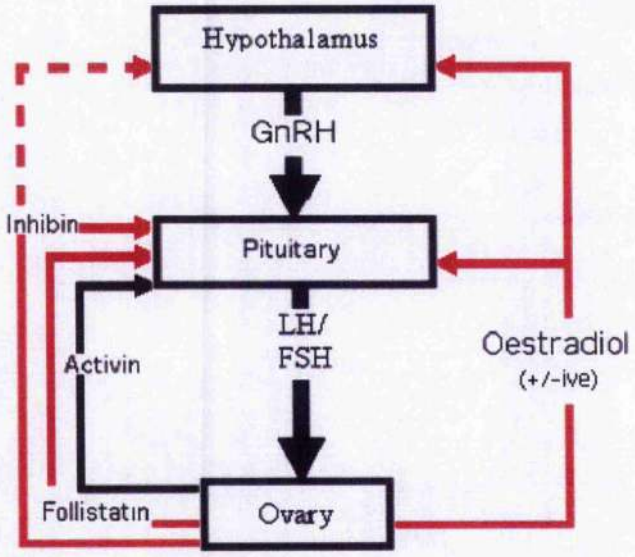


Figure 1.2 Summary of the hypothalamic-adenohypophyseal-ovarian axis. The gonadal steroids exert negative or positive (depending on the stage of the ovarian cycle) feedback at the pituitary level by blocking GnRH action and at the hypothalamic level by inhibiting GnRH release. Separate gonadal products either suppress (inhibin and follisatin) or stimulate (activin) FSH release. (red arrow represents negative effect, black arrow positive effect).

The physiological and behavioural changes associated with the oestrus cycle depend on a complex interaction of endocrine and local factors under the integrative control of the hypothalamus. This hypothalamic-adenohypophyseal-ovarian axis is the system under which normal ovarian function is driven by the pituitary hormones, whose secretions are in turn controlled via the hypothalamic-hypophysiotrophic hormones, and a feedback mechanism of steroid and peptide hormones (figure 1.2). Therefore hypothalamic hormones stimulate the production of hormones from the anterior pituitary that ultimately stimulate

follicle development and ovulation in the ovary. Steroid hormones from the ovary provide a feedback loop at both the hypothalamic and adenohypophyseal level to control hormone secretion.

The oestrus cycle is divided into luteal and follicular phases. The luteal phase in mice starts after ovulation and copulation when the corpus luteum is formed from the wall of the collapsed follicle. Progesterone is the dominant hormone of the luteal phase. The follicular phase starts after regression of the CL, Oestrogen levels increase gradually in the follicular phase in association with follicle growth. The preovulatory follicle produces high levels of oestradiol leading to behavioural changes during oestrus and stimulating gonadotrophin release to induce ovulation.

The main hormones regulating reproductive function that are released from the anterior pituitary are follicle stimulating hormone (FSH) and luteinising hormone (LH), they regulate ovarian function and steroid hormone secretion. FSH is the key regulatory hormone involved in follicle growth and development, LH is the key hormone involved in terminal preovulatory follicle maturation for ovulation and then luteinisation.

Control of gonadotrophin secretion is through release of gonadotrophin releasing hormone (GnRH) from neurones in the hypothalamus. They represent the final output component of the neuronal network that regulates reproductive hormone secretion. The episodic release of GnRH into the hypophyseal portal system creates a pulsatile pattern of LH secretion (Knobil 1980, Levine et al 1991). Fluctuations in this pattern generate the marked changes in the LH secretion profile observed over the course of the ovarian cycle (Freeman 1994, Goodman 1994). The midcycle LH surge, responsible for initiation of ovulation, results from an abrupt and massive increase in hypophyseal GnRH secretion in a number of species (Sarkar et al 1976, Ching 1982, Caraty et al 1989, Moenter et al 1990, Pau et al 1993, Karsch et al 1997). Oestrogen is one of the principle determinants of GnRH neurone function and is critical in enabling these cells to exhibit fluctuating patterns of biosynthetic and secretory activity. For most of the ovarian cycle oestrogen serves to restrain LH secretion partly by inhibition of GnRH secretion (Caraty et al 1989, Sarkar and Kink 1980, Chongthammakun

and Terasawa 1993, Evans et al 1994) and also by direct action on the pituitary gland (Freeman 1994, Goodman 1994, Shupnik 1996). Oestrogen also exhibits positive feedback influence on GnRH neurones and pituitary gonadotrophs to generate the LH surge. The rising follicular concentrations of circulating oestradiol can on their own, or in combination with circadian input, trigger an LH surge (Moenter et al 1990, Karsch et al 1997, Sarkar and Fink 1980, Xia et al 1992, Everett and Sawyer 1950, Legan et al 1975).

The gonadal peptide hormones, inhibins and activins, produced by the granulosa cells in the female and sertoli cells in the male, comprise an endocrine feedback loop to the pituitary. Inhibins and activins are named for their effects which inhibit and activate respectively pituitary production of FSH. Their importance in regulating reproductive function is demonstrated by mice null for the inhibin α subunit, and thus deficient in inhibins, developing mixed granulosa/sertoli cell tumours in the ovaries and testes (Matzuk et al 1992).

The pattern of events that occur during ovulation is initiated in a responsive preovulatory follicle by a surge of LH, which induces both theca cells and granulosa cells to stimulate cAMP production and activate selected protein kinase signalling cascades (Richards 1994, 2001, Richards et al 2000). These pathways induce the transient transcription of specific genes prior to follicle rupture that induce ovulation and promote follicular remodelling to form a corpus luteum. Although the principal effects of the hormones LH, FSH, oestrogen and progesterone on the ovary are known, their precise functions and interplay are still not clear. Moreover, it is becoming increasingly apparent that other hormones, growth factors and cytokines are involved in the fine-tuning of ovarian function. A complete intraovarian paracrine system is implied in follicular growth and maturation (Findlay 1994, Chabbert-Buffet et al 1998). Local regulation of ovulation and luteinisation involves the interaction of LH and intrafollicular factors including steroids, prostaglandins and peptides derived from endothelial cells, leukocytes, fibroblasts, and steroidogenic cells.

1.3 The Gonadotrophic hormones and receptors

Follicle stimulating hormone (FSH) and luteinising hormone (LH) are glycoproteins whose release from the gonadotroph cells of the anterior pituitary is stimulated by gonadotrophin releasing hormone (GnRH) from the hypothalamus. The pleiotrophic effects of gonadotrophins are manifest in various cells of the reproductive system including LH and FSH in ovarian granulosa cells, LH in theca interna cells, FSH in testicular Sertoli cells, and LH in Leydig cells (Sprengel et al 1990, Amsterdam et al 1992, Segaloff and Ascoli 1993). Both hormones are heterodimers that contain a common α subunit and dissimilar β subunits that confer biological specificity on the individual hormones. The two subunits are linked by non covalent interactions stabilised by a β cysteine loop (Lapthorn et al 1994, Wu et al 1994). Structurally these hormones are members of the superfamily of cysteine-knot growth factors which also include transforming growth factor β (TGF β). The gonadotrophic receptors bind only the intact heterodimeric hormone, the individual subunits having no binding activity (Catt et al 1973)

Follicle stimulating hormone plays a central role in the regulation of follicle growth and survival (Hillier 2001), it is essential for normal folliculogenesis and fertility (Kumar et al 1997 and 1998). The hormone interacts with receptors expressed exclusively on granulosa cells in the ovary and it initiates cytodifferentiation and proliferation which ultimately results in the development of the preovulatory follicle (Camp et al 1991, Erickson 1983, Hirshfield 1991). The physiological importance of this can be demonstrated by the death of follicles by apoptosis when FSH action is restricted (Hseuh et al 1994, Tilly 1996). Follicle growth from the secondary to late antral stage is dependant on FSH. In the ovary, FSH induces LH receptor expression in small and medium follicles, the LH surge promotes the maturation of follicular cells and enhances the subsequent stages of follicular development and steroidogenesis in granulosa and luteal cells (Richards and Hedin 1988), acting to induce ovulation and luteinisation of the mature antral follicle.

The ability of FSH to modulate ovarian function depends not only on the circulating levels of hormone, but also on the expression of appropriate receptor proteins by the granulosa cells of the ovary. Both FSHR and LHR are structurally related members of the seven transmembrane domain G-protein associated receptor superfamily (Sprengel et al 1990, MacFarland et al 1989) The gene encoding the FSHR protein is encoded by the first 9 exons of the FSHR gene giving rise to a highly specific FSH binding protein (Huhteniemi 1994). Gonad specific expression of FSHR mRNA (Camp et al 1991, Zeleznik et al 1974, Nimrod et al 1976) and in situ hybridisation has shown FSHR mRNA localised exclusively within the granulosa cells of healthy follicles, persisting throughout preovulatory folliculogenesis.

The gene encoding the LHR protein contains 11 exons and variable transcription leads to multiple gene splice variants (Segaloff et al 1990 and 1993). The LHR is expressed primarily in gonadal tissues and has been studied extensively in Leydig and ovarian cells and in cell membrane preparations from several species, including rat, mouse and pig (Richards and Hedin 1988, Dufau 1988, Saez 1994). The presence of LHR on thecal/interstitial cells has been demonstrated as has their induction in granulosa cells by FSH (Zeleznik et al 1974, Magoffin et al 1982). In–situ hybridisation has shown LHR mRNA to be located in the thecal cells of immature follicles but in both thecal and granulosa cells of mature antral follicles (Camp et al 1991). The receptor is composed of two functional units, the extracellular hormone binding domain (Tsai-Morris et al 1990, Xie et al 1990) and the seven membrane/transmembrane cytoplasmic module, which is the anchoring unit that transduces the signal initiated in the extracellular domain and couples to G proteins (Tsai-Morris et al 1993, Wang et al 1993).

The connections between the gonadtrophin receptors and specific signal transduction pathways within the granulosa cell will be discussed later (section 1.5.2.2).

1.4 Endocrine feedback from the granulosa cell.

1.4.1 Oestrogen

The endocrine actions of oestrogen are wide ranging and are manifest in a number of tissues outwith the reproductive system including bone, liver, and brain. The influence of oestrogen is known to be important for processes such as bone metabolism, cardiac and vascular function, neuroprotection, and the modulation of immunity (Srivastava et al 2001, Mendelsohn and Karas 1999, Migliaccio et al 1996, Simoncini et al 2000, Wilder 1998, Manolagas 2000, Mathews et al 2000, Wise et al 2001).

Its influence on the reproductive process is profound, via direct actions on the hypothalamic neurones and pituitary cells (McEwan and Alves 1999) it can control the rate of synthesis and secretion of nearly all pituitary hormones, including FSH and LH (Fink 1988, Gharib et al 1990). As already mentioned positive oestrogen feedback, which drives the ovulatory process, occurs at the time of the preovulatory surge of LH, while negative feedback is shown in the luteal and early follicular phases of the reproductive cycle. Other reproductive functions include its actions on mammary tissue, both directly (Migliaccio et al 1996, Razandi et al 2000) and via prolactin to promote mammary development and lactation (Maurer et al 1990), and on the female reproductive tract where it acts to stimulate cell proliferation and progesterone receptor synthesis (Horowitz et la 1978).

1.4.2 Inhibin/Activin/Follistatin

Inhibin and activin are members of the transforming growth factor β family and have a considerable role in the regulation of FSH production. The inhibin α, β_A, and β_B subunits dimerise to give rise to the inhibin and activin isoforms (Ying 1988, Knight and Glister 2001). Inhibins suppress FSH and activins stimulate FSH (Ying 1988). A role for inhibins in regulating FSH release is supported by evidence that shows, in cattle, immunoneutralisation of inhibin is associated with elevated circulating FSH and increases in follicular development and ovulation

(Kaneko et al 1995, Kaneko et al 1997, Glencross et al 1994, Bleach et al 1996, Morris et al 1995). Activin secretion in known to increase as follicles increase in size (Newton et al 2002) and has been shown to stimulate GnRH-induced LH secretion from rodent pituitary cells (Weiss et al 1993). Follistatins are the binding proteins of activins and inhibins, binding to activin negates the activity of that ligand (De Winter et al 1996, Phillips and De Krester 1998) while inhibins, bound or unbound, retain their biological activity. Inhibins have the ability to antagonise activin by binding both activin receptors and inhibin binding proteins (Martens et al 1997, Lebrun and Vale 1997, Pangas and Woodruff 2000, Chong et al 2000) and then interfering with activin signalling.
In addition to the gonad, these proteins are also produced at the pituitary level and act in a paracrine manner to regulate gonadotrophin secretion (DePaolo et al 1991, Bilezikjian et al 1994, Mather et al 1992, Padmanabhan 2001)

1.4.3 Gonadotrophin surge attenuating factor (GnSAF)

As early as the 1980s several research groups had identified a product with the ability to alter responsiveness of pituitary cells to GnRH (Ferraretti et al 1983, Littman and Hodgen 1984, Spoelak and Hodgen 1984, Messinis and Templeton 1986). It is now clear that this is a gonadotrophin-stimulated ovarian product emanating specifically from the granulosa cell (Fowler et al 2002). In rodents GnSAF production is FSH-dependant and at its highest levels in early antral follicles. LH has no apparent effect on GnSAF production (Fowler and Spears 2004). In rodents, the wave of atresia due to follicle dominance appears to occur around the mid antral stage of development (Baker et al 2001). The fall in GnSAF production in the antral follicle occurs immediately after that (Fowler and Spears 2004), alongside a rise in the production of oestrogen as follicles mature towards the preovulatory stage (Spears et al 1998). It has been proposed that the role of GnSAF is to regulate LH pulsatility (Fowler et al 2003).

1.5 The Antral Follicle: development, structure and physiology.

1.5.1 Follicular development: primordial to antral follicle

The first primordial follicles are formed in mice around the day of birth, these are the non-renewable dormant follicle stock from which follicle recruitment occurs and, consequently, is depleted throughout the reproductive lifespan. There are between 5750 and 8250 resting follicles per ovary in the mouse (Hirshfield 1991). There is little information about the mechanisms controlling primordial follicle development but it has been established that the transcription factor, the factor in the germ line alpha (FIGα), is required for follicular formation in mice (Soyal et al 2000). From birth to menopause follicles will continually exit the primordial follicle pool and enter the growth phase. A variety of growth and paracrine factors have been indentified during early follicular growth and shown to be expressed in a cell- and stage-specific manner (McNatty et al 2000), although their precise roles are not clear. The transition from follicular quiescence to follicular growth does not appear to be dependant upon gonadotrophins (Peters et al 1973) although hypogonadal mice with a deletion of the gonadotrophin releasing hormone (GnRH) gene have a reduced number of early growing follicles, an abnormality which can be reversed by FSH administration (Haplin and Charlton 1988) and, equally, chronically elevated LH levels cause accelerated depletion of the ovarian reserve (Flaws et al 1997). This implicates some gonadotrophic involvement in follicular recruitment despite the evidence for continued early follicle development in absence of FSHR mRNA (Dierich et al 1998). Local paracrine factors, by contrast, are heavily involved in follicular recruitment. Kit ligand (KL) from granulosa cells, acts on receptors produced by both the oocyte and thecal cells and has been shown to enhance *in vitro* initiation of follicular growth (Parrott and Skinner 1999) while keratinocyte and hepatocyte growth factor produced by thecal cells can stimulate granulosa cell proliferation (Parrott and Skinner 1998). Basic fibroblast growth factor (bFGF) has been localised to the oocyte of primordial and primary follicles (Van Wezel et al 1995) with miminal levels of receptors in the granulosa cells of

primordial follicles rising to maximal levels in the granulosa cells of primary follicles before declining thereafter as the follicles increased in size (Wandji et al 1992), consistent with the idea that secretion of bFGF from the oocyte could activate receptors on adjacent granulosa cells. Follicular development from the primordial follicle stage is a process that involves oocyte enlargement, granulosa cell proliferation and basement membrane synthesis, processes which bFGF has been shown capable of stimulating in vitro (Nuttnick et al 1993, Lavranos et al 1994, Rodgers et al 1995). Another potential mechanism regulating follicle development involves neurotrophic molecules such as nerve growth factor (NGF) and brain-derived neurotophic factor (BDNF) the absence of which has been shown to impair folliculogenesis and delay puberty (Lara et al 1990, Ojeda et al 2000). Equally as important as the production of activating substances is the removal of potential inhibitors of early folliculogenesis. Transforming growth factor β2 is expressed in quiescent but not early growing follicles in primates and has been suggested as an inhibitor of the kit ligand system and, subsequently, of follicular recruitment (Gougeon and Busso 2000, Heinrich et al 1995). Antimullerian hormone (AMH) is highly expressed in the granulosa cells of non-atretic preantral and small antral follicles and has been shown to exert an inhibitory effect on recruitment of primordial follicles into the pool of growing follicles with AMH-null females showing rapid depletion of their primordial follicle stock (Durlinger et al 1999).

When a follicle does begin to grow it will continue to grow and either succeed to ovulation or degeneration (Peters and McNatty 1980). It takes three weeks to grow from the primordial stage to maturity in the mouse (Pedersen 1970). During this time the oocyte enlarges and the surrounding layer of granulosa cells become cuboidal and proliferate to develop into an intermediary and then primary follicle (Fortune and Eppig, 1979). The secondary follicle is characterised by the appearance of a second layer of granulosa cells. Primary and secondary follicular development can take place in the absence of gonadotrophins but the follicles are responsive to, and optimal follicular growth may require, the presence of these hormones (Fortune and Eppig, 1979). Mice lacking growth differentiation factor 9 or bone morphogenic protein 15 form

primordial follicles but do not progress past the primary follicle stage and females are sterile (Dong 1996, Galloway 2000). Somewhat later in folliculogenesis, induced mutations in connexins that are involved in somatic cell interactions and somatic-germ cell interactions arrest development at the primary and early antral stages (Simon et al 1997, Juneja et al 1999)

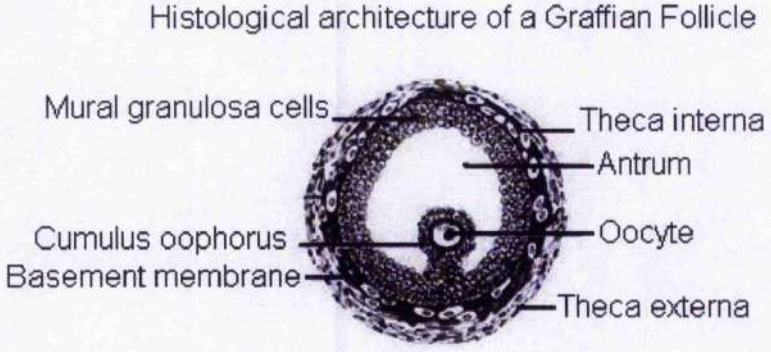


Figure 1.3 The follicular wall consists of three distinct layers, mural granulosa, theca interna and theca externa. The follicular basement membrane separates the granulosa and theca layers.

During the preantral stage, follicles undergo progressive differentiation in addition to substantial growth. The oocyte completes growth and the zona pellucida becomes prominent, whereas theca cells condense and become vascularised outside the developing basement membrane. Granulosa cells proliferate and begin to differentiate relative to their proximity to the basement membrane or the zona pellucida. FIGα has been implicated in the coordinated expression of the three genes (Zp1, Zp2, Zp3) that encode the mouse egg coat (Liang et al 1997) since FIGα null females do not express these genes and the persistence of FIGα expression through folliculogenesis suggest it may play a regulatory role in the expression of multiple oocyte specific genes and regulate additional pathways critical for ovarian development (Soyal et al 2000).

Paracrine signalling within the follicle, which is crucial to emergence of the dominant follicle in species such as humans, brings about the sensitisation of follicles to FSH and LH. Sex steroids, IGF's, and TGF-β are important

components of the follicular paracrine system. Development beyond the antral stage is dependant on gonadotrophins (Halpin et al 1986); in their absence follicles become atretic and disappear.

Differentiation of granulosa cells first occurs when the primordial follicle is recruited to initiate growth. Profound alterations in granulosa cells occur concurrent with morphological transformation. The once flattened epitheloid granulosa cell assumes a cuboidal shape and the cells are shifted into prominent mitoses. At the secondary or tertiary follicular stage, differentiation of granulosa cells becomes FSH dependant, in contrast to the pituitary independent differentiation of earlier stages. Recent studies have demonstrated the expression of another type of FSH receptor other than the widely accepted G_s coupled entity, and its upregulation under hormonal (PMSG) influence during follicular growth (Babu et al 2001). It has been proposed that the receptor may be coupled to a signalling pathway that induces rapid calcium mobilisation and extracellular regulated kinase (ERK) 1 / 2 activation without elevating cAMP (Babu et al 2001). This receptor may serve to stimulate the rapid cell proliferation required for follicular expansion

Some of the physiologically important genes that are induced by FSH signalling in the granulosa cell include cytochrome P450 aromatase (cyp19) luteinising hormone receptor (LHR), steroidogenic acute regulatory protein (StAR), P450 side chain cleavage enzyme (cyp11a1), 3β hydroxysteroid dehydrogenase (3βHSD) and inhibin subunits. (Steel and Leung 1993, Richards et al 1995). This physiological importance is underlined by the fact that when FSH action is restricted the developing follicles die by undergoing apoptosis and there are no ovulations (Hseuh et al 1994, Tilly 1996).

Gonadotrophin stimulation of the granulosa cells in larger follicles is responsible for the secretion of the angiogenic factor VEGF that regulates the development of the blood vessel network within the thecal compartment. Those follicles, which activate VEGF synthesis early, increase their blood vessel extension and activate steroidogenesis, thus pointing to VEGF production as a crucial event in the control of follicular dynamics (Mattoli et al 2001).

The critical role of FSH in follicular development is well established but the effect of the hormone itself is subject to numerous growth factors produced by the follicle that modulate FSH action through autocrine, paracrine and endocrine mechanisms. Increasing evidence supports a role for various systemic growth factors, intraovarian growth factors, or both, including insulin-like growth factors I and II (IGF-I and IGF-II) and their binding proteins (IGF-BPs), epidermal growth factor (EGF) family members (including transforming growth factor alpha: TGFα), and TGFβ superfamily members (including inhibins and activins) as co-regulators of follicle development and will be discussed at length later (section 1.5.2.3). There is increasing evidence that FSH also plays a role in the resumption of meiosis (Dekel and Beers 1978, Downs et al 1998, Singh et al 1997). In the antral follicle, FSH alters the enviroment of the maturing oocyte by stimulating granulosa cell divison and differentiation (Themmen and Huhtaniemi 2000), modulating steroidogenesis (Zhang and Armstrong 1989, Moor et al 1985, Szoltys et al 1994), granulosa cell metabolism (Downs and Utecht 1999, Downs et al 2002) protein synthesis (Singh et al 1997, Moor et al 1985) and inducing cumulus expansion (Eppig 1979). At this time the oocyte undergoes critical cellular events that enable the egg to resume meiosis and complete cytoplasmic maturation.

During late FSH-induced granulosa cell development LH has a stronger effect on cAMP than FSH (Richards 1994, Yong et al 1992b), which would suggest increased LHR density or more effective coupling to cAMP generation. The LH surge triggers the expression of 'high-tone' cAMP response genes leading to terminal granulosa cell differentiation and ovulation (Hillier 2001). The genes activated by LH include interleukin 1β, interleukin 1βR (Adashi 1998a), prostaglandin endoperoxidase synthetase 2 (COX-2) (Morris and Richards 1995), VEGF (Ravindranath et al 1992), proteases (Liu et al 1998), neurotrophin receptors (Mayerhofer et al 1996), StAR (Ronen-Fuhrmann et al 1999), and progesterone receptors (Natraj and Richards 1993, Duffy et al 1996, Clemens et al 1998). LH also suppresses granulosa cell division (Yang et al 1992a) and follicle growth; the genes activated by LH create the conditions for follicle rupture, luteinisation and progesterone secretion. Many in vivo models have

linked the failure of these LH-activated processes to different degrees of female infertility (Matzuk and Lamb 2002).

In the maturing follicle, granulosa cells are not a homogenous tissue but rather a specialised subpopulation that consists of corona radiata, cumulus, mural and antral granulosa cells. The two populations of granulosa cells, those around the oocyte, the cumulus oophorus, and those lining the follicle wall, the mural granulosa cells, influence each other through paracrine effects. In the pre-antral follicles with a single layer of cuboidal granulosa cells, these cells contact both the growing oocyte and the basal lamina. By the antral follicular stage the granulosa cell population has become more heterogeneous with distinct separate mural and cumulus cell phenotypes. The subpopulation of cumulus cells that surrounds the oocyte, in direct contact with the oocyte through cytoplasmic extensions across the zona pellucida are known as the corona radiata (De Loos et al 1991). During folliculogenesis, differentiation of these cell types becomes more marked. Prior to ovulation, cumulus cells, under gonadotrophin stimulus secrete hyaluronic acid in a process called mucification (Eppig 1979), mural cells however, under the same stimulus undergo luteinisation. The subpopulations of granulosa cells differ in the distribution of receptors and steroidogenic characteristics (Rouillier et al 1994, 1996, and 1998). This variation has been demonstrated to involve differences in LH receptor distribution, steroidogenic capability, and mRNA encoding cholesterol side chain cleavage cytochrome P450, cytochrome P450 aromatase, IGF-1, Mullerian inhibiting substance, lectin binding, and other uncharacterised molecules (Latham et al 1999). Using high resolution 2D-PAGE analysis Latham et al 1999 showed that protein synthesis patterns of mural and cumulus cells differ at a level of about 10.5% of the proteins having a 2 fold or greater difference. The result of this is that in antral follicles the mural granulosa cells closest to the basal lamina are highly differentiated, as demonstrated by their high steroidogenic potential and LH receptor content (Amsterdam et al 1975, Lawerance et al 1980, Peng et al 1991, Whitelaw et al 1992). In contrast, the cumulus cells surrounding the oocyte are less differentiated, showing lower steroid production and LH receptor levels. The undifferentiated status of the

cumulus cells is necessary for optimal oocyte development (Eppig et al 1998), whereas the highly differentiated mural cells are essential for steroidogenesis as well as follicle rupture induced by the preovulatory LH surge (Nekola et al 1982).

Much of the variation between mural and cumulus cells is thought to be as a result of oocyte-cumulus interactions already described. A concentration gradient of paracrine factors established by the oocyte within the follicle could explain the stratification of granulosa cells in antral and preovulatory follicles, with the oocyte itself responsible for the development of two distinct subpopulations of granulosa cells. The Gdf-9 inhibition of FSH induced differentiation (Vitt et al 2000) of granulosa cells is a striking example of this (discussed in section 1.5.2.3).

The basement membrane is an acellular layer between the thecal and granulosa cells containing several forms of collagen as well as fibronectin, laminin and proteoglycans (Rodgers et al 1999). The follicular antrum contains the follicular fluid made up from blood exudates modified by local secretions and metabolism (Gordon and Lu 1990). Although meiotically competent at the antral stage, oocytes are held in arrest by their interactions with granulosa cells until the preovulatory follicle stage, at which time they progress to metaphase II in anticipation of ovulation and subsequent fertilization (Pincus and Enzmann 1935).

The preovulatory gonadotrophin surge is responsible for the resumption of meiosis in oocytes and induces marked changes in the mural granulosa and cumulus cells. Cumulus expansion occurs via the secretion, from the granulosa cells, of hyaluronic acid (a non-sulphated glycosaminoglycan bound to the cells by linker proteins) which when hydrated embeds the cells in a mucified matrix (Epigg 1979, Salustri et al 1999). Suppression of this process greatly reduces ovulation rate. The mural granulosa cells undergo further differentiation and luteinisation under the same influence.

In addition to granulosa cell development, folliculogenesis is characterised by recruitment and growth of the thecal cell layers. Theca cells are derived from the mesenchymal tissue surrounding the follicles (Hirshfield 1991), and although a

clear thecal cell layer cannot be distinguished in early follicle stages, the secondary follicle is surrounded by theca cells, which proliferate during follicle progression.

It has been shown that it is the oocyte that coordinates and orchestrates the rate of follicular development (Eppig et al 2002). The rate of follicular development can be doubled by the transfer of mid growth stage oocytes to primordial follicles.

In primates and rodents dominant follicles develop only during the follicular phase and are thus destined for ovulation (Fortune 1994). In contrast the bovine, ovine and equine species show the development of dominant follicles outwith this phase. Dominant follicles influence the development of other follicles (Ginther 1996). Since dominant follicles are more vascular it has been proposed that greater vascularity may play a part in the acquistion of dominance (Reynolds and Redmer 1998). Dominant follicles have a more vascular theca than other antral follicles, and as a result display increase uptake of serum gonadotrophins (McNatty et al 1981). Equally it has been reported that decreased proliferation of thecal capillary endothelial cells leads to reduced thecal vasculature, one of the earliest events in follicular atresia. Atresia of primordial follicles may be due to reduced blood supply (Greenwald 1989, Jablonka-Shariff et al 1994). During follicular development, the majority of ovarian follicles are destined to undergo atresia (Tilly et al 1991, Kaipai and Hseuh 1997). Apoptosis of follicular cells occurs at all stages of follicle development, and is regulated by several intraovarian factors, including tumour necrosis factor (TNF)-α, interleukin (IL)-6, and gonadotrophin releasing hormone (Gorospe 1992, Billig et al 1993, Santana et al 1995, Kaipai et al 1996). In addition, overexpression of Bcl-2 in follicular cells inhibits cell apoptosis and increases the frequency of germ cell tumours in ageing animals (Hsu et al 1996). Therefore, follicular cell apoptosis is also important for normal ovarian development.

Development of the ovarian follicles is accompanied by qualitative and quantative changes in the microvascular bed. Primordial follicles are surrounded by single capillary loops and growth of these follicles can be initiated by locally sprouting microvessels (Banks 1992). At a later stage of development the thecal

layers differentiates into two, the theca externa, an outer layer of connective tissue cells and the theca interna, which is an inner vascular layer with cuboidal secretory cells (banks 1992). Entering the theca interna the arterioles break up into a rich network of capillaries that builds a basket like network around the avascular stratum granulosm (Köing et al 1988). The mature Graffian follicle itself is avascular, nourished by diffusion, and its growth gradually leads to inner hypoxia (Neeman et al 1997). After the surge in LH prior to ovulation, vasodilation of capillaries is prominent and the cross-sectional areas of vasculature lumina increase. There is evidence of increased vascular permeability, tissue oedema and ischaemia. Capillaries develop perforations through which blood cells and platelets escape when ovulation occurs. Shortly before ovulation, blood flow stops in a small area of the ovarian surface overlying the bulging follicle, this area then ruptures (Findlay 1986, Rüsse et al 1987).

1.5.2 Follicular signalling mechanisms.

Paracrine and autocrine factors produced within the ovary have the ability to regulate folliculogenesis. Some of these factors are synthesised and secreted by the oocyte (Eppig et al 1997), and act as morphogens to control follicular growth as well as differentiation (Erickson and Shimasaki 2000). Others are produced by the granulosa cells and can have varied effects on follicle growth and development. Communication between the granulosa cells and the oocyte is bi-directional, involves paracrine factors and gap junction mediated signals (Eppig et al 1994 and 1997) and is governed by a complex interplay of regulatory factors. The gonadotrophins are likely to affect the function of this loop by driving the pathways of granulosa cell differentiation, although it is thought that the oocyte controls the direction of differentiation (Eppig 2001) with the mural cell phenotype and ultimate luteinisation the default pathway. The oocyte has the ability to promote the development of the cumulus cell phenotype, although *in vitro* this has been shown to require the presence of FSH (Eppig 1991).

1.5.2.1 Communication pathways

To efficiently allow the paracrine interactions described above the oocyte and companion cumulus and mural granulosa cells have developed into a functional syncytium connected via gap junctions, and have established adherence junctions that are specialised zones of cell-cell contact (Eppig 1991, Eppig et al 1996, Amsterdam et al 1976, 1981, 1989a, 1989b, Amsterdam and Rotmensch 1987, Sommersberg et al 2000). This coupling starts at the formation of primordial follicles in the foetus or neonate and expands during later folliculogenesis (Mitchell and Burghardt 1986). As well as paracrine signals, metabolites, amino acids and nucleotides are passed to the developing oocyte. Multiple connexins are involved in these pathways; Cx32, Cx37, Cx43, Cx45 and Cx57 have been identified in rodents (Acket et al 2001). Gap junctions consist of two hemi-channels present in the opposing plasma membranes of adjacent cells formed from the oligomerisation of six gap junction proteins. Granulosa cell proliferation beyond the unilaminar stage has been shown to be blocked in Cx43 deficient mice and it has been hypothesised that the ability of granulosa cells to maintain their response to certain paracrine stimulants depends on intercoupling via Cx43 channels. Cx43 null mice also show impaired oocyte development (Acket et al 2001). Cx32 null female mice are both viable and fertile (Nelles et al 1996), Cx37 null mice are viable but follicle development cannot proceed beyond the antral stage and ovulation does not occur. Instead the granulosa cells appear to undergo premature luteinisation (Simon et al 1997). Cx45 knockouts suffer early foetal death (Kumar et al 2000). This variation in phenotype indicates specificity in the gap junction coupling, possibly involving different permeant molecules, and it is therefore incorrect to assume a free passage of paracrine signals throughout the granulosa-oocyte syncytium.

It seems that the integrity of gap junctions plays an important role in the survival of granulosa cells. This conclusion is drawn from the fact that gap junctions become larger and appear in higher incidence subsequent to culturing of the cells on native ECM and/or in the presence of LH, FSH or glucocorticoids (Sasson and Amsterdam 2002, Amsterdam et al 1989). Following stimuli for apoptosis,

integrity of the junctions is interrupted (Sasson and Amsterdam 2002), but it is not yet clear whether apoptotic signals cause the breakdown of gap junctions or whether breakdown of gap junctions initiates and accelerates the apoptotic process. Cx43 is a major component of gap junctions, and its expression is clearly elevated both by gonadotrophins/cAMP (Sommersberg et al 2000) and glucocorticoids (Sasson and Amsterdam 2002). Adherence type, junction size and frequency were also found to be elevated by glucocorticiods, concomitantly with the elevation of cadherin expression (Amsterdam et al 2003). Therefore, integrity of adherence and gap junctions may also play a role in the resistance of granulosa cells to apoptotic signals.

1.5.2.2 Intracellular receptor mediated signalling pathways

Gonadotrophins exert their stimulatory activity via interaction with their specific transmembrane receptors. Upon binding of the ligands both receptors stimulate the G_s-protein via contact sites on the intracellular loops with the specificity of the receptor/G protein interaction depending on the appropriate configuration of the intracellular surface of the receptor (Birnbaumer 1995, Schwartz 1996, Strader et al 1994). This, in turn, activates membrane associated adenylyl cyclase, causing an elevation of intracellular cAMP (Cooke 1999, Hunzicker-Dunn and Birnbaumer 1976, Zeleznik et al 1977). Activation of alternative signalling pathways by gonadotrophin receptors include calcium ion mobilisation (Flores et al 1992), the MAPK pathway (Das et al 1996), the PKA and PKB pathways (Gonzalez-Robayna et al 2000), Sgk signalling (Richards et al 2002) and stimulation of chloride ion influx (Amsterdam et al 1999). FSH is known to regulate glucose uptake via the PI3-kinase pathway (Roberts et al 2004) and signalling via this same pathway has been demonstrated in rat granulosa cells by increased phosphorylation of protein kinase B (PKB/Akt) in the presence of FSH (Gonzalez Robayna et al 2000, Shimada et al 2003). Further FSH stimulated responses can be inhibited by PI3-kinase inhibitors (Sun et al 2003, Shimada et al 2003).

In the ovary, FSH and LH stimulate the A-kinase pathway and thereby control the growth and differentiation of the ovarian follicle (Walsh et al 1968). Tonic FSH stimulation of immature granulosa cell induces low levels of intracellular cAMP formation and activation of genes required for proliferation and differentiation. (Hillier 2001). Genes such as serum- and glucocorticoid-induced kinase (Sgk) (Alliston et al 1997, Webster et al 1993) and serum-induced kinase (Snk) (Walsh et al 1968), as well as the cell cycle regulatory molecule, cyclin D2 (Sicinski et al 1996, Robker and Richards 1998), are induced in these cells in an immediate early expression pattern. In contrast, other genes exhibit a more delayed response to hormone stimulation and do not peak until 24-48 hrs after exposure to FSH when granulosa cell function has reached the preovulatory stage. Genes induced at this time include aromatase (Hickey et al 1990, Fitzpatrick and Richards 1991), inhibin α (Pei et al 1991), LH receptor (Segaloff et al 1990, Richards 1994), and the secondary rise of Sgk (Natraj and Richards 1993). In response to the LH surge granulosa cells generate high levels of cAMP and rapidly initiate a program of terminal differentiation in which proliferation ceases (Richards 1994, Robker and Richards 1998). LH dramatically down-regulates genes associated with follicular function such as aromatase (Richards 1994, Fitzpatrick and Richards 1991) and cyclin D2 (Robker and Richards 1998) and rapidly, but transiently, induces genes required for ovulation such as progesterone receptor (Natraj and Richards 1993, Park and Mayo 1991, Richards et al 1998, Clemens et al 1998, Lydon et al 1995), prostaglandin synthetase-2 (Wong and Richards 1991, Sirois and Richards 1993, Dinchuk et al 1995, Morham et al 1995) and CAAT enhancer binding protein (Sirois and Richards 1993, Sterneck et al 1997). During the process of luteinisation granulosa cells appear to become refractory to further cAMP stimulation. Genes such as P450scc (Richards et al 1986, Oonk et al 1989) are constitutively expressed at elevated levels. Neither cAMP stimulation, nor A-kinase inhibition, alters expression of P450scc in luteinised cells (Oonk et al 1989).

In the testis and ovary agonist activation of LHR via G protein coupling leads to stimulation of adenylate cyclase and phosphorylation of intracellular proteins via activation of protein kinase A (Catt and Dufau 1991, Dufau 1988), as discussed

below. The high surge concentrations of LH up-regulate PKA signalling and alter the expression of other genes that coordinate the final stages of follicular development and ovulation (Hillier 2001). In the ovary the receptor also promotes PI hydrolysis and calcium signalling (Davis et al 1984, Kosugi et al 1996). PI hydrolysis probably results from stimulation of phospholipase C (Herrlich et al 1995, Rajagopalan-Gupta et al 1997). There is a wide difference in gonadotrophin concentrations (ED_{50} 50-100pM versus 2500 pM hCG respectively) needed for stimulation of adenylate cyclase and PI hydrolysis in Leydig cells expressing LHR (Guderman et al 1992) confirming the predominace of the adenylate cyclase pathway in this cell type. In the ovary, however, the increased hormonal stimulation at certain stages of the ovarian cycle brings the PI pathway into operation (Dufau 1998). The diverse pathways of gene expression that are regulated by FSH in granulosa cells are dramatically altered by the LH surge-induced transition to luteal cells. LH also regulates the expression of PRL receptors (Richards and Williams 1976, Ormandy et al 1997, Gibori 1992) which, in response to cytokine, activates the Jak/Stat signalling pathway in rat luteal cells (Dajee et al 1996 and 1998, Russell et al 1996).

Protein Kinase A

Gonadotrophin signalling via PKA/adenylyl cyclase is one of the most extensively documented granulosa cell signalling pathways. Activation of the gonadotrophin receptors activates adenylyl cyclase, and in turn leads to the induction of PKA. A-kinase not only regulates its own pathway by controlling levels of its regulatory subunits (Richards et al 1987), LH receptors (Segaloff et al 1990), and the phosphorylation of transcription factors such as cAMP response element binding protein (Carlone and Richards 1997, Mukherjee et al 1996) but it is also known to regulate other cellular signalling pathways that control cell proliferation and differentiation. These include the cyclin-dependant kinases controlling mitosis (Sherr 1996, Elledge 1996, Hunter and Pines 1994), MAPK (Das et al 1996, Maizels et al 1998), PRL receptors (Richards and Williams 1976, Ormandy et al 1997, Gibori 1992) and several members of the serine/threonine kinase family including Sgk and Snk (Webster et al 1993,

Simmons et al 1992). Apoptosis is rapidly induced in cultured granulosa cells from hen preovulatory follicles when treated with the pharmacologic PKA inhibitor, H-89 (Chun et al 1994 and 1996).

Thus the A-kinase pathway controls the expression of numerous genes in granulosa cells at distinct stages of differentiation and by specific molecular events.

Protein Kinase B

The PKB pathway consists of three isoforms that are activated following phosphorylation. It is well established that several growth factors such as insulin-like growth factor -I (IGF-I) and transforming growth factor α (TGFα) initiate PKB activation and promote granulosa cell survival (Westfall et al 2000, Johnson et al 2001). FSH can activate PKB signalling in a PKA independent manner (Gonzalez-Robayna et al 2000) mediated by PI3 kinase (Zeleznik et al 2003), with inhibition of this activation by a PI3 kinase inhibitor actively promoting apoptotic cell death (Asselin et al 2001) and blocking the survival promoting effects of IGF-1 and TGFα treatment (Westfall et al 2000, Johnson et al 2001). Significantly, this apoptosis is reversed when cells are co-cultured in the presence of cAMP or LH (Johnson et al 2001). PKB is obligatory to FSH-stimulated granulosa cell differentiation since it is required for the expression of aromatase and LH receptor mRNA (Zeleznik et al 2003).

Protein Kinase C

In primary rat granulosa cell culture the effect of ovulatory concentrations of LH can be mimicked by subovulatory concentrations of LH/hCG used in conjunction with a PKC activator, suggesting that the PKC pathway serves to mediate the actions of LH (Morris and Richards 1993 and 1995). However Salvador et al 2002 demonstrated that activation of PKC is not a direct consequence of LH receptor activation and its precise role as a mediator of gonadotrophic signals has yet to be determined.

Serum and glucocorticoid-induced kinase (Sgk)

Up-regulation of Sgk signalling is promoted by FSH and LH in granulosa cells from preovulatory follicles, and upon activation Sgk is known to translocate to the nucleus where it can modulate the activity of transcription factors (Richards et al 2002). However there is a distinct transition of Sgk protein from the nucleus in immature, proliferative granulosa cells to punctate sites within the cytoplasm of differentiated, non proliferative, luteinising granulosa cells (Gonzalez-Robayna et al 1999). Its expression in both proliferating granulosa cells, terminally differentiated luteal cells as well as resting oocytes suggests Sgk may have multiple functions in controlling cell cycle progression and differentiation (Alliston et al 2000).

Mitogen activated protein kinase (MAPK)

The mitogen-activated protein kinase cascade consists of multiple pathways, including those that signal through c-Jun N-terminal kinases (JNKs), p38 MAP kinases (p38 MAPK) and extracellular signal related kinases (ERKs). Whilst JNK and p38MAPK pathways are linked to promoting cell apoptosis (Gebauer et al 1999), the ERK pathway is indirectly linked to promoting cell survival (Gebauer et al 1999, Johnson et al 2001) and has a firmly established role in the modulation of gonadotrophin induced differentiation (Seger et al 2001). FSH has been shown to regulate the mitogen activated protein kinases (Das et al 1996, Maizels et al 1998). It has also been demonstrated that ovarian granulosa cell ERK is activated (2-5 fold) in response to LH and FSH (Cameron et al 1996, Das et al 1996) and that this activation is generated via the PKA pathway in gonadotrophin-induced granulosa cells (Seger et al 2001). The ERK cascade can activate G-protein-coupled receptor kinase 2 which, in turn, induces down regulation of the gonadotrophin receptors (Pitcher et al 1999).

1.5.2.3 Intrafollicular paracrine and autocrine signalling factors

Over the last few years a number of growth factors have been identified as being involved in folliculogenesis. Those secreted by the oocyte and acting on granulosa cells: GDF-9, BMP-15, BMP-6, TGFβ-2, FGF-8. Other factors

produced by the granulosa cells include components of the IGF system, PAPP-A, inhibin, activin, follistatin and kit ligand. Thecal cells produce IGF, EGF, KGF, TGFα, BMP-4 and BMP-7, which also target the granulosa cell (for reviews see Knight and Glister 2001 and 2003).

GDF-9

Evidence is available to indicate that oocyte-secreted factors can influence FSH action (Eppig et al 1997, Matzuk 2000, Erickson and Shimasaki 2000). *In vitro* experiments have shown that oocyte-derived factors can act to inhibit FSH-induced expression of P450scc, progesterone (P4) production (Goldschmit et al 1989), urokinase plasminogen activator (Canipari et al 1995), and LH-R mRNA (Eppig 1997), while acting to stimulate mitosis (Vanderhyden et al 1992), hyaluronic acid (Buccione et al 1990, Salustri et al 1990, Vanderhyden et al 1990), and oestradiol production (Vanderhyden 1995). GDF-9, which is exclusively expressed in the oocyte (Dong et al 1996), is one oocyte factor involved in the regulation of these proliferative and differentiation responses (Hayashi et al 1999, Elvin et al 1999, Elvin et al 2000, Vitt et al 2000). Gdf-9 production starts in mice at the primary follicle stage but in other species can be expressed by primordial oocytes. In Gdf-9 null mice, follicle development ceases at the primary pre antral stage, despite continued oocyte growth and zona pellucida formation (Dong et al 1996). The oocyte growth in this case being correlated with increased expression of another paracrine signalling molecule, MGF (mast cell growth factor), by the granulosa cells (Elvin et al 1999b). Study of Gdf-9 deficient mice has shown that it has an important role in the stimulation of granulosa cell proliferation during pre antral and primary follicle development and stimulates DNA synthesis in preantral and dominant secondary follicles. It also reduces FSH-induced differentiation in granulosa cells as reflected by the suppression of steroidogenesis and LH receptor expression (Vitt et al 2000).

Kit Ligand

Granulosa cells produce kit ligand (KL), which induces oocyte growth or development and theca cell proliferation (Parrott and Skinner 1997 and 2000).

Expression of the KL gene encoding KL mRNA, important for initiation of follicular growth, antrum formation and oocyte maturation (Driancourt et al 2000), is increased in granulosa cells of Gdf-9 null mice (Elvin et al 1999). This indicates that Gdf-9 suppresses KL in granulosa cells. Gdf-9 null animals also fail to express c-kit receptors on the theca cells (as well as 17-alpha-hydroxylase and LH receptors) (Haung et al 1993). Immature oocytes *in vitro* have been shown to reduce kit ligand expression by granulosa cells (Joyce et al 1999), therefore, early follicle progression could be coordinated through combined actions of oocyte derived Gdf-9 and granulosa cell derived kit ligand.

The importance of c-kit receptor and KL interaction was initially found in mouse strains with a homozygous mutation in either c-kit or KL encoding genes which were fertile. There are two membrane bound forms, KL-1 and KL-2, which can be cleaved to produce soluble forms of KL (Williams et al 1992). In the ovary c-kit is expressed in oocytes (Horie et al 1991, Manova et al 1991) and KL produced by granulosa cells affects oocyte growth in a paracrine manner (Clark et al 1996, Motro and Bernstein 1993, Tisdall et al 1997). Expression of c-kit is found in oocytes at as early as the primordial follicle stage in newborn mice (Manova et al 1993) and in the mouse inhibition of the interaction between KL and c-kit by specific antibody prevents transition from primordial follicles to primary follicles without blocking the formation of primordial follicles (Haung et al 1993, Yoshida et al 1997). Administration of anti- c-kit antibody to mice has even stopped the proliferation of granulosa cells (Yoshida et al 1997). Therefore KL/c-kit interactions appear to be essential for the growth initiation of mouse oocytes. It has also been shown that KL promotes the growth of oocytes in vitro (Packer et al 1994) and induces significant development of primordial follicles (Parrott and Skinner 1999), as well as having a promotional effect on antrum formation (Reynaud et al 2000).

Bone morphogenic proteins

Another member of the same transforming growth factor-β superfamily, BMP-15 (or GDF-9B) has been implicated in modifying FSH action by suppressing FSH receptor expression (Otsuka et al 2001) without affecting adenylate cyclase

activity, as well as stimulating granulosa cell proliferation and selectively inhibiting FSH induced P4, but not E2, production (Otsuka et al 2000). BMP15 achieves this by lowering the steady state levels of FSH-induced mRNA including StAR, P450scc, 3β-HSD, LH-R, inhibin/activin subunits and FSH-R (Otsuka 2001). It is considered to be a negative regulator of the major actions of FSH in the ovary and consequently the oocyte can play an important role in determining granulosa cell proliferation and FSH sensitivity in developing follicles. BMP15 has an essential role in folliculogenesis and fertility by promoting early folliculogenesis while preventing the later stages of follicular maturation. A recent genetic study with a naturally occurring BMP15 defect homozygous mutant in sheep shows follicular development which has arrested at the primary stage resulting in infertility. In contrast heterozygotes exhibit increased ovulation rate and multiple pregnancies (Galloway et al 2000), an effect which may possibly be related to the inhibitory action of BMP15 on FSHR expression.

BMP6 unlike Gdf-9 and BMP15 lacks mitogenic activity on granulosa cells and has a selective modulation of FSH action on steroidogenesis, suppressing FSH induced progesterone P4 production but not oestradiol production. It also has similar actions to BMP15 as it attenuates steady state mRNA levels of FSH-induced StAR and P450scc, without an affect on P450 aromatase mRNA level. It is thought to work by down-regulating adenyl cyclase activity without altering FSH-R mRNA levels (Otsuka et al 2000). This factor does not appear to have an essential role in fertility since BMP6 null mice are fertile with normal sized litters (Tilly et al 1992). The highly prolific Booroola strain of Merino ewes have a point mutation in a gene encoding the bone morphogenic protein receptor IB (Mulsant et al 2001, Wilson et al 2001, Souza et al 2001) which confers increased fertility through higher ovulation rates and litter sizes in both heterozygotes and homozygotes. It has been suggested that this phenotype may be caused by the inability of the granulosa cell to elicit BMP6 signalling. (Otsuka 2001). This would prevent inhibition of cAMP synthesis causing an increased sensitivity to FSH.

Other members of the BMP system are known to be active within the follicle, BMP3 is expressed in human granulosa-lutein cells (Jaatinen et al 1996) and BMP receptors are expressed in both oocytes and granulosa cells throughout follicle development (Shimasaki et al 1999). BMP4 and BMP7 are produced by the thecal cell and exert a paracrine influence on granulosa function (Glister et al 2004).

Inhibin, acitivin and follistatin

Granulosa cells are a major site of inhibin, activin and follistatin (FS) expression. FSHR activation is required for granulosa cells to transcribe the inhibin βA, βB and alpha subunits above basal levels (Hirst et al 2004). While increased levels of FSH increase production of inhibin B in granulosa cells it is suggested that LH stimulation may be responsible for the late follicular rise in inhibin βA subunit expression (Woodruff et al 1996, Hirst et al 2004). The endocrine role played by inhibins in the negative feedback regulation of pituitary FSH secretion has already been mentioned, however activins, FS, and inhibins are also firmly implicated as intraovarian autocrine/paracrine regulators of follicle function. For instance, activin A has been shown to promote granulosa cell proliferation, up regulate FSH receptor expression and enhance granulosa cell steroidogenesis and inhibin production. Through its role as an activin binding protein, FS can neutralise these actions of activins. Follistatin is highly expressed by granulosa cells of developing follicles (Shimasaki et al 1988 and 1989, Nakatani et al 1991) and binds activin A, AB, and B with high affinities (Roberts et al 1993, Nakamura et al 1990, Shimonaka et al 1991, Kogaua et al 1991, Schneyer et al 1994) blocking the biological action of those molecules including stimulation of FSH receptor expression and inhibin secretion (DePaolo et al 1991, Xiao and Findley 1991, Nakamura et al 1992, Xiao et al 1992, Cataldo et al 1994, Eramaa et al 1995). It also binds to BMP-15 to form an inactive complex inhibiting its bioactivity (Otsuka et al 2001).

It is debatable whether inhibins exert autocrine actions to modulate granulosa cell function, although they have a potent paracrine effect on thecal cells to enhance LH-induced androgen production, while activin acts to inhibit thecal

androgen synthesis (Hseuh et al 1987, Hillier 1991, Hillier et al 1991b). Activin predominates in immature follicles where it promotes FSH-induced mitosis and FSH-induced steroidogenic differentiation (Miro and Hillier 1996). Inhibin and follistatin are produced in progressively greater amounts relative to activin by granulosa cells as follicles mature (Nakatani et al 1991). Thereby the stimulatory action of inhibin on thecal androgen synthesis gains sway during late preovulatory follicular development, when androgen is required in increasing amounts as a substrate for oestrogen synthesis (Hillier 1991).

Insulin-like Growth Factors

Of the many growth factors which have varied effects on follicle growth and development the IGF system is among the most extensively studied. Granulosa cells express type 1 IGF receptors (Perks et al 1995, Spicer et al 1994, Armstrong et al 2000) and it has a proven link to FSH action via IGFBP-4, which is a potent inhibitor of FSH-induced oestradiol production in granulosa cells. Gene expression of IGFBP-4 has been demonstrated in atretic follicles *in vivo* and is thought to be involved (through its FSH antagonistic activities) in the regulatory pathways that lead to follicle atresia. The production of an IGFBP-4 protease in granulosa cells has been reported to be stimulated by FSH. Pregnancy associated plasma protein-A (PAPP-A) has been identified as an IGFBP-4ase and demonstrated to be present in follicular fluid. Recent evidence suggests that expression of the PAPP-A gene is restricted to granulosa cells of healthy Graafian follicles and luteal cells of healthy CL's (Erickson and Shimasaki 2001).

Although physiological concentrations of insulin, IGF-I and IGF-II can stimulate thecal androgen production, inhibin greatly enhances the action of all three factors *in vitro* (Nahum et al 1995). Thus, regardless of the contributions made by insulin or IGF's to the control of follicular androgen production, paracrine regulation by inhibin could be of overriding importance during preovulatory follicular development. Antral follicles, too immature to secrete oestrogen, do not need androgen as an aromatase precursor (Hillier et al 1994). At such early stages of development, androgen synthesis may be suppressed due to a

preponderance of activin relative to inhibin, possibly aided by granulosa cell derived IGF-BPs that sequester and inhibit the actions of IGFs (Ling et al 1993). Granulosa cells express type 1 IGF receptors and it is well established in a range of species, that IGF can act both alone, and in synergy with FSH, to modulate granulosa cell proliferation, differentiation, and steroidogenesis. IGF-1 has been found to induce mitosis and enhance steroidogenesis of bovine granulosa and thecal cells (Schams et al 1988, Spicer et al 1996). Deletion of the IGF-I gene leads to a failure of ovulation although follicle development occurs to the preantral stage (Baker et al 1996). It is thought that the role of IGF-I is to amplify FSH action. Transcripts of IGF-II, -IR, and IGFBP-2 to –5 have also been shown to be expressed in the mouse ovary (Wandji et al 1998), with only the expression of IGF-I and IGFBP-4 and –5 changing during the course of follicular development. IGF-I levels increase within the follicle as it progresses to the antral stage and it is known to regulate antral development and FSH action (increased aromatase activity) (Baker et al 1996, Zhou et al 1997). Early follicular development with low growth rate is associated with low IGF-I levels, whereas high IGF-I levels are found in rapidly growing large preantral and early antral follicles and it has been suggested that IGF-I may be rate limiting for preantral follicle development (Wandji et al 1998). Expression of IGFBP-4 transcripts in granulosa cells have been found to precede the morphological appearance of atresia, and it has been hypothesised that IGFBP-4 promotes follicular atresia by sequestrating IGF-1, thereby reducing its ability to interact with IGF-IR in granulosa cells (Baker et al 1996). In the rat ovary, FSH can stimulate the production of IGF-I by granulosa cells, this suggests a paracrine role for granulosa cell derived IGF-I in the regulation of thecal androgen synthesis (Hernandez et al 1992, Adashi 1998b). Thecal cells have been shown to possess receptors for insulin and IGFs, (Poretsky et al 1985) and both insulin and IGF stimulate thecal/stromal androgen synthesis in vitro.

Connective tissue growth factor

Connective tissue growth factor (CTGF) is expressed during the predifferentiated stage of granulosa cell development in the rat ovary. FSH

stimulus on granulosa cells induces down regulation of granulosa cell CTGF mRNA although it remains expressed in the most antrally located granulosa cells up to and after ovulation. It is suggested that it may have a role in establishing or maintaining the early follicular cell phenotype and also as a thecal cell mitogen or luteotrophic factor (Harlow and Hillier 2002).

1.5.2.4 Intraovarian role of the steroid hormones

The paracrine activities of ovarian steroid hormones have important roles in the regulation of local ovarian function as well as their feedback regulation of pituitary function.

Oestrogen

FSH-induced expression of the enzyme cytochrome P450arom leads to increased conversion of androgen to oestrogen in preovulatory granulosa cells (Richards 1980, Hillier et al 1994). Granulosa cells also express oestrogen receptors, which may mediate autocrine action within the granulosa cell layer (Richards 1975a and 1975b). The effect of oestrogen on the granulosa cell is to amplify the overall actions of FSH (Richards 1994). Oestradiol has been shown to be obligatory for synthesis of specific proteins in rat granulosa cells in response to FSH and LH (Richards 1980, Hseuh et al 1984)

Oestrogen synthesis by the granulosa cell can exert diverse local and systemic physiological effects. Oestrogens stimulate the proliferation of granulosa cells and facilitate the LH- and FSH-induced differentiation of these cells by increasing LH receptor level and enhancing gap junction formation (Rosenfield et al 2001). The autocrine actions of oestradiol 17β (E2), the principle bioactive oestrogen, are mediated via two nuclear factors, oestrogen receptor (ER)-α and -β, in the granulosa cell. Effects are principally via ER-β, although both receptors are present (Couse et al 1997).

Oestrogens increase follicular expression of both FSH and LH receptors and in bovine follicles acts on thecal cells to provide positive feedback on androgen synthesis (Fortune 1986, Roberts and Skinner 1990). It can influence gap

junction formation (Burghardt and Anderson 1981) via regulation of connexin 43 (Yu et al 1994) and inhibit granulosa cell apoptosis (Lund et al 1999). Oestrogen is known to increase the CYP17 expression in theca cells (Johnson and Crane 1998), an essential enzyme foe catalysis of the 14-demethylation of sterol precursors, and the consequent increase in progesterone production which is thought to promote corpus luteum formation and maintenance through luteal progesterone receptors (Duffy and Stouffer 1995).

Oestrogens are pro-angiogenic *in vivo*. They are known to moduate angiogenesis, and have been demonstrated to augment angiogenesis induced by FGF. Equally however, the endogenous oestrogen metabolite 2-methoxyestradiol is known to be a potent anti-angiogenic factor. It induces apoptosis in endothelial cells and inhibits angiogenesis (Yue et al 1997).

Glucocorticoids

Glucocorticoids are not true ovarian paracrine factors since the ovarian cells do not express the enzymes necessary for glucocorticoid synthesis. They do however express 11β hydroxysteroid dehydrogenase (11βHSD), a microsomal short chain alcohol dehydrogenase (Krozowski 1992), that interconverts cortisone and cortisol and thereby control the access of cortisol to ovarian corticosteroid receptors (Micheal et al 1997). Evidence for glucocorticoids having a physiological role in the regulation of ovarian folliculogenesis has been strengthened with the knowledge that granulosa cell expression of 11βHSD is developmentally regulated. High affinity glucocorticoid receptors are present in rodent granulosa cells (Schreiber et al 1982) and glucocorticoids have been shown to modify gonadotrophin action on these cells in vitro. Gonadotrophic regulation of 11βHSD genes in the ovary has been demonstrated-LH induces 11βHSD1 and down regulates 11βHSD2-both enzymes catalysing cortisol-cortisone conversions (Tetsuka et al 1997, 1999a, 1999b). This favours local accumulation of anti-inflammatory cortisol to aid rapid postovulatory healing and quickly restore normal ovulatory function (Hillier and Tetsuka 1998). Glucocorticoids have been shown to stimulate FSH-induced progesterone production and tissue-type plasminogen activator (Tetsuka et al 1999). The

physiological importance of this is that the follicle is likely to operate a cortisol-cortisone (corticosterone-11-dehydrocorticosterone in the rat and mouse) shuttle based on differential expression of 11βHSD, the follicle can alternate between inactivation via 11βHSD2 catalysed oxidation to activation via 11βHSD1 catalysed reduction. This allows ovulatory follicles to gain exposure to glucocorticoids at levels required for involvement in oogenesis, or the process of follicular rupture, possibly as an anti-inflammatory modulator.

Glucocorticoids such as dexamethasone and hydrocortisone enhance steroidogenesis in granulosa cells while co-stimulation with gonadotrophin/cAMP enhances formation of progesterone (Hosokawa et al 1998, Sasson et al 2002, Barkan et al 1999). Glucocorticoids have been found to exert protective effects on apoptosis induced by serum deprivation, cAMP, p53 and TNF-α (Hosokawa et al 1998, Sasson et al 2001 and 2002, Barkan et al 1999,). The protective effects are exerted by upregulation of BCL2 and/or attenuation of its degradation (Barkan et al 1999, Sasson and Amsterdam 2002). Glucocorticoids, therefore, may play an important role *in vivo* by accelerating the healing process of the ruptured follicle subsequent to ovulation and during formation of the corpus luteum (Sasson and Amsterdam 2002, Amsterdam et al 2002)

Progesterone

Progesterone is produced in increasing amounts in differentiated granulosa cells through FSH induction of cytochrome P450scc, which catalyses the rate limiting conversion of cholesterol to pregnenolone (Richards et al 1998). Progesterone is one of the major steroids synthesised and secreted by the ovary (Monniaux et al 1997). It is synthesised by preantral follicles with just two to four layers of granulosa cells (Roy and Greenwald 1987), with the rate of progesterone secretion increasing as follicle development proceeds (Roy and Greenwald 1987, Fujii et al 1983). Although the concentration of progesterone fluctuates throughout the oestrus cycle, the concentration of progesterone within antral follicles is always within the micromolar range (Fujii et al 1983). The importance of these high progesterone levels in regulating ovulation has been

emphasised by several studies (Curry and Nothnick 1996, Robker et al 2000). These investigations have shown that the nuclear PR does not appear in granulosa cells until the onset of the LH surge. It is then transiently expressed before ovulation and reappears later in the corpus luteum (Natraj and Richards 1993). Further treatment with PR antagonists or genetic ablation of the nuclear PR interferes with gonadotrophin-induced ovulation (Curry and Nothnick 1996, Robker et al 2000, Pall et al 2000, Conneely et al 2002, Lydon et al 1996) demonstrating the progesterone receptor gene to be essential for ovulation. Based on the expression pattern of the PR it is predictable that progesterone inhibits apoptosis of granulosa cells isolated during the periovulatory period (Svensson et al 2000). Progesterone also prevents apoptosis of granulosa cells isolated from immature rats prior to the gonadotrophin surge (Peluso and Pappalardo 1998, Peluso et al 2001) and inhibits apoptosis of spontaneously immortalised granulosa cells (Peluso et al 2001). This is suprising considering that granulosa cells isolated prior to the LH surge do not express the classic nuclear PR (Natraj and Richards 1993, Park and Mayo 1991). Recent work has suggested that this anti-apoptotic action is mediated via a 60kDa progesterone binding protein which serves to regulate calcium homeostasis and ultimately granulosa cell viability (Peluso 2003).

Progesterone synthesis during the oestrus cycle can play an important role in the fertilisation of the oocyte following ovulation. It has been suggested that early progesterone production in the preovulatory follicle impairs the quality of the mature egg during fertilisation (Lindheim et al 1998, Fanchin et al 1997, Urman et al 1999). In contrast, proper timing of progesterone production and the duration of its secretion seems to be critical for maintaining functional granulosa-lutein cells, subsequent to the LH surge and maintenance of the corpus luteum during early pregnancy. Progesterone interaction with its cytosolic receptor may play a part in the survival activity of the granulosa-lutein cells (Svensson 2001)

Androgens

Synthesis of androgens occurs in thecal cells under LH control (Smyth et al 1993). Granulosa cells express androgen receptors (AR) throughout antral development, permitting paracrine androgenic stimulation (Tetsuka et al 1995 and 1996). The main effect of this is the up-regulation of cAMP formation, possibly through inhibition of cAMP metabolism (Hillier and de Zwart 1982). Amplification of FSH induced PKA signalling by androgen appears to be a means of sensitising granulosa cells to tonic stimulation by FSH. During late preovulatory development, transcription of the granulosa AR gene and AR protein levels decline which may serve to diminish granulosa cell responsiveness to gonadotrophins and delay terminal differentiation (luteinisation) until exposure to the LH surge (Hillier and Tetsuka 1997, Tetsuka et al 1995).

The role of androgens in influencing the process of folliculogenesis has been examined. They have two roles, firstly as metabolic precursors for androgen synthesis, and secondly as ligands for androgen receptors. Androgens are the predominant steroids produced in early follicular development and are present at high concentrations in follicular fluid at all stages of follicular growth, although the ratio of androgens to oestrogens changes as the follicle advances and dominant follicles engage in aromatase activity (McNatty et al 1979). Studies using 5α-dihydrotestosterone (DHT) have shown that androgens have a direct effect on ovarian function (Louvet et al 1975, Nandedkar et al 1981, Rao 1975, Armstrong and Dorrington 1976, Daniel and Armstrong 1980, Hillier and De Zwart 1981), an effect thought to be mediated by the androgen receptor (AR) which has been detected in ovarian cells from all vertebrate species studied to date (Ito et al 1985, Hild-Petito et al 1991, Horie et al 1992, Yoshimura et al 1993, Hirai et al 1994, Sperry and Thomas 1999, Touhata et al 1999, Slomczynska et al 2001, Lutz et al 2001, Vermeirsch et al 2001), suggesting a conserved receptor mediated role for androgens in folliculogenesis. Granulosa cells display the strongest AR immunoreactivity and are exposed to both testosterone and DHT sourced from thecal cells and internal production from conversion of androgen precursors (McNatty et al 1979). Both testosterone and DHT promote *in vitro* follicle growth in mice (Murray et al 1998, Wang et al 2001) and many of the differentiative actions of FSH on granulosa cells are

augmented by AR agonists including, cholesterol mechanism, progesterone secretion, expression of steroidogenic enzymes, and induction of aromatase activity (for review see Hillier and Tetsuka 1997)

Other steroid metabolites

One product of lanosterol 14α-demethylase (CYP51) mediated lanosterol 14 demethylation has been identified as a meiosis activating steroid (MAS). Ovarian CYP51 is expressed in mature ovarian follicles and corpa lutea, and is suppressed in hypophysectomised animals. Induction of CYP51 activity with PMSG is due to synergism of marked growth of follicles and elevation of CYP51 levels in each follicle. The expression of CYP51 is dependant on pituitary gonadotrophins and its expression in follicles, triggered by FSH, has been shown to be higher in cumulus cells than in mural granulosa cells supporting a local elevation of MAS required for resumption of meiosis (Yamashita 2001, Rozman et al 2002).

Ovarian steroid hormones may also play an important role in controlling ovarian cell death. Oestradiol acts as a survival factor in both corpus luteum and granulosa cells (Goodman et al 1998). Progesterone was suggested to maintain genomic viability through non genomic mechanisms (Peluso 1997). Glucocorticoids, such as hydrocortisone and dexamethasone, were also found to protect against apoptosis and it has been reported that both oestradiol and dexamethasone can increase the synthesis of Bcl-2, which serves as an ovarian survival factor (Goodman et al 1998, Sasson et al 1999). Androgens are reported to antagonise the protective effect of diethylstilbestrol (DES), which has oestrogenic activity in granulosa cells (Billig et al 1993). In contrast testosterone was found to reduce spontaneous follicular apoptosis in immature rats, leading to the development of polycystic ovaries (Gold et al 1999).

1.5.3 Thecal role in follicular function.

Communication between the granulosa cells and the oocyte is necessary for follicle development (Tsafriri 1997), but interaction between granulosa and theca cells is also critical. Factors secreted by preantral granulosa cells enhance differentiation of theca cells before expression of LH receptors (Gelety et al 1997), and co culture of theca and granulosa cells increases the proliferation and steroidogenesis of both cell types (Kotsuji et al 1994). Thus paracrine factors secreted between theca and granulosa cells are likely to play a crucial role in follicle development. In vitro studies suggest that these two somatic cell types modulate each other's responsiveness to gonadotrophins in a reciprocal manner (Kotsuji et al 1994). Signalling of c-kit receptors on theca cells is modulated by granulosa cell derived kit ligand, and theca cells produce keratinocyte and hepatocyte growth factor, which influence granulosa cell physiology (Parrott and Skinner 1998, McGee et al 1999). Furthermore, following stimulation by LH, theca cells secrete androgens to serve as substrates for the oestrogen producing granulosa cells.

EGF, and its closely related homologue TGFα, are expressed by various cell types; they both interact with the same cell surface receptor (EGF receptor), which is expressed by numerous cell types including granulosa and thecal cells. Exposure of granulosa cells to EGF/TGFα promotes cell proliferation and this is associated with a loss of differentiated function, exemplified by a marked reduction in E2 production in vitro and in vivo. Theca cells have been identified as a key site of TGFα expression in the bovine ovary and evidence suggests that TGFα of thecal origin exerts a local paracrine action on neighbouring granulosa cells to modulate their proliferation and responsiveness to gonadotrophins and other regulatory factors.

Keratinocyte growth factor derived from the thecal cell may play a role in early folliculogenesis by promoting granulosa cell survival, increasing the rate of follicle growth, and enhancing early granulosa cell differentiation (McGee et al 1999).

BMP-7 is expressed in the thecal cell, and the BMP-7 receptor is expressed by the granulosa cell (Shimasaki et al 1999). BMP-7 has been shown *in vitro* to reduce FSH-induced progesterone production while enhancing FSH induced oestradiol production by granulosa cells, this has been shown to be mediated through decreasing levels of StAR mRNA and increasing P450arom resulting in an enhanced conversion of androstenedione to oestradiol and inhibition of cholesterol transport from the outer to inner mitochondrial membrane. BMP-7 has also been found to stimulate folliculogenesis while inhibiting ovulation and luteinisation (Lee et al 2001). BMP-7 null mice die shortly after birth with severe bilateral renal dysplasia (Dudley et al 1995) and conditioned knockouts have not yet been produced which are targeted at thecal cells.

Stanniocalcin (STC) is a glycoprotein hormone first discovered in bony fish. In rodents STC 1 is widely expressed in a range of tissues with the ovary showing the highest level (Chang et al 1995, Chang et al 1996, Varghese et al 1998). Treatment of granulosa cells with STC1 decreases gonadotrophin stimulation of progesterone production, Cyp11a mRNA expression and LH receptor formation without affecting gonadotrophin-induced oestradiol secretion (Luo et al 2004). In addition, gonadotrophin treatment suppresses STC1 transcripts in thecal cells (Luo et al 2004), suggesting the induction of granulosa cell differentiation by gonadotrophins may involve the suppression of the biosynthesis of theca cell-derived STC1. Transgenic mice overexpressing STC1 are sub-fertile as reflected by reduced litter sizes, and a possible defect in the ovulatory mechanism has been suggested (Varghese et al 2002). STC1 may, therefore, be a potential luteinisation inhibitor sourced from thecal cells in the developing follicle.

1.5.5 Ooctye growth and development

The observation that no follicle can be formed in the absence of oocytes had been made as long as 50 years ago (Coulombre et al 1954), while pharmacological ablation of oocytes in rats is known to result in defective folliculogenesis (Merchant 1975, Hirshfiled 1994). It has also been known for several decades that oocytes prevent the spontaneous luteinisation of granulosa

cells (El-Fouly et al 1970, Nekola and Nalbandov 1971). More recently, however, the level of influence that the oocyte has over normal development of its own follicle is much better understood. It has been shown that it is the oocyte that coordinates and orchestrates the rate of follicular development (Eppig et al 2002). The rate of follicular development can be doubled by the transfer of mid growth stage oocytes to primordial follicles. Oocytes participate in the modulation of steroidogenesis by maintaining oestradiol production and inhibiting progesterone production (Vanderhyden and Tonary 1995). In the presence of oocytes, proliferation of granulosa cells is accelerated (Vanderhyden et al 1992), and expression of LH receptor mRNA is inhibited (Eppig et al 1997 and 1998).

Although the zona matrix physically separates the oocyte and somatic cells, close associations are maintained throughout follicular development via paracrine factors and cellular processes that form gap junctions with the oocyte membrane (Eppig 1991). These interactions provide the two-way communications required for oocyte growth and maintain the oocyte in meiotic arrest. Oocytes unable to form interactions with surrounding granulosa cells, either because of ectopic displacement (Zamboni and Upadhyay 1983), or genetic mutation (Soyal et al 2000) do not survive.

The growth of the oocyte has been shown to be positively correlated with the number of adherent granulosa cells and extent of metabolic cooperation between these two cell types (Brower and Schultz 1982, Herlands and Schultz 1984). The establishment of gap junctions allows the accumulation of materials essential for fertilization and pre-implantation development and increased protein content is known to be related to growth in oocyte size (Schultz and Wassarman 1977). Granulosa cells actively transfer low molecular weight substances such as nutrients and metabolic precursors (Heller et al 1981, Colonna and Mangia 1983, Eppig 1977) to the oocyte which also appears to incorporate externally produced macromolecules (Glass 1961). Granulosa cells convert cystines to cysteines which are then made available for oocyte utilisation (De Matos 1997). Gonadotrophins are known to stimulate glucose consumption by cumulus cells via the PI3-kinase pathway leading to pyruvate production (Roberts et al 2004,

Hillier et al 1985, Zuelke and Brackett 1992). Pyruvate as an energy source is obligatory for resumption of meiosis (Biggers et al 1967, Eppig 1976) and is supplied by surrounding cumulus cells through the glycolysis of glucose (Leese and Barton 1985, Donahue and Stern 1968). Levels of these energy substrates can have a profound effect on oocyte maturation (Downs and Mastropolo 1994) and adequate levels of pyruvate and glucose are important for progression to metaphase II. (Downs and Hudson 2000, Rose-Hellekant et al 1998). This serves to demonstrate the supportive role played by the granulosa cell in maintaining oocyte viability and growth.

Cumulus-enclosed oocytes can be prevented from undergoing spontaneous maturation using inhibitory agents such as cyclic AMP and purines (Dekel and Beers 1978, Downs et al 1988). FSH overcomes this inhibition, resulting in ligand-induced maturation (Downs et al 1988). FSH has been shown to significantly increase the proportion of oocytes reaching metaphase II after 15-16 hours of in vitro culture, suggesting that FSH has the ability to accelerate maturation (Roberts et al 2004). FSH also increases hexokinase activity in the cumulus oophorus (Downs et al 1996), facilitating glucose uptake and its conversion to glucose-6-phosphate. It is the pentose phosphate pathway that is the metabolic route that mediates ligand-induced resumption of meiosis (Downs and Utecht 1999, Downs et al 1996) and which provides precursors for the de novo purine pathway. This in turn is involved in meiotic induction (Downs et al 1998). Additionally, ATP is known to induce Ca^{2+} release from intracellular stores in cumulus cells, which is then transmitted via gap junctions to the oocyte (Webb et al 2002) and it has been suggested that FSH-induced glucose uptake may result in increased glycotically produced ATP, leading to Ca^{2+} release and stimulation of maturation (Roberts et al 2004).

The correct hormonal support is fundamental to achieving adequate development competence by increasing the number and permeability of gap junctions between oocyte and cumulus cells, thus leading to a better interaction between the germinal and somatic components of the follicle (Mattioli et al 1990 and 1991). Both cytoplasmic and nuclear maturation are important when determining the developmental competence of the germ cell (Lucidi et al 2003). Properly

matured oocytes no longer affect progesterone production but acquire the ability to inhibit oestrogen production (Lucidi et al 2003, Glister et al 2003). Thus, properly matured oocytes not only control the activity of cumulus cells but also modify their messengers in order to favour the functional luteinisation of granulosa cells after the gonadotrophin surge.

1.5.5 Steroidogenic activity

Steroid hormone secretion by ovarian tissues is episodic, tightly regulated, and crucial to the coordination of reproductive cyclicity. Biochemically, steroid hormone biosynthesis is modulated by cholesterol availability and expression of specific steroidogenic enzymes. In the developing follicle, oestradiol is the main steroid product synthesised by granulosa cells and has major endocrine and intraovarian roles to regulate the oestrus cycle, follicle development and ensuring granulosa cell survival

In order that steroids, including oestradiol, can be synthesised by steroidogenic cells, they must first acquire cholesterol either via *de novo* synthesis or by the uptake of lipoprotein-carried cholesterol (Brown and Goldstein 1997, Gwynne and Strauss 1982, Strauss et al 1981). The actual biosynthesis of oestradiol incorporates both the theca and granulosa cell layers. These 2 layers must integrate fully to facilitate the conversion of cholesterol to oestradiol. The conversion of the various precursors depend entirely upon many enzymes, in particular several members of the large cytochrome P450 family of heme-containing enzymes, and hydroxysteroid dehydrogenases (HSD) (Strauss et al 1981) (figure 1.4).

The first rate-limiting step in steroid synthesis is the conversion of cholesterol to pregnenolone, and cyp11a catalyses this conversion. Cyp11a is localised to both granulosa and theca layers in the follicle (Huet et al 1997). However, the next 2 steps take place almost exclusively in thecal cells. Firstly, cyp17 catalyses the conversion of pregnenolone and progesterone to dehydroepiandrosterone and androstenedione. Secondly, 3β-HSD catalyses the conversion of pregnenolone into progesterone, 17α-hydroxypregnenolone into 17α-hydroxyprogesterone, and

dehydroepiandrosterone into androstenedione (Fortune and Quirk 1988; Strauss and Penning 1999). 17β-HSD catalyses the conversion of the weak androgen, androstenedione, to the stronger androgen, testosterone (Strauss and Penning 1999). Finally, testosterone and/or androstenedione are aromatised into oestradiol in the granulosa cells by Cyp19 (Strauss and Penning 1999, Huet e al 1997). Therefore, the whole process of oestradiol synthesis in the follicle is a two-cell two-gonadotrophin system (Fortune and Quirk 1988) whereby, under the direction of FSH and LH, theca cells essentially produce androgens that can be used as a substrate for oestradiol synthesis in the granulosa cells. In addition, there is evidence that the increase in oestradiol secretion positively feeds back to stimulate more androgen secretion from the theca cells(Bao et al 1998, Fortune 1986, Fortune and Quirk 1988, Roberts and Skinner 1990).

During the follicular phase of the cycle, FSH promotes the differentiation of granulosa cells of the growing follicles and consequently renders them competent to produce copious amounts of oestradiol, but nearly no progesterone (Smith et al 1975, Richards 1994). In contrast when granulosa cells from early antral follicles are cultured in vitro in the presence of FSH they secrete both oestradiol and progesterone (Shimasai et al 1999, Otsuka et al 2000). Although the stimulation of steroidogenesis by FSH has been studied extensively, at present little is known about the regulation of the divergent steroidogenic properties of granulosa cells. Bone morphogenic proteins (BMP-7 and BMP-4) have been identified as factors that act on granulosa cells to augment FSH induced P450 aromatase expression and subsequent oestradiol secretion and to attenuate FSH-induced StAR expression and subsequent progesterone secretion (Shimasaki et al 1999, Lee et al 2001). Mitogen activates protein kinase molecules (MAPK), particularly extracellular signal related kinases 1 and 2 (ERK 1 and 2) have been directly implicated in the modulation of steroid biosynthesis by granulosa cells (Moore et al 2001).

The periovulatory period is accompanied by dramatic changes in ovarian follicular steroidogenesis as the LH surge triggers follicular luteinisation, a process during which the predominant steroid produced switches from 17β-oestradiol to progesterone.

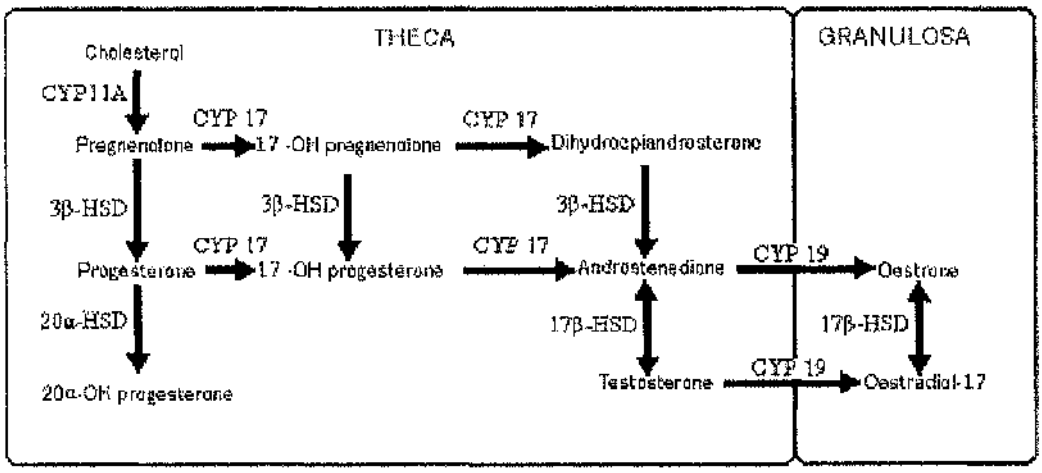


Figure 1.4 Pathways of synthesis of steroid hormones in ovarian somatic cells. (CYP11A; cytochrome P450 side chain cleavage enzyme: CYP 17; cytochrome p450 17α-hydroxylase 17,20 lyase: CYP 19; cytochrome P450 aromatase: 3β-HSD; 3β-hydroxysteroid dehydrogenase: 20αHSD; 20α hydroxysteroid dehydrogenase)

1.5.6 Extracellular matrix.

Dynamic remodelling of the ovarian extracellular matrix is characteristic of follicle growth and atresia, ovulation, and corpus luteum development and regression. Adhesion to the extracellular matrix regulates the survival, proliferation and differentiation of numerous cell types in many tissues (Rusolahti 1990, Adams and Watt 1993, Lelievre e al 1996 ,Streuli 1999). In vivo, ovarian follicles are surrounded by a typical basement membrane containing components such as laminin, type IV collagen, heparin sulphate proteoglycans and fibronectin, whose composition has been shown to change during follicular growth and atresia (Rodgers et al 1999 and 2003). Individual ECM components such as type I collagen, fibronectin, laminin and heparin are able to alter granulosa cell function, even in the absence of exogenous growth factors. Moreover these components regulate granulosa cell shape, survival,

proliferation and steroidogenesis in a specific and controlled manner (Huet et al 2001).

It would appear therefore that the ovarian ECM does more than just provide a framework to support ovarian structure, it serves to provide a specialised microenvironment for specific ovarian cells. Changes in this microenviroment can influence gene expression and cell migration, proliferation, differentiation and atresia (Smith et al 1999).

One of the means by which components of the ECM are able to regulate cell function is through interaction with integrins (specific receptors for ECM components on the cell surface). Culture of rat granulosa cells on fibronectin or laminin, but not type 1 collagen, promotes differentiation into a luteal phenotype whereas an antibody to the integrin β1 subunit blocks granulosa cell differentiation (Aten et al 1995). In addition, many growth factors are constitutively secreted and sequestered in an inactive form in the ECM or in association with specific binding proteins where they can subsequently be liberated by proteolysis. Examples include fibroblast growth factor, transforming growth factor β, platelet derived growth factor, hepatocyte growth factor and IGFBP 3 (Smith et al 1999). The follicular basal lamina also has a function in controlling the selective filtration of molecules in and out of the follicular fluid both by means of electrical potential (Hess et al 1998), and physical size (Shalgi et al 1973), allowing it to determine, in part, the milieu of factors to which granulosa cells and the oocyte are exposed.

Remodelling of the ECM is carried out by two main groups of enzymes, the matrix metalloproteinases (MMP) and the plasminogen activator/plasmin family. In the bovine it has been shown that specific MMP and plasminogen activator system components are regulated in a temporally unique and cell specific fashion in the preovulatory follicle in response to a ovulatory stimulus (Bakke et al 1999, 2000, Dow et al 1999, 2000a, 2000b). Throughout follicular growth the composition of the basal lamina changes. Collagen type IV declines (Frojdman et al 1998) during development while laminin β1 is transiently expressed at the preantral stage, and α1, β2, and γ1 appear to be more highly expressed in preantral and antral follicles (Van Wezel et al 1998). Nidogen 1 and perlecan are

not detectable in primordial follicles but are expressed later in follicle development (MacArthur et al 2000). The significance of the decreased collagen and increased laminin content of the basal membrane is not known, but the upregulation of perlecan may be significant since it has the ability to bind a number of growth factors.

The follicular fluid of antral follicles contains proteins and soluble ECM molecules, including proteoglycans. Among those proteglycans identified are versican, decorin (MacArthur et al 2000, Eriksen et al 1999) and hyaluronan (Salustri et al 1992, Schoenfelder and Einspanier 2003). Their roles in follicular fluid are as yet poorly understood, although the role of hyaluronan in the expansion of the cumulus oophorus complex in ovulating follicles has been well studied (Richards 2005).

1.6 Ovulation and Luteinisation

1.6.1 Morphological changes associated with luteinisation

Ovulation is the unique process by which mature ovarian follicles respond to the surge of luteinising hormone and rupture to release fertilisable oocytes. The LH induced transition of a preovulatory follicle to one that can ovulate is a complex multi-step, multi-gene process. The gonadotrophin surge is the physiological trigger that stimulates ovulation of preovulatory follicles, LH activates adenylyl cyclase via its receptor and stimulates the A kinase pathway, inducing in granulosa cells of preovulatory follicles the rapid and transient expression of specific genes that have been shown to be critical for ovulation (Natraj and Richards 1993). During ovulation functional changes occur in multiple ovarian cell types within the follicle (granulosa and thecal cells) and the stroma (fibroblasts and endothelial cells), as well as the ovarian surface epithelium (Espey and Lipner 1994). In addition ovulation is characterised by the recruitment and invasion of inflammatory cells, such as leukocytes (neutrophils) and macrophages (Norman and Brännström 1994), and can be regulated by cytokines that are secreted by, and act on, immune cells and ovarian cells (Norman and Brännström 1994).

Expansion of the cumulus oocyte complex is a prelude to ovulation which has been examined in many species (Dekel et al 1978, Dekel and Phillips 1979, Flechon et al 1986). Although an extracellular matrix appears to accumulate gradually during the preovulatory interval, complete coverage of the cumulus cells with hyaluronan is achieved only a few hours before ovulation. Expansion of the cumulus oophorus is associated with the interruption of gap junctions and a reduction in the physical integrity of the cumulus oocyte complex (Anderson and Albertini 1976). These modifications of the cumulus oocyte complex alter the cellular mechanisms of the granulosa-oocyte interface affecting the bi-directional exchange of paracrine signals to and from the oocyte (Buccione et al 1990). These signals are primarily transferred by means of granulosa cell transzonal projections (TZPs) that transverse the zona pellucida and terminate on

the oocyte cell surface (Motta et al 1994). Cumulus cell uncoupling from the oocyte involves TZP retraction and remodelling during maturation of cumulus oocyte cell complexes (Suzuki et al 2000, Albertini and Rider 1994).

Prior to ovulation, but after the preovulatory LH surge, follicular diameter increases and the follicular wall becomes slightly folded (Priedkalns et al 1968, McClellan et al 1975). In addition gap junctions among granulosa cells decrease, the cells disperse, and the cumulus oocyte complex becomes free floating (Murdoch 1985, Murdoch and Cavender 1987, LeMarie 1989). After ovulation in sheep there is no evidence of mitotic figures among luteinised granulosa cells; however, evidence of mitosis has been observed in theca interna cells, endothelial cells and fibroblasts (McClellan et al 1975). The majority of cells undergoing mitosis include endothelial cells and fibroblasts, which are reported to migrate into the developing corpus luteum (Pedersen 1951). The granulosa cells undergo ultrastructural changes associated within increased steroidogenic activity. The cells hypertrophy and differentiate into the large granulosa lutein-cells, as discussed previously (Fawcett et al 1969). Cytostructural changes include expansion of the smooth endoplasmi reticulum, increased size of the golgi apparatus and an increased number and complexity of mitochondria (Priedkalns et al 1968, Cavazos et al 1969, Fawcett et al 1969, Enders 1973, McClellan et al 1975).

The remodelling process of ovulation also requires structural changes within the extracellular matrix, where regulated dissolution of matrix proteins occurs. At the time of ovulation a precise area of the ovarian surface adjacent to the apical region of the ovulatory follicle disintegrates to allow release of the oocyte. Subsequent structural reorganisation and remodelling of the follicle occurs as the granulosa and thecal cells luteinise. The basement membrane between the granulosa and thecal layers undergoes dissolution and rupture and collapse of the follicular wall results in the mural granulosa forming folds which protrude into the residual follicular lumen (O'Shea et al 1980). Thecal capillaries expand by sprouting into the avascular granulosa compartment to form a dense network of sinusoidal capillaries surrounding the luteinised granulosa cells. The majority of dividing cells in the developing CL are endothelial with the proliferation rate

being even more intense than that found in malignant neoplasms (Stouffer et al 2001, Machelon and Emilie 1997). The folding of the follicular wall allows migration of cells into the more central areas of the developing corpus luteum. Connective tissue and endothelial cells from the theca interna rapidly migrate into the fibrin rich ovulation site to form a primitive network of neovasculature (Goede et al 1998). Interstital (type 1) collagen constitutes the primary fabric of the follicular theca and tunic albuginea. Basement membranes that circumscribe thecal capillary beds and support mural granulosa and ovarian surface cells are comprised of type IV collagen (Eyre et al 1984). Type IV collagen forms a flexible mesh like scaffold to which matrix consitituents attach (Murdoch 2000). Collagen breakdown and cellular death (apoptosis and inflammatory necrosis) within the apex of the preovulatory follicle are the hallmarks of the impending ovulation (Andreasen et al 2000). After ovulation, growth within the corpus luteum can exceed the growth rate of most rapidly growing tumours (Reynolds and Redmer 1998). The degeneration of the follicular basement membrane over the periovulatory period allows theca cells and vascular elements, as well as connective tissue cells, to invade the previously avascular membrana granulosa (Van Blerkholm and Motta 1978, Mori et al 1983, Murphy 2000). The subsequent consolidation of these cells results in a functional corpus luteum. In a mature CL the capillary network pervades the organ, such that each luteal cell is in direct contact with two to four capillaries (Goede et al 1998). These anatomical observations show that CL formation requires the development of an extensive blood vessel network and suggests that mediators of angiogenesis must play an important role in its formation.

1.6.2 Granulosa cell differentiation: follicular lineage of luteal cells

Corpora lutea are a continuation of follicular maturation and form from the remaining follicular cells after ovulation. It has been known for decades that the preparation of luteal cells for the synthesis of progesterone begins before ovulation (McNatty and Sawers 1975, McNatty 1979), therefore the mechanisms

associated with luteinisation are not dependant on follicular rupture, and neither does successful ovulation guarantee normal luteal development and function.
The corpus luteum is made up of a heterogenous population of cells with distinct morphological appearances. These cell types include endothelial cells, fibroblasts and pericytes, but the steroidogenically active cells are the small and large luteal cells, responsible for luteal steroidogenesis. It is generally accepted that in mammals granulosa cells differentiate into large luteal cells and theca cells into small luteal cells (O'Shea 1987). The fate of granulosa cells has been investigated in studies utilising both morphological and immunological approaches. In sheep, the number of granulosa cells within preovulatory follicles (O'Shea et al 1985) approximates the number of large luteal cells (Rodgers et al 1984, O'Shea et al 1986). Because ovine granulosa cells undergo very little or no mitosis after ovulation (McClellan et al 1975) this suggests the differentiation of granulosa cells into large luteal cells. In addition theca and granulosa cells incubated in the presence of forskolin plus insulin exhibit the morphological and function characteristics of small and large luteal cells isolated from the corpus luteum (Meidan et al 1990). In the case of the granulosa cells this includes the high basal secretion of progesterone, reduced LH-induced progesterone secretion, and secretion of oxytocin, similar to large luteal cell function. Finally monoclonal antibodies against surface antigens of bovine granulosa cells have been shown to bind primarily to large luteal cells (Alila and Hansel 1984)

1.6.3 Molecular and genetic response to luteinisation hormone

The LH surge rapidly initiates the terminal differentiation of granulosa cells to luteal cells. Beginning within 4 h and complete by 12 hrs of exposure to LH, granulosa cells cease to divide (Robker and Richards 1998). During this time granulosa cells are completely programmed to become luteal cells. They acquire and maintain a stable luteal cell phenotype, as characterised by the constitutively elevated expression of genes such as P450scc (Richards et al 1986, Oonk et al 1989).

Although the LH surge simultaneously initiates the processes of ovulation and luteinisation, these events are functionally dissociated. In fact, it is critical that the events associated with and controlling ovulation preceed those that dictate and finalise the genetic program for luteinisation. If the events of luteinisation occur too rapidly, as in PDE4D (a cAMP specific phosphodiesterase involved in feedback regulation of cAMP levels) null mice (Jin et al 1999), or if the events associated with ovulation are impaired or delayed, as in PR null mice (Lydon et al 1995), oocytes can be trapped within a functional corpus luteum. To this end it has been noted that in species with differing ovulatory time spans following the LH surge, expression of COX-2 occurs at a similar time prior to follicle rupture (Liu et al 1997, Boerboom and Sirois 1998).

It has been well documented that the effect of the LH surge is evident only in preovulatory follicles while the remaining pool is apparently unaffected. The acquisition of the correct receptor is involved in this selective effect of LH (Peng et al 1991), but differential distribution of the gonadotrophin due to increased follicular blood vessel development and permeability is also involved (Zeleznik et al 1981), a condition strictly dependant on the ability of the follicle to sustain a local production of VEGF.

1.6.3.1 Follicular dissemination of the LH signal

It is perhaps surprising that there is an absence of LH receptors on the cumulus oocyte complex (Mattioli et al 1994), consequently alternative means of signalling have to be generated by LH within this region. A series of signals appear within the COC with a precise time schedule following the LH surge. The first of these appears to be an immediate rise in intracellular Ca levels within follicular somatic cells (Davis et al 1987), followed within minutes by a Ca rise within the oocyte (Mattioli et al 1998).

Following this another second messenger, cAMP, rapidly shows increased levels within the COC (Moor et al 1981, Yoshimura et al 1992, Mattioli et al 1994). This messenger has a complex regulatory role. Levels of cAMP within cumulus cells and oocytes, determine the transcriptional response to this messenger (Mattioli et al 1996). Activation of oocyte PKA mediates the suppressive effect

on germinal vesicle breakdown (GVBD), while the activation of PKA that results within cumulus cells, resulting from gonadotrophin stimulation, is responsible for the induction of maturation (Mattioli and Barboni 1998).

The next event to occur is the depolarisation of the cumulus-oocyte complex, happening a few hours after exposure to LH (Mattioli et al 1996). The LH-induced activation of PKA/PKC results in cumulus-corona cells undergoing progressive depolarisation of membrane potential (Barboni and Mattioli 1996), a response peculiar to cumulus cells (Mattioli et al 1991). The coupling of cumulus cells and the oocyte by gap junctions ensures that depolarisation originating in the somatic compartment is rapidly extended to the oocyte (Mattioli et al 1990). This depolarisation can act through a number of methods to influence oocyte maturation. Firstly it can induce the translocation of PKC from the plasma membrane to the nuclear membrane where it could contribute to the process of GVBD (Kong et al 1991). Secondly its effect on voltage gated ion channels may provide a mechanism for producing a sustained rise in intracellular calcium. The presence of Ca channels on the oolemma (Mattioli et al 1998a) and their requirement for gonadotrophin-induced maturation has been demonstrated by the ability of Ca channel blockers to reversibly arrest meiotic progression of the oocyte (Mattioli et al 1998a, Mattioli and Barboni 2000). Finally, the depolarisation also serves to influence the permeability of the junctional network between the follicular cells. Prior to the LH surge there is a permanent electrical gradient of positive ions from the oocyte to the cumulus cells (Mattioli et al 1990). Depolarisation eliminates this transjunctional potential leading to a transient increase in both metabolic and electrical coupling (Mattioli and Barboni 2000).

One of the more puzzling aspects of LHR expression is its restriction to the mural granulosa cells (Mattioli 1994); cumulus cells and oocytes expressed few or no LHRs and are insensitive to direct LH stimulation. The connection between mural and cumulus granulosa cells and the oocyte via gap junctions may allow the flow of intracellular mediators from the periphery to the core of the follicle (Tasfriri and Dekel 1994). Alternatively factors released by mural granulosa cells may convey the LH stimulus to cumulus cells and the oocyte.

The epidermal growth factor (EGF)-related proteins amphiregulin (AR), epiregulin (EPI), and betacellulin (BTC) are three growth factors which have been shown to mediate LH activity with the follicle via EGF receptor (Park et al 2004). They have functions as short range mediators in tissue remodelling and cell growth and are potential intermediates in G protein-coupled receptor signalling (Prenzel 1999). They show rapid and transient expression within 1-3 hours following an ovulatory dose of hCG, with EPI mRNA remaining elevated for up to 12 hours (Park et al 2004). Although their expression is limited to the mural granulosa cells they have been shown in vitro to be potent stimulators of oocyte maturation and cumulus expansion in cumulus oocyte complexes (COCs) which have been denuded of mural granulosa cells and shown to be insensitive to LH (Park et al 2004). This confirms their role as paracrine mediators of LH signals during ovulation.

The junctional area between mural and cumulus cells is rapidly lost after LH stimulation while heterologous junctions between the corona radiata and the oocyte remain unaltered during most of the maturation period (Larsen et al 1987). This indicates that the persistence of cell support provided by the corona cells represents an essential prerequisite for the maturation of the oocyte (Mattioli et al 1988)

1.6.3.2 Genomic response to the LH signal

LH acts on mature follicles to terminate the program of gene expression associated with folliculogenesis. The transcription of genes that control granulosa cell proliferation, IGF-1 (Zhou et al 1997), FSHR (Richards 1979), oestrogen receptor β (Sharma et al 1999), cyclin D2 (Robker and Richards 1998a and 1998b), and others (Richards 2001) is rapidly turned off. Expression of genes encoding steroidogenic enzymes for oestrogen synthesis is also rapidly terminated (Richards 1994). Not surprisingly the targeted disruption of genes obligatory for follicle maturation precludes ovulation or luteinisation. Specifically in mice null for FSHR, FSHβ, LHR, LH, IGF-1, IGF-1R, leptin, c-fos, cyclin D2, Erβ, Erα, aromatase, or the corepressor RIP 140, either follicular growth is arrested at a developmentally immature stage or further growth results

in the formation of cystic follicles (Richards 2001,Elvin and Matzuk 1998, Orly 2000, Couse and Korach 1999, Hasegawa et al 2000, White et al 2000, Johnson et al 1992). In conjunction with the termination of specific gene expression in mature follicles, LH induces genes involved in ovulation. These include the genes for progesterone receptor (Park and Mayo 1991, Natraj and Richards 1993), cycloxygenase-2 (Sirois and Richards 1993), CAAT enhancer binding protein β (C/EBPβ) (Sirois and Richards 1993), early growth regulatory factor (Egr-1) (Espey et al 2000), and pituitary adenylyl cyclase activating peptide (PACAP) (Park J. et al 2000, Park H. et al 2000). Genes involved in luteinisation are then also induced by the LH surge. Some of these include the cell cycle inhibitors p21 CIP and p27KIP, steroidogenic enzymes StAR and P450scc, specific transcription factors Fra2/JunD, protein kinases, and other factors (Richards 2001, Sharma and Richards 2000).

Egr-1, C/EBPβ, and progesterone receptor (Espey et al 2000, Sirois and Richards 1993, Park and Mayo 1991, Natraj and Richards 1993) are induced rapidly but expressed only transiently, with peak levels of message and protein occurring approximately 4 hours after the LH surge. Other transcription factors, such as the activator protein-1 family members (eg. C-fos, c-jun, Fra2, and JunD), are induced rapidly and remain elevated during the postovulatory luteal phase (Sharma and Richards 2000). Each of these mediators appears to be involved in the functional activity of the granulosa cells of ovulating follicles.

The LH-induced induction of progesterone receptor (PR) (Park and Mayo 1991, Natraj and Richards 1993) and prostaglandin endoperoxide synthase-2 (PGS-2) or cyclooxygenase-2 (COX2) (Richards 1994, Sirois and Richards 1992, Wong and Richards 1991) has led to them being implicated in ovulation. This was initially based on the timing of their synthesis following the LH surge (Richards 1994, Espey and Lipner 1994, Espey 1980) and by the effects of specific inhibitors and antagonists on their synthesis or action (Espey and Lipner 1980, Espey 1980). More recently inhibitors of prostaglandin synthesis and progesterone action have been used to block the rupture of follicles in vitro (Mikuni et al 1998, Rose et al 1999). It has also been observed that the expression of PR (Park and Mayo 1991, Natraj and Richards 1993) and PGS-2

(Sirois and Richards 1992, Wong and Richards 1991) is selectively induced by LH in preovulatory follicles. The fate of the oocytes in both PR and PGS-2 null mice is to remain within morphologically and functionally normal corpora lutea which have failed to ovulate due to the absence of the proteolytic cascade (Robker et al 2000). Mice null for PR fail to ovulate even when stimulated by exogenous hormones, findings which support other studies implicating progesterone as a key player in the ovulatory process (Lydon et al 1995, Rose et al 1999, Pall et al 2000). Despite this failure of ovulation, the expression of COX-2, cumulus expansion, and luteinisation proceed normally (Robker et al 2000). Thus the molecular targets of PR appear to be those controlling rupture of the follicle, rather than those of luteinisation.

The expression of several molecules including ECM components such as fibronectin and collagen have been reported to change in luteinising granulosa cells during corpus luteum formation (Amsterdam et al 1989, Honda et al 1997, Yamada et al 1999). The expression of these molecules is regulated by gonadotrophin and/or cytokines. For example, the expression of low density lipoprotein receptor, integrin 5α and collagen type IV is enhanced by hCG in luteinising cell cultures (Honda et al 1997, Yamada et al 1999, Golos et al 1986, Golos and Strauss 1987) whereas dipeptidyl peptidase IV and leukocyte functional antigen-3 are induced, not by hCG, but by inflammatory cytokines such as interleukin (IL)-1α and tumour necrosis factor α (Fujiwara et al 1994, Hattori et al 1995). On the other hand the expression of endothelin converting enzyme-1, which is a cell surface endopeptidase and activates proendothelin peptide, is promoted by hCG, IL-1α and TNF-α.

1.6.3.3. Transcription factors implicated in luteinisation

Early growth response 1(Egr1)

As discussed above the genomic response usually includes the induction of immediate-early transcription factor genes such as early growth response protein-1 and/or the *c-fos* and *c-jun* genes (Gashler and Sukhatme 1995, McMahon and Monroe 1996). Egr-1 is a zinc finger transcription factor. It often

binds overlapping sequences with Sp1, an important transcription factor for several ovarian expressed genes, such as Sgk, P450scc, MMP 14 and p21CIP (Pardali et al 2000, Alliston et al 1997, Prowse et al 1997, Haas et al 1999). Egr-1 can exert positive transcriptional events or negative regulation of Sp1 (Richards et al 2002). Mice null for Egr-1 have impaired synthesis of LH and fail to ovulate or form corpora luteal, although whether this is due to a lack of pituitary LH or ovarian defects is not known (Lee et al 1996). Ovarian expression of Egr-1 mRNA and its protein product appears to peak after approximately 4 hours following the ovulatory gonadotrophic surge and does not return to basal level until 12-24 hours later (Espey et al 2000). This transcriptional factor has the unique ability to regulate the transcription of some genes positively while affecting other genes negatively (Gashler and Sukhatme 1995, Beckmann and Wilce 1997). Among the genes induced by Egr-1 is the slightly delayed induction of NGFI-A-binding proteins, a family of co-repressors that bind directly to Egr-1 and repress Egr-1 mediated transcription (Qu et al 1998, Swirnoff et al 1998, Silverman et al 1999), thus ensuring that the Egr-1 mediated cascade is a transient effect. Some of the physiologically relevant genes that are now recognised as targets for Egr-1 include fibroblast growth factor, tumour necrosis factor, platelet derived growth factor, the interleukin genes, cell surface adhesion proteins and matrix metalloproteinases (Gashler and Sukhatme 1995, McMahon and Monroe 1996, Dorn et al 1999, Fitzgerald and O'Neill 1999, Haas et al 1999, Bourguignon et al 1998, Takahashi et al 1999, Yu and Stamenkovic 1999). This has led to Egr-1 being proposed as a mediator of the transient events that cause degradation and rupture of a follicle.

Nerve growth factor induced B (NGFI-B)

NGFI-B (also called Nur77), an orphan nuclear receptor of transcription factor, is rapidly and transiently induced by the LH surge in granulosa cells in the rat (Park et al 2001). The induction of NGFI-B is mediated through both ERK1/2 (Stocco et al 2002) and PKC (Park et al 2003). It is an immediate early response gene whose expression is regulated by a variety of extracellular stimuli and it encodes transcription factors regulating the expression of other genes, ultimately

culminating in phenotypic changes (Herschman 1991). It has been implicated in the regulation of expression of steroidogenic enzymes (Wilson et al 1993, Havelock et al 2005) and mediation of apoptosis (Liu et al 1994). In the ovary, expression has been reported in corpora lutea (Richards 1994) and in granulosa cells following the LH surge (Stocco et al 2000). It has also been shown to induce 20α-HSD expression during prostaglandin mediated luteolysis (Stocco et al 2000) and to regulate the expression of 3βHSD2 (Havelock et al 2005). Because the spatial pattern of NGFI-B matches with the expression patterns of P450scc (Zlotkin et al 1986) and StAR (Ronen-Fuhrmann et al 1998) in cells engaged in steroidogenic activity, it has been suggested that NGFI-B may be correlated with terminal commitment of cells for steroidogenic differentiation (Park et al 2003).

Two other members of the NGFI-B subfamily, Nurr1 and Nor1 are also induced by LH in the granulosa cells of preovulatory follicles, although, unlike NGFI-B, they are not induced in the theca cells at the same stage (Park et al 2003). Whether they perform a similar function as NGFI-B is unknown.

CAAT enhancer binding protein beta (C/EPBβ)

C/EPBβ is another transcription factor known to be induced by LH (Sirois and Richards 1993). All of the C/EPB family members are expressed in the ovary (Piontkewitz et al 1993, Sirois and Richards 1993). Mice null for C/EPBβ exhibit impaired ovulation and luteinisation (Sterneck et al 1997, Pall et al 1997) with the ovaries displaying abnormal vascular morphology and haemorrhagic follicles as well as entrapped oocytes. Null mice also lack corpora lutea (Dekel et al 1988, Sterneck et al 1997). A possible target gene for C/EBPβ is prostaglandin synthase-2 (PGS-2) (Sirois and Richards 1993).

Wnt and Frizzled family

Wnt-4, a member of the Wnt family of extracellular signalling proteins, has already been mentioned as having an important role in the regulation of foetal gonad development. Several members of the Wnt and Frizzled (Fz) families and downstream components of the Wnt-Fz signalling pathway are known to be

expressed within the mature rodent ovary (Hsieh et al 2002, Ricken et al 2002). Wnt-4, Fz-4 and Fz-1 have been found to be regulated by gonadotrophins and steroids with Fz-1 specifically induced by the LH surge, as indeed is the Fz-1 receptor, both appearing at high level in granulosa cells 8-12 hours after exposure to LH/hCG. Wnt-4 and Fz-4 are present in terminally differentiated luteal cells. These signalling factors are involved in processes such as cell fate specification, proliferation, differentiation and tissue patterning (Cadigen and Nusse 1997, Miller et al 1999, Richards et al 2002). Secreted frizzled related protein 4 (sFRP-4) expression is also induced in granulosa cells under the influence of LH and maintained in luteinised cells by stimulation from progesterone receptor ligand (PRL) (Hsieh et al 2003). Interestingly, the expression pattern for sFRP-4 in cultured luteinised granulosa cells is similar to the expression pattern of P450scc (Hsieh et al 2003). A potential role for Wnt/Fz regulation of steroidogenesis is indicated because Wnt-4 and Fz-4 are expressed in luteal cells. In addition gonads of female mice null for Wnt-4 misexpress the steroidogenic enzymes 3β HSD and 17α-hydroxylase (Vainio et al 1999). The Wnt/Frizzled pathways and the BMP pathways have been shown to impact and antagonise each other in many aspects of development (Schneider and Mercola 2001, Marvin et al 2000) and, thus, may act to modify the actions of various BMP like molecules (GDF-9, TGF-β, BMP 15) or FGF molecules (Kawakami et al 2001) in the ovary.

1.6.3.4. Cytokines, role and regulation

After ovulation, the basement membrane is destroyed and immune cells and endothelial cells rapidly invade the luteinising granulosa cell layer in a process resembling tissue inflammation (Espey 1980). Indeed ovulation has many features in common with an inflammatory reaction, including the central participation of leukocytes and classical inflammatory mediators such as eicosanoid, histamine and bradykinin (Espey 1980). Detection of leukocytes in ovarian tissue has revealed accumulation of some specific subsets of these cells in the preovulatory follicle at the time of ovulation (Brännström and Norman

1993). It has been reported that peripheral blood leukocytes increase the number of LH-induced ovulations in rat ovaries in vitro (Hellberg et al 1991). A function for these cells at ovulation is likely because depletion of circulating neutrophils in rat ovaries decreases the ovulation rate (Brännström et al 1995). In addition, the cellular content of human follicular fluid has been shown to consist of 5-15% macrophages (Loukides et al 1990) which are involved in the production of interleukin 1β (Machelon et al 1995). There is evidence that cytokines are involved in the inhibition and stimulation of follicular responsiveness to gonadotrophins (Gougeon 1994). Cytokines, therefore, seem to play a pivotal role in the regulation of the development and atresia of follicles in the ovary (Jasper et al 1996, Kaipai and Hsuch 1997).

Tumour necrosis factor α

One of the most frequently studied cytokines in the ovary is tumour necrosis factor α (TNFα), which has the capacity to elicit inflammatory responses (Kondo and Sauder 1997). Because ovulation has similarities to the inflammatory process (Espey 1994), and TNFα is produced in the ovary (Zolti et al 1990), its function there is usually discussed with respect to ovulation (Murdoch et al 1999). However it also has roles in steroidogenesis, proliferation, apoptosis, and luteolysis in several species (Terranova and Rice 1997), and its effects on follicular development and differentiation are due to a receptor-mediated pathway (Balchak and Marcinkiewicz 1999, Veldhuis et al 1991, Roby et al 1999). In several species TNFα has been shown to decrease gonadotrophin-upregulated progesterone production (Terranova and Rice 1997) and suggests that it has an inhibitory effect on luteinisation. It also has the ability to induce apoptosis in mouse granulosa cells (Quirk e al 1998) through a pathway that involves modulation of Bcl-2 (Sasson et al 2002), and yet can also increase human granulosa-lutein cell number (Yan et al 1993). The coexistance of the effects of TNFα on proliferation and apoptosis has been suggested to be due to the differential expression of TNFα receptors I and II (Prange-Kiel et al 2001, Tartaglia et al 1993). It has recently been suggested that the luteolytic effect of TNFα may be mediated by inhibition of StAR, the key regulatory protein in

progesterone production (Lin et al 1996) or by indirectly decreasing LHR expression in addition to stimulation of PGF2α production (Lin et al 1996, Chen et al 1999)

In human and rat granulosa-lutein cells, glucocorticoids can attenuate the apoptotic action of TNFα (Sasson et al 2002) and may therefore play a part in the healing process of the ovarian follicular tissue during follicular rupture and corpus luteum formation.

Interleukins

Interleukins are best known for their immune and inflammatory functions but a growing body of evidence has implicated their involvement in the periovulatory follicle. Interleukin 1α and interleukin 1β mRNA have been localised to the theca, cumulus and oocyte of the murine follicle. In the mouse, ovarian synthesis of IL-1α and IL-1β is first observed in the theca interna of growing follicle and the oocyte (Simon et al 1994, Terranova and Rice 1997). At the time of preovulatory maturation, after the LH surge, high levels of IL-1α and IL-1β are observed in cumulus cells (Simon et a 1994). Type I interleukin receptor (IL-1R1) is synthesised by thecal cells from growing follicles. Before ovulation it is expressed by cumulus and granulosa cells, and is abundantly expressed in the mouse oocyte throughout follicular development (Simon et al 1994). The potential functions of interleukins in the ovary are wide ranging, IL-1β in the rat has been shown to induce ovulation and potentiate the inductive ovulatory effect of LH (Brännström et al 1993), they are also involved in the production and activation of proteolytic enzymes, prostaglandin production, nitric oxide production, cellular metabolism, and steroidogenesis (for review Gerard et al 2004), frequently in a species-specific manner.

Ovarian granulosa luteal and stromal cells also express interleukin (IL)-8 mRNA and produce IL-8 protein with the protein level being increased by hCG administration (Arici et al 1996). Its importance in follicular growth and ovulation has been shown by the inhibition of hCG induced ovulation following administration of anti-IL-8 antiserum (Ujioka et al 1998). IL-8 is a chemotatic cytokine secreted by a variety of cells in response to inflammatory stimuli such

as IL-1, TNFα, or lipopolysaccharide and acts in the recruitment and activation of neutrophils as well as in angiogenesis (Herbert et al 1991, Clark-Lewis et al 1993, Strieter et al 1995). It has been shown to induce ovarian vasodilation, follicle development (Goto et al 1997) and to increase the density of capillary vessels around developing follicles (Goto et al 2002) this suggests a role as a potent angiogenic factor in neovascularisation of the developing/luteinising follicle. Interleukin 6, whose production is induced by both TNFα and IL-1, has also been detected in follicular fluid (Büscher et al 1999)

An interleukin 1 receptor antagonist (IL-1-ra) has also been found in follicular fluid in concentrations comparable to serum concentrations or higher (Büscher et al 1999). This could serve to suppress the IL-1α and IL-1β mediated reactions of the immune system against further ovulatory tissue damage, and may represent an attempt to limit the reaction cascade.

Melanoma cell adhesion molecule

Human luteinising granulosa cells express melanoma cell adhesion molecule (MCAM), which is upregulated by LH/hCG and cytokines during luteinisation (Yoshioka et al 2003). MCAM has been shown to mediate cell-endothelial cell interaction (Xie et al 1997) and may play a role in neovascularisation during corpus luteum formation in the human ovary. Its expression can be induced by TNFα (Yoshioka et la 2003) which is known to be an angiogenic and inflammatory cytokine (Ferrara 2000, Kim et al 2002), and by IL-1α (Yoshioka et al 2003). These cytokines have also been reported to stimulate other angiogenic factors such as VEGF and angiopoietin (Jung et al 2001, Scott et al 2002). The production of inflammatory cytokines by luteinising granulosa cells plays a role in promoting the production of soluble angiogenic factors during lutcal development.

Granulocyte colony stimulating factor

Granulocyte colony-stimulating factor (G-CSF) is known to have specific effects on the proliferation, differentiation and activation of haematopoietic cells (Mielcarek et al 1996, Visani and Manfroi 1995). It exerts these biological

effects through binding to specific, high affinity receptors (Nagata and Fukunaga 1993, Fukunaga et al 1990, Demetri and Griffin 1991, Shimoda et al 1993, Nicholson et al 1994, Nagata and Fukunaga 1991) that have been reported on cells of the granulocytic lineage, platelets (Shimoda et al 1993), monocytes and lymphocytes (Nicholson et al 1994, Nagata and Fukunaga 1991, Avalos 1996). Its expression in human luteinised follicular granulosa cells has also been demonstrated, indeed both G-CSF and G-CSF receptor (G-CSFR) are expressed (Salmassi 2004). Granulosa cells therefore seem to represent one of the sources and targets of G-CSF around the periovulatory peroid, acting via autocrine or paracrine mechanisms.

Ovulation, therefore, appears to be a LH-induced, cytokine-regulated inflammatory process, followed by an anti-inflammatory response mediated by interleukin receptor antagonists and glucocorticoids.

1.6.3.5. Steroidogenesis

Terminal granulosa cell differentiation, like differentiation in other cells, is accompanied by cessation of proliferation, altered gene expression, and morphological changes (Nanbu-Wakao 2000). A hallmark of terminal granulosa differentiation after ovulation is the rapid loss of cytochrome P450 aromatase, the rate-limiting enzyme that converts testosterone to oestradiol (Fitzpatrick et al 1997, Hickey et al 1998). The process of follicular luteinisation is also associated with marked changes in the expression patterns of other steroidogenic enzymes, most notably an increase in StAR and P450scc which promote enhanced progesterone synthesis (O'Shaughnessy et al 1990, Fortune 1994, Richards 1994, Sandhoff and Maclean 1996, Ronen-Fuhrmann et al 1998). Progesterone biosynthesis requires only two enzymatic steps; the conversion of cholesterol to pregnenolone, catalysed by P450 side chain cleavage enzyme (P450scc) located on the inner mitochondrial membrane, and its subsequent conversion to progesterone, catalysed by 3β-hydroxysteroid dehydrogenase (3β-HSD) present in the smooth endoplasmic reticulum. Both these organelles show increased size

and/or number and complexity in periovulatory granulosa cells (Priedkalns et al 1968, Cavazos et al 1969, Fawcett et al 1969, Enders 1973, McClellan 1975).

The loss in 17β oestradiol biosynthetic capacity after the LH surge has been explained by this marked decrease in the expression of the key steroidogenic enzymes involved in the follicular production of active oestrogen. However, it is also now known that members of the 17βHSD family, in particular 17βHSD4, which are key regulators of the biological potency of androgens and oestrogens in mammals, show an increase in expression that accompanies hCG induction of ovulation in preovulatory follicles (Brown 2004). This upregulation occurs both in theca and granulosa but is clearly more pronounced in the granulosa cell compartment (Brown 2004). The 17βHSD family reversibly catalyse the interconversion of less active 17-ketosteroids such as androstenedione and oestrone into the more active 17β-hydroxysteroids such as testosterone and 17β-oestradiol (Penning 1997, Peltoketo et al 1999). The induction of an 17β-oestradiol inactivating enzyme such as 17βHSD4 may represent a novel and complementary mechanism that can contribute to the reduction in active oestrogens within the follicle during luteinisation.

The steroidogenic process first requires the coordinated regulation of cellular uptake, transport and utilisation of cholesterol (Miller 1988). Blood borne low density lipoprotein (LDL) taken up by low density lipoprotein-receptor (LDL-R) is the primary source of intracellular sterol substrate in some mammals such as primates and pigs (Chaffin et al 2000, Grummer and Carroll 1988, LaVoie et al 1997, Soumano and Price 1997). LDL-R expression increases in preovulatory granulosa-luteal cells and remains elevated in the corpus luteum (Chaffin et al 2000, Yamada et al 1998, Garmey et al 2000). In vitro FSH, LH, insulin and IGF-1 all drive the LDL-R gene and protein expression and concomitant progesterone production (Golos and Strauss 1987, Grummer and Carroll 1988, LaVoie et al 1999, Sekar et al 2000, Veldhuis 1988, Veldhuis et al 1986).

In cattle and rodents high density lipoprotein (HDL) is regarded as the more important for maintaining luteal steroidogenesis (Bao et al 1995, O'Shaughnessy and Wathes 1985, Temel et al 1997) with the selective uptake of HDL being particularly active in rats and mice (Glass et al 1985, Stein et al 1983) mediated

through specific binding to scavenger receptor class B, type1 (SR-B1) (Temel et al 1997). Mice null for SR-B1 show a significant increase in plasma cholesterol levels and decreased adrenal gland cholesterol content (Rigotti et al 1997). Li et al (1998) demonstrated that SR-B1 expression within the ovary increases dramatically during PMSG induced follicle maturation but is localised primarily to the thecal cells. As luteinisation progresses there is a shift in the cellular localisation of SR-B1 with an increased intensity of the hybridisation signal in the corpus luteum, demonstrating its inducibility in the granulosa cell by hCG.

The StAR protein is known to regulate acute transport of cholesterol from the outer to the inner mitochondrial membrane for conversion to pregnenolone by P450scc (Stocco and Clark 1996). Prior to the LH surge StAR is virtually absent from granulosa cells which are unable to metabolise and synthesise progesterone from cholesterol precursors (Chaffin et al 2000, Kiriakidou et al 1996, Pescador et al 1996). Regulation of StAR gene transcription is the primary mechanism for regulating StAR activity in granulosa cells (Kiriakidou et al 1996) and thereby controlling progesterone production.

Peroxisome proliferator-activated receptors (PPARs) are key regulators of lipid metabolism and cell differentiation (Maloney and Waxman 1999). All known isoforms of PPAR are expressed in the ovary (Braissant et al 1996) but their functions in this tissue remain unclear. While PPARγ is highly expressed in preovulatory granulosa cells, it is down regulated after ovulation, suggesting it may be involved in the differentiation of oestrogen producing granulosa cells, to progesterone producing luteal cells (Komar et al 2001, Lohrke et al 1998). Targeted loss of PPARγ in oocytes and granulosa cells decreases progesterone levels and impairs fertility, consistent with a role for PPARγ in normal ovarian function (Cui et al 2002). PPARγ ligands also stimulate progesterone secretion in luteal cells, evidence for a role of PPARγ in developing and maintaining a differentiated phenotype in luteal cells (Lohrke et al 1998).

A link between activation of PPARγ and suppression of aromatase has been established. The PPARγ ligand troglitazone inhibits aromatase activity and mRNA levels in human ovarian granulosa cells (Mu et al 2000). Recent studies show that activation of both PPARα and PPARγ suppresses aromatase mRNA

and oestradiol levels, but has no effect on P450scc mRNA, (Lovekamp-Swan et al 2003). Both pathways upregulate fatty acid binding protein (FABP) (Lovekamp-Swan et al 2003), a protein associated with the differentiation of oestrogen-producing granulosa cells into progesterone producing granulosa cells (Iseki et al 1995). Levels of FABP have been shown to be differentially altered after hCG induction of ovulation (Leo et al 2001). Only PPARα activation induces 17β-HSD IV, aryl hydrocarbon receptor (AhR), cytochrome P450 1B1 (CYP1B1), and epoxide hydrolase (Lovekamp-Swan et al 2003) all of which have involvement in regulation of oestrogen synthesis (Corton et al 1996, Murray et al 2001, Hattori et al 2000) and are generally thought of as being involoved in xenobiotic activation and metabolism (Murray 2001). Induction of these enzymes likely contributes to decreased oestradiol levels and increased estrone levels produced by granulosa cells in vitro and in vivo (Davis et al 1994a and 1994b). AhR is upregulated after the ovulatory gonadotrophin stimulus in primate granulosa cells, and may be involved in terminal differentiation (Chaffin et al 1999). These observations suggest that PPARα and γ function as regulators of metabolism and differentiation in the granulosa cell demonstrating specific effects on steroidogenesis.

1.6.3.6 Angiogenesis

Apart from during tumour growth and wound healing the adult vascular endothelial epithelium is generally quiescent. The exception to this occurs within the female reproductive system which undergoes cyclical tightly controlled angiogenesis and angiogenic regression regulated by endogenous stimulatory and inhibitory factors (Pepper 1997, Risau 1997, Plendl and Sinowatz 1999). It is mediated by the same proangiogenic factors as tumour angiogenesis but is highly controlled (Smith et al 1993, Nicosia and Villaschi 1999). The development of new ovarian blood vessels is essential to guarantee the necessary supply of nutrients and hormones to promote follicular growth and corpus luteum formation. During follicle formation the granulosa compartment is avascular but the theca layer acquires a vascular sheath that consists of capillary

networks in the theca interna and externa (Stouffer et al 2001). The acquisition of a vascular supply is probably a rate limiting step in the selection and maturation of dominant follicles (Stouffer et al 2001).

As follicles mature angiogenesis becomes accompanied by vasodilation, a functional adaptation to impending ovulation and to the developing thecal endocrine function (Jiang et al 2003). Although angiogenesis prior to ovulation occurs in the thecal layer, granulosa cells exert an important role in this process in that they produce several angiogenic factors that act in the theca. Granulosa cells from pig follicles, when co-cultured with aortic endothelial cells have been shown to significantly enhance endothelial sprouting and capillary elongation (Grasselli et al 2003). The maturation of preovulatory follicles, as a prelude to ovulation and CL formation, require sufficiently well developed microvasculature to deliver adequate levels of hormones and lipoprotein bound cholesterol (Davis et al 2003). During corpus luteum formation, endothelial cells migrate into regions containing luteinising granulosa cells and then form vascular networks among luteal cells. When labelling cells with 3H-thymidine, no less than 30% of the endothelial cells in the forming CL show active DNA synthesis (Gaede et al 1985). An increasing body of evidence suggests that endothelial migration among luteinising granulosa cells is promoted by secretion of soluble angiogenic factors such as bFGF and VEGF (Phillips et al 1990, Yan et al 1993, Reynolds and Redmer 1998). Several works have demonstrated that the production of some angiogenic factors is regulated by oxygen concentration with the induction of VEGF and angiogenin being induced by hypoxic stress in cultures of human luteinising granulosa cells (Friedman et al 1997, Koga et al 2000). The initial growth of the follicle after gonadotrophin stimulation results in inner hypoxia that triggers elevated expression of VEGF in the cumulus and inner granulosa cells. In response to VEGF, vessel permeability increases along with neovascularisation of the follicular periphery, thereby supplying a larger effective dose of LH to that particular follicle (Neeman et al 1997).

Among the angiogenic factors produced by the ovary are basic fibroblast growth factor (Gospodarowicz et al 1985), angiopoietins (Stouffer et al 2001) and VEGF (Robker and Richards 1998).

Basic fibroblast growth factor (bFGF) stimulates endothelial cell proliferation (Bikfalvi et al 1998) and its expression in granulosa and thecal cells of the pig ovary has been shown to be enhanced by eCG administration (Shimizu et al 2003). In the bovine ovary, thecal expression of bFGF has been shown to increase during final follicular maturation, while remaining weak in granulosa cells (Shimizu et al 2003).

In the mouse three angiopoietins have been identified and serve to destabilise existing vessels, loosening the supporting cell matrix and allowing angiogenic factors such as VEGF to stimulate endothelial cell proliferation and migration. They also recruit peri-endothelial support cells to promote vessel maturation and maintain vessel integrity (Stouffer 2001, Tamanini and DeAmbrogu 2004). In the mouse, angiopoietin 1 is expressed in the thecal layer of the preovulatory follicle and then in the granulosa derived luteal cells, angiopoietin 2 expression precedes invading blood vessels, firstly in the theca and then in the granulosa layer (Stouffer 2001).

Vascular endothelial growth factor (VEGF) is a critical regulator of angiogenesis in CL formation (Ferrara and Davis-Smyth 1997). With multiple isoforms (Tamanini and DeAmbrogu 2004) it is expressed during the angiogenic growth phase of CL formation (Ferrara et al 1998, Shweiki et al 1993). VEGF has been localised in the preovulatory follicle and early CL and shown to be stimulated by both LH and IGF-1 in bovine granulosa cells (Schams et al 2001). The VEGF protein in this species is found within granulosa, theca, and some endothelial cells in the preovulatory follicle and early CL (about 24hrs after ovulation) (Schams et al 2001). VEGF mRNA and protein in the primate are not present in granulosa cells of primordial and pre-antral follicles but become evident in the theca layer of antral follicles and in the cumulus granulosa of preovulatory follicles (Stouffer et al 2001). The ovine preovulatory follicle only shows VEGF mRNA expression in the theca interna and theca derived areas of the CL (Redmer et al 2001). In the marmoset VEGF expression starts at the end of the development of secondary follicles and is maximal in tertiary follicles, especially in granulosa cells (Wulff et al 2001). VEGF-A null mice die during early embryonic development, whereas VEGF-B null mice develop with cardiac

abnormalities but are fertile (Argraves and Drake 2005) suggesting isoform specific roles during vasculogenesis.

In the pig and bovine hypoxia will increase granulosa VEGF expression (Bianco et al 2003, Berisha et al 2000), but not in the primate (Martinez-Chequer 2003). LH administration upregulates bovine and primate granulosa VEGF mRNA (Berisha et al 2000, Martinez-Chequer 2003), an effect mimicked by eCG in pigs (Shimizu et al 2002) and hCG in humans (Laitinen et al 1997). In the pig, however, although eCG induced follicular maturation causes granulosa cells to actively secrete VEGF into follicular fluid, this activity is rapidly switched off by an ovulatory dose of hCG (Barboni et al 2000). In this species the organisation for VEGF production is completely reprogrammed after hCG administration when theca cells remain the only source of this angiogenic factor in preovulatory follicles. There is, therefore, considerable variation between species in the localisation and regulation of VEGF expression in the ovary.

Other angiogenic factors expressed within the ovary include nitric oxide (for review Tamanini et al 2003) and epidermal growth factor (Shimizu et al 2002) but many potential angiogenic factors can also be found among the numerous cytokines known to be produced within the ovary (Gaetje et al 1994). As previously stated there is evidence that ovulation shares many features of an acute inflammatory reaction, and pro-inflammatory cytokines contribute to the rupture of the follicle at ovulation and amplify the ovulation rate. Just before the time of ovulation the number of neutrophils increases markedly in the thecal layer. Interleukin-8 (IL-8), a neutrophil-activating factor and potent angiogenic agent which is modulated by steroid and trophic hormones, has been proposed to have a role in periovulatory physiology, particularly in the neovascularisation of the CL (Arici et al 1996). TNF-α is an angiogenic molecule suggested to regulate CL function (Okuda et al 1999, Sakumoto et al 2000). Other factors which are known to have effects on endothelial cell function include interferons, platelet derived growth factor, TGF alpha and beta, nerve growth factor and angiogenin (Pepper 1997).

In order to ensure control, anti-angiogenic factors have been suggested as a potential mechanism of balancing of angiogenic proliferation (Hanahan and

Folkman 1996, Plendl 2000). One potential anti-angiogenic factor may be metalloproteinase inhibitor. The principal step in angiogenesis is degradation of the basement membrane, consequently protease inhibitors are anti-angiogenic because they prevent the breakdown of matrix proteins and thus maintain the integrity of the endothelium (Auerbach and Auerbach 1994). Tissue inhibitors of metalloproteinases (TIMPs) have been indentified as being secreted by granulosa cells of preovulatory follicles under gonadotrophic stimulation (Smith and Moor 1991, Smith et al 1993).

1.6.3.7. Final oocyte maturation: meiotic resumption

The oocyte resumes meiosis in response to the preovulatory LH surge (Callesen et al 1986, Channing et al 1978, Dieleman et al 1983, Ireland and Roche 1982, Masui an Clarke 1979, Peng et al 1991). The question of how the preovulatory gonadotrophin surge acts to trigger resumption of meiosis has yet to be properly answered. The general opinion is that it acts to overcome a molecular inhibitor maintaining meiotic arrest, or alternatively generates local trophic signals within the follicle, or a combination of both. During the meiotic process the nuclear membrane starts to fold, the nuclear pores disappear and then nuclear membrane fragments before disappearing (Kubelka et al 1988, Szollosi et al 1972). These events are known as germinal vesicle breakdown (GVBD) and are the first visible sign of meiotic resumption. Oocyte maturation also involves transformations at the cytoplasmic level that prepare the cell to support fertilisation and early embryonic development. The completion of nuclear maturation alone does not guarantee subsequent embryo development (Sirard et al 1989, Yang et al 1998). Resumption of meiosis in vitro can be instigated spontaneously by separation of the oocyte from its surrounding follicle cells and is associated with a drop in intraoocyte concentrations of cAMP (Dekel 1996). However this ability to resume meiosis is not shared by all ovarian oocytes, and is progressively acquired during oocyte growth (Sorensen and Wasserman 1976, Jelinkova et al 1994).

The mitogen-activated protein kinases are known to have a role in regulating meiosis. In oocytes a MAKP pathway has been implicated as a cell cytostatic factor responsible for the second metaphase arrest in conjunction with a germ cell exclusive kinase, Mos (Masui and Market 1971, Sagata et al 1989). MAKP interacts during the meiotic cell cycle with another regulatory enzyme, maturation promoting factor (MPF). MPF was originally identified as a factor that triggers reinitiation of meiosis in frog oocytes (Masui and Market 1971) and has subsequently been observed in a number of meiotically and mitotically dividing cells. In mammalian oocytes an MPF-dependant regulatory step of MAKP activation at reinitiation of meiosis involving the expression of Mos has been identified, and it has been suggested that the upstream regulator of this cascade is cAMP (Josefsberg et al 2003). The expression of Mos is subject to negative regulation by a protein kinase A mediated cAMP action (Lazar et al 2002)

The role of cAMP in the initiation of meiosis only becomes active after maturation of the oocyte. Goren et al (1994) have demonstrated that meiotic arrest in incompetent oocytes is independent of intra-oocyte cAMP concentrations suggesting that other factors responsible for the meiotic incompetence of immature oocytes must be involved.

Another paracrine system influencing meiotic activity in the oocyte involves Leydig insulin-like 3 (Insl3). Female INSL3-null mice exhibit impaired fertility associated with increases in follicular atresia and premature luteolysis (Nef and Parada 1999, Spanel-Borowski 2001). Recent studies indicate that testis Insl3 acts as an endocrine factor to activate a G-protein coupled receptor LGR8 (leucine-rich repeat-containing G protein-coupled receptor 8) with consequent increases in cAMP production (Kumagai et al 2002), LGR8 is expressed by the germ cells of both sexes and Insl3 has been shown to have a paracrine role in initiating oocyte maturation (Kawamura et al 2004). Insl3 is expressed in the thecal cells of the ovary (Bathgate et al 1996) with transient stimulation of this expression being induced by LH/hCG (Kawamura et al 2004). Although LH stimulates cAMP production in follicular somatic cells, a decrease in intraoocyte cAMP is required for meiotic resumption (Tsafriri and Pomerantz 1986), this

being an evolutionary conserved mechanism for regulating meiotic progression (Maller 1985). Insl3 has been shown to suppress intraoocyte Ca levels and to induce oocyte maturation as early as 1 hour after administration (Kawamura et al 2004), indicating that the Insl3-LGR8 pathway may be important for germ cell meiotic progression.

In cultured, intact follicles both LH and FSH have been observed to induce oocyte maturation (Hillensjö 1976, Tsafriri et al 1972, 1998, and 2002, Dekel et al 1988, Tornell et al 1995). LH has the ability to induce meiosis in oocytes from large antral follicles from eCG primed rats, but FSH can induce meiosis in large and small follicles from unprimed rats (Dekel et al 1995). It had been proposed that cumulus cells secrete a substance after stimulation by FSH which influences meiotic resumption, and this has led to the identification of meiosis-activating sterols (MAS) (Byskov et al 1995 and 1997). Since then several studies have revealed that MAS play a role in the mouse oocyte meiotic resumption (Lu et al 2000, Grøndhal et al 1998 and 2000). Recent studies have shown that MAS levels increase rapidly in response to LH in the rabbit ovary in vivo (Grøndhal et al 2003) and that MAS binding sites can be detected at the surface of the mouse oocyte membrane (Faerge et al 2001). Further work using a serum-free culture model for intact mouse follicles has also concluded that gonadotrophins employ MAS as a downstream signal transduction molecule for the initiation of oocyte maturation (Xie et al 2004).

During the phase when resumption of meiosis is becoming apparent there is also a decrease in the availability of granulosa cell aromatase as the shift to progesterone dominance progresses. Progesterone titres can become particularly high in follicular fluid with levels of 6400ng/ml being recorded in domestic farm animals (Hunter et al 1976). Progesterone has been shown to influence oocyte quality and maturation independent of the gonadotrophin surge (Borman et al 2004). It has also been shown to be able to prevent oocyte atresia and promote oocyte nuclear maturation in primate follicles.

1.6.3.8. Cell survival

During follicular growth and development the majority of the follicles selectively degenerate. This atresia is characterised primarily by granulosa cell apoptosis. In cultured, fully differentiated preovulatory follicles (following FSH-induced LH receptor expression) LH is proposed to provide continued support for follicle survival until the time of ovulation (Chun et al 1994) and an increased resistance to apoptosis has been observed in granulosa cells of preovulatory follicles following the LH surge in cattle (Porter et al 2001) and in rats (Svensson et al 2000). If preovulatory gonadotrophin surges are blocked or serum gonadotropins are decreased following hypophysectomy, follicles undergo atresia (Braw and Tsafriri 1980, Braw et al 1981). However, studies using cultured rat granulosa cells have shown that treatments with FSH, LH/hCG or insulin growth factor (IGF-I) are ineffective in the prevention of apoptosis, despite their apoptosis-suppressing action in cultured rat follicles (Billig et al 1996). This indicates the importance of neighbouring theca cells and local factors produced in the ovary for regulation of follicle growth and atresia. Other follicle survival factors, e.g. epidermal growth factor/transforming growth factor, basic fibroblast growth factor (Tilly et al 1992), interleukin-1β (Chun et al 1995) and growth hormone (Eisenhauer et al 1995) as well as pro-apoptotic factors, e.g. tumour necrosis factor-α (Kaipai et al 1996), Fas ligand (Quirk et al 1995) and GnRH (Billig et al 1994) have been characterized

One of the most extensively documented granulosa cell survival pathways involves PKA/adenylyl cyclase signalling (Johnson et al 2003). Mechanisms implicated in this gonadotrophin-mediated survival pathway include the suppressed expression of proapoptotic factors such as Bax (Tilly et al 1995), Apaf-1 (Robles et al 1999), and caspase 3 (Boone and Tsang 1998). Progesterone, acting through its nuclear receptor, is also reported to exert antiapoptotic effects on isolated granulosa cells collected from periovulatory follicles (Svensson et al 2000). In addition peptide hormones such as vasoactive intestinal peptide (VIP) and pituitary cyclase-activating polypeptide (PACAP) also act via PKA to support survival. Progesterone has the ability to upregulate PACAP and the PACAP receptor in granulosa cells during the periovulatory period.

Serum glucocorticoid regulated kinase (Sgk) expression is rapidly reduced by the LH surge but this decrease is transient. The expressions of Sgk mRNA and protein are increased as the cells begin to luteinise (Gonzalez-Robayna 1999, Alliston 2000). Upon activation Sgk is known to translocate to the nucleus where it can modulate the activity of transcription factors, including at least one member of the Forkhead family (Richards et al 2002) which has been shown to induce cell cycle arrest and apoptosis (Brunet et al 2001). Sgk is known to prevent apoptosis in mammary epithelial cell lines (Mikosz et al 2001) and in a human embryonic kidney cell line (Brunet et al 2001).

A number of cellular anti-apoptotic proteins are also involved. Many of the members of the Bcl-2 family have been isolated in the ovary. The antiapoptotic Boo (Bcl-2 homologue of ovary) expression is highly restricted to the ovary and the epididymis (Song et al 1999) and Bok (Bcl-2 related ovarian killer) is highly expressed in the ovary, testis and uterus (Hsu et al 1997). Members of the Bcl-2 family have been shown to be upregulated by gonadotrophins via PKA signalling (Tilly et al 1995) and it has been suggested that the production of excess levels of these proteins enables efficient binding and neutralisation of other pro-apoptotic proteins such as Bad, Bax, and Apaf-1 (Tilly et al 1995, Hsu and Hsueh 2000). Bcl-2 has been shown to function as a survival factor in primary human granulosa cells (Sasson and Amsterdam 2002). Sasson et al (2004) identified a number of genes related to cell death and/or survival expressed in luteinised human granulosa cells in response to gonadotrophic stimulation. Among them is BAX inhibitor 1, Bcl-2 antagonist of cell death (BAD), Bcl-2 associated athanogene (BAG1). Bax null mice have been shown to have reduced ovarian apoptosis (Perez et al 1997). Another protein, apoptotic repressor (ARC), thought to be unique to heart muscle, is now known to be expressed in granulosa cells (Neuss et al 2001) and to be induced by gonadotrophin stimulation (Sasson et al 2004). ARC contains caspase recruitment domains which interact with and inactivate caspase activites thus helping to preserve mitochondrial integrity and function (Neuss et al 2001, Shelke and Leeuwenburgh 2003).

The Myc/Max/Mad family of transcription factors is linked closely to proliferation, differentiation and apoptosis (Grandori et al 2000). The protooncogene *c-myc*, which typically facilitates movement of cells into DNA synthesis (Nasi et al 2001), is antagonised by Mad proteins which compete for access to promoter sites and are thus associated with cell cycle arrest and differentiation (McArthur et al 1998). Granulosa cells from rodents and primates express *c-myc* transiently after a gonadotrophic ovulatory stimulus and it has been suggested that that this gene may act as a switch mechanism between proliferating and luteinising follicles (Agarwal et al 1996, Piontkewitz et al 1997, Fraser et al 1995). C-Myc is also a potent inducer of apoptosis (Piontkewitz et al 1997) with both reduction and inappropriate overexpression associated with apoptosis. C-Myc is rapidly induced (within 1 hour) in rat granulosa cells following an hCG stimulus (Piontkewitz et al 1997), suggesting an increased proliferation of cells during the very initial steps of CL formation. This matches well with data from primates showing that the acquisition of the luteal phenotype by granulosa cells is preceeded by a proliferative burst driven in part by a transient increase in the ratio of c-myc to Mad (Chaffin et al 2003). Yet in the mouse within 4 h and complete by 12 hrs of exposure to LH, granulosa cells cease to divide (Robker and Richards 1998). The cessation of cell division is associated with the rapid loss of cyclin D2 and the increased expression of the cell cycle inhibitors, $P21^{CIP1}$ and $p27^{KIP1}$ (Robker and Richards 1998). Cyclin D2 regulates cell cycle kinase cascades that are obligatory for entry of cells into the G1 phase of the cell cycle. In the ovary, cyclin D2 is expressed selectively in proliferating granulosa cells of the growing follicle (Robker and Richards 1998). Mice null for cyclin D2 fail to ovulate; however, the granulosa cells within the small follicles can be stimulated to differentiate and to express genes associated with ovulation (PR, COX-2) and luteinisation (P450scc) (Sicinski et al 1996). It could be hypothesised that this final proliferative activity is a necessary component of the terminal differentiation of granulosa cells, which is brought to an effective stop by the increasing concentrations of the cell cycle inhibitors $P21^{CIP1}$ and $p27^{KIP1}$.

Integrity of cell-cell adherence and gap junction communication also has a role in increasing the resistance of granulosa cells to apoptotic stimuli. The expression of connexin 43 is clearly elevated by gonadotrophins (Sommersberg et al 2000). Following stimuli for apoptosis, integrity of the gap junctions is interrupted (Sasson and Amsterdam 2002), although whether this is a cause or effect of an apoptotic process is not known.

1.6.3.9 Follicular remodelling

The mature ovarian follicle contains granulosa cells with a number of luteinising hormone receptors (Espey and Lipner 1994, Richards 1994). The signal transduction processes that are initiated by these receptors at the time of the ovulatory surge of LH induce several dynamic changes in follicular cell function. Along with resumption of meiotic activity in the oocyte, there is induction of granulosa cell differentiation into progesterone secreting lutein cells. The fibroblasts in the thecal layers around the periphery of a follicle undergo a transformation from quiescence to motility, as they proliferate through the membrana propria towards the interior of the follicle, where they lay down a connective tissue framework to support the developing luteal tissue (Espey and Lipner 1994). Thus acute hormonal stimulation of a mature ovarian follicle leads to substantial cellular changes that convert a cavernous ovulatory follicle into a solid mass of luteal cells within 24-48 hours. This transformation of an ovarian follicle into a corpus luteum involves distinct ovarian cell types, diverse signalling pathways and temporally controlled expression of specific genes (Richards et al 1998).

The characteristics of both the theca and granulosa cells change markedly during luteinisation. The cells increase in size, acquire a polyhedral shape, accumulate lipids and produce increased amounts of progesterone (Van Blerkholm and Motta 1978, Mori et al 1983). In some species the theca- and granulosa-lutein cells are separated in layers and can be distinguished from one another (Mori et al 1983, Greenwald and Rothchild 1968), but in rodents the two types of luteal cells become intermingled (Greenwald and Rothchild 1968, Pedersen 1951).

Cell-cell adhesion

Clearly changes in cell adhesion molecules (CAMs) must occur during this remodelling process to permit the migration of theca cells and to facilitate the association of luteinising theca and granulosa cells into a functional corpus luteum.

The cadherins are a family of calcium dependant CAMs that have been studied extensively and shown to be important regulators of reproductive tissue structure, function and viability (Peluso 1997, Makrigiannakis et al 2000, Peluso 2000, Rowlands et al 2000) and to have a role in maintaining the viability of granulosa cells (Trolice et al 1997). Cell adhesion affects the ability of granulosa cells to acquire LH receptors (Farookhi and Desjardins 1986) and also influences their responsiveness to gonadotrophin signals. Both E- and N-cadherin expression have been reported in preovulatory follicles and during formation of the corpus luteum, and E-cadherin is expressed during the functional luteinisaton of isolated rat granulosa cells in vitro (Machell and Farookhi 2003).

The neural cell adhesion molecule (NCAM) has been localised to granulosa cells and luteal cells of ovaries from both the rat and mouse. Granulosa cells from preovulatory follicles of the human ovary and cultured GCs that underwent luteinisation in vitro, both expressed NCAM mRNA and protein (Mayerhofer et al 1991), suggesting it may be involved in corpus luteum formation. NCAM has been reported to downregulate the expression of matrix metalloproteinases (MMP1 and MMP9) (Edvardsen et al 1993).

Integrins build up cell matrix connections and act as receptors for ECM proteins. Several groups have been found, in particular integrin α6, but also α2, α3, and β1, that are expressed during follicular development and corpus luteum formation in time and cell specific manners, suggesting specific roles during these processes (Aten et al 1995, Honda et al 1995, Giebel et al 1996, Nakamura et al 1997, Fujiwara et al 1998).

Proteolytic mechanisms

Two principle families of enzymes, plasminogen activators/plasmin and matrix metalloproteinases (MMPs), govern tissue dissolution and remodelling during ovulation and luteinisation. In response to the LH surge plasminogen activator expression is increased differentially at the apices of preovulatory follicles within the ovarian surface epithelial cells (Carmeliet et al 1994). Both urokinase and tissue plasminogen activators contribute to ovarian plasmin production and ovulatory efficiency in rodents (Hägglund et al 1996, Curry and Osteen 2001, Murdoch et al 1986). That ovarian/follicular MMPs are increased and collagens are degraded during ovulation has been established (Murdoch and McCormick 1992). In preovulatory ovine follicles, there is a direct association of apical plasmin accumulation with the onset of collagenolysis (Fukumoto et al 1981, Bjersing and Cajander 1975). Morphological observations indicate that preovulatory connective tissue disruption begins at the ovarian surface and advances inward to encompass the ovarian follicular wall (Reich et al 1991, Tadakuma et al 1993). Tunica/thecal fibroblasts and follicular steroidogenic (theca, granulosa) cells are sources of procollagenase (Ichikawa et al 1983, Reich et al 1985).

Tumour necrosis factor (TNF) α is known to be expressed by preovulatory follicles (Black et al 1997, Johnson et al 1999, Murdoch 1994 and 1995) and is secreted, within a limited diffusion radius, into the progenitor site of follicular rupture (DeMola et al 1998). TNFα induces collagenase production in the follicle (Murdoch et al 1999, Brännström et al 1995) and it therefore appears that it potentiates ovulatory collagenolysis by assuring that sufficient quantities of (pro) MMPs are synthesized. Secretion into the follicular fluid of low levels of TNFα by the oocyte cumulus complex evidently facilitates collagen breakdown throughout the follicular wall (Murdoch et al 1999).

MMP 2 production during the follicular transition to the luteal phase is upregulated under the transcriptional control of TNFα (Murdoch and Gottsch 2003) and its localisation within the connective tissue strands that extend into the substance of the corpus luteum (Brännström et al 1995) is consistent with the concept that follicular type IV collagen is remodelled into an anchoring

infrastructure for blood vessel development and cellular migration (Sato and Seiki 1996, Stack et al 1998, Nelson et al 2000, Woessner 1991). MMP 2 is essential for ovulatory rupture and remodelling and normal angiogenesis within the developing corpus luteum (Baibin et al 1996).

In addition, other proteolytic enzymes have been found to be involved in periovulatory connective tissue dynamics; MMP-9 is elevated in the luteinising granulosa cells of rodents (Boujrad et al 1995), MMP-13 is elevated in preovulatory follicles (Balbin et al 1996, Komar et al 2001) as are cathespin L and ADMATS-1 (Robker et al 2000).

Net proteolysis during ovulation is controlled by the relative balance of enzymes to inhibitors. Increased expression of TIMP-1 and α2-macroglobulin by granulosa cells of periovulatory follicles apparently serves to confine the extent of ovulatory tissue destruction and assure that a viable corpus luteum is formed (Liu et al 1998 and 1999).

There is also evidence that MMPs and TIMPs influence luteal emergence independent of ramifications on the extracellular matrix. Matrix metalloproteinases, by liberating growth factors from inhibitory binding proteins, can trigger cellular expansion (Liu et al 1999). Progesterone production by rat granulosa cells is stimulated by TIMP-1 when complexed with cathespin L (Boujrad et al 1995).

Two proteases that are induced in granulosa cells of preovulatory follicles by the LH surge and whose expression is impaired in PR null mice are cathespin L and ADAMTS-1. Cathespin L is a lysosomal cysteine protease which degrades types I and IV collagen, fibronectin and laminin (Kirschke et al 1998). It has been associated in vivo with metastatic potential of transformed cells (Ishidoh and Kominami 1998), suggesting that it is important for tissue remodelling and cell migration/invasion. ADAMTS-1 is a member of the ADAMs family of proteases (Kuno et al 1997) characterised by a multifunctional structure and zinc binding domains (Black and White 1998). The peak in ADAMTS-1 transcripition occurs 8-12hrs after exposure of the ovaries to an ovulatory dose of hCG (Robker et al 2000, Espey et al 2000), after the peak of PR expression but before ovulation (usually observed at 14-16 hrs). It has several possible

functions in the ovary, firstly it is a potent active proteases which may serve to initiate one or more proteolytic cascades (Kuno e al 1999). Secondly as a protease it may serve to control the amount and cellular location of various proteoglycans. It has been shown to degrade aggrecan and brevican both of which are present in follicular fluid (MacArthur et al 2000, Nakamura et al 2000, Tortorella et al 2000, Kuno et al 2000) and to proteolyze the cell surface ectodomain of the syndecans, which may release potent biological peptides into the follicular matrix (Park et al 2000). Proteoglycans appear to play a critical role in the physical composition of follicular fluid and are important for cell migration and for other cell functions (Salustri et al 1999). A lack of ADMATS-1 might prevent the activation of one or more potent bioactive factors in the follicular fluid by preventing their release from proteoglycans. Finally, its ability to interact with specific cellular signalling molecules suggest its third putative function as a cell signal regulator (Kuno et al 1999, Bigler et al 2000). At least one class of G protein coupled receptors are activated by proteolytic cleavage of their extracellular domain (Nakanishi-Matsui et al 2000).

In addition at least two other ADAMs family member are expressed in the rodent ovary, ADAMTS-4 and ADAMTS-9, but the specific source cell type has not been identified (Abbaszade et al 1999).

1.7 Rationale and aims for the present study.

Although much is known about the molecular mechanisms responsible for follicular development comparatively little study has been carried out to analyse the control of, and genetic response to, luteinisation. The extensive morphological and functional changes described above during luteinsiation involve the regulation of gene and protein expression responsible for the cessation of proliferation and differentiation of the individual granulosa cells. The formation of the functional corpus luteum and secretion of progesterone is essential for the establisment of pregnancy following ovulation. However, as already described, the differentiating granulosa cell also functions in an endocrine manner, as a paracine mediator of thecal function and oocyte

maturation, as the driving force behind follicle formation and subsequent corpus luteum remodelling, and as a provider of nutritional and metabolic support for the oocyte. The control of such an array of functions depends on the tight regulation of protein expression within the cell. Initiation of gene transcription depends on the activity and cooperation of transcription factors and other controlling elements binding to a gene specific promoter region on the chromosome and activating synthesis of the mRNA. Following this, transcript processing, transport, translation and post translation modification/activation or inactivation will all impact on the eventual role of the transcribed gene.

In order to investigate the mechanisms underlying these processes we embarked on a time- and cell-specific analysis of gene expression in the granulosa cell during late follicle development and early luteinisation. Our aims were to provide a comprehensive record of gene expression within the cell during this fundamental transformation and in so doing identify some of the core regulatory systems controlling luteinisation.

The advantages of the experimental approach chosen to perform this analysis will be discussed in Chapter 3.

1.8 Tissue generation and mouse model

The mouse is an excellent animal for the study of follicle development and luteinisation. The first follicles are formed in mice around the day of birth (Peters 1970) so that the newborn mouse ovary contains only primordial follicles. At 2-3 weeks after birth the follicles have developed to antral follicle stage. Neonatal mouse ovaries therefore contain uniform follicle populations of similar sizes as determined by the number of days after birth (Sorensen et al 1976; Epigg 1991).

To attain a more complete knowledge of the mechanism of cell function, it is necessary to investigate the full range of molecules involved. Analysis of mixed cell populations may miss significant transcripts expressed in a cell type that has a low frequency in the population, or over emphasise the importance of a transcript expressed in multiple cell types compared to those with more limited

expression. By isolating follicular somatic cells from thecal and interstitial tissue this problem is largely overcome and results in a significant improvement over those gene expression studies (Leo et al 2001; Espey and Richards 2002) based on the whole ovary without regard for variation between specific cellular compartments. The value of this approach is illustrated by the work of Virlon et al 1999 who microdissected kidney tubules and identified transcripts dramatically enriched in specific histological regions. The fact that several of the most highly expressed transcript tags in this study had no matches to cDNA databases suggests that existing cDNA libraries made from entire kidneys did not contain important genes expressed in small subpopulations.

The gonadotrophin-induced mouse model using PMSG (pregnant mare serum gonadotrophin; mainly FSH activity) and hCG (human chorionic gonadotrophin; mainly LH activity) at 20 days old, before the animal has entered its own oestrus cycle, simplifies the staging and collection of uniform populations of cells. It is also a considerable advantage that the timing of ovarian events are well known in this model, which allows tissue sampling and pharmacological treatments at defined time points during follicular development and ovulation. The PMSG treatment stimulates follicular growth and development and the subsequent hCG administration induces ovulation between 12 and 14 hours later (Wilson and Zarrow 1962; Zarrow and Wilson 1961).

Chapter 2

Introduction to basic laboratory methods, mouse model and tissue collection.

Chapter 2. Overview of Basic Laboratory Procedures

The main objective of this project were the identification of genes with altered expression during luteinisation. This was largely achieved using serial analysis of gene expression (SAGE) but a number of molecular biology techniques such as RNA extraction, DNAse treatment of RNA, reverse transcription, normal and 'hot start' PCR, gel electrophoresis, cDNA cloning and sequencing were also used and require a brief explanation. Unless specifically stated later in the text these are the protocols followed.

2.1. RNA extraction

Messenger RNA within the cell is the intermediate coding stage between the gene and the protein. The initial stage in RNA formation is the production of heteronuclear RNA, a direct copy of a sequence of genomic DNA, containing both introns and exons. The noncoding exons are removed by splicing, leaving the coding exon sequence mRNA as the template for protein synthesis.

RNA extraction was carried out by addition of the tissue sample to the appropriate volume of Trizol (Invitrogen, UK), usually 400µl. When dealing with aspirated granulosa cells from ruptured follicles no homogenisation was used other than vortexing the sample for 15 seconds. In the case of samples with significant structural organisation homogenisation was carried out using the Ribolyser (Hybaid, UK) for a 20 second period or as required for complete disruption of tissue structure. A one fifth volume of chloroform (usually 80µl) was added and the contents mixed by vortexing. The tube was left on ice for 3 minutes then centrifuged for 7 minutes at 14,000 rpm (18,000g). The top aqueous layer was removed and transferred to another tube. An equal volume of isopropanol was added along with 1µl of RNAase free glycogen (Invitrogen, UK) and the tube left at room temperature for 20 minutes before centrifugation for 20 mins at 18,000g. The centrifugation formed an RNA pellet. The supernatant was decanted and the pellet washed in 750µl 75% ethanol, vortexed for a few seconds and then recentrifugated for 15 minutes. The ethanol was

removed and the pellet allowed to air dry before resuspension in 20-50µl rtH_2O (UV treated) and storage at -70°C or in liquid nitrogen.

2.2 DNAse Treatment of RNA samples

Contamination of RNA samples with genomic DNA can produce a false positive signal, particularly in highly sensitive real time PCR processes. This will occur if the amplicon does not span an intron/exon boundary. While false positives can be detected using a control which has undergone the reverse transcription protocol without active enzyme, removal of contaminating DNA aids detection and quantification of RNA species. This can be carried out efficiently by using a DNAse treatment such as DNA-*free* (Ambion Inc UK) which breaks down DNA into short oligo and mononucleotides but leaves RNA unaffected.

To DNAse treat one sample 1µl of 10x DNAse buffer (100mM Tris, pH 7.5; 25 mM $MgCl_2$; 5 mM $CaCl_2$) and 0.5µl DNAse (Ambion Inc) was added to 5µl of the RNA sample along with 3.5µl rtH_2O (total volume of reaction 10µl) and incubated at 37°C for 30 minutes and then placed on ice. DNase inactivation reagent (4µl) was added to each sample and left to stand at room temperature for 2 minutes. Centrifugation at 18,000g for 2 minutes pelleted the inactivation reagent and DNAse and allowed removal of the supernatant containing DNA-free RNA. This was stored at -70°C or in liquid nitrogen.

If real time PCR showed DNA contamination still present then a second cycle of DNAse treatment was performed.

2.3 Reverse Transcription

The conversion of unstable RNA to the more easily manipulable cDNA by RNA dependant DNA polymerases is the starting point for many molecular biology techniques. Recent evidence has shown that some types of reverse transcriptase such as RnaseH RT (Superscript II, Invitrogen, Paisley UK) may generate up to four time more cDNA from an identical amount of starting material (Virlon et al 1999) when compared to some of the more traditionally used enzymes such as

the Moloney murine leukaemia virus reverse transcriptase, making it the obvious choice for use.

These enzymes act by extending an oligo dT or random hexamer primer in the 3' direction by the incorporation of deoxynucleotides (dNTPs)

Reverse transcription reactions were usually performed in a 10µl total volume containing 2µl 5x RT buffer, 1µl 10mM DTT, 0.25µl dNTPs (20pmol/L), 0.25µl random hexamers (20pmol/L) or oligodT primer (20pmol/L), 0.15µl of RNAse inhibitor (RNasin, Promega, UK), 0.15µl of Superscript II RT (Invitrogen, UK), 5.2µl of water and 1µl of RNA template. The mixture was incubated at 42°C in a water bath for 1 hour then cooled and stored at -20°C.

2.4 Polymerase Chain Reaction

The polymerase chain reaction allows amplification of specific target sequences of DNA using oligonucleotide primers each complimentary to one end of the DNA target sequence. These primers are extended in a 3' direction by a thermo stable DNA polymerase in a three-step reaction involving a high temperature denaturation step (95°C), a low temperature annealing step (54-65°C) and an extension step (72°C).

The primers are usually 17-28 bases long, with 45-60% GC content and minimal self complementarity. The specific primers used in various reactions will be listed in the text.

Each PCR reaction usually contained 10x buffer (750mM Tris-HCl pH8.8, 200mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20®.) , 0.1µl taq polymerase (5iu/µl), $MgCl_2$ (2mM) (All from Abgene, UK), dNTPs (0.2mM), 1µl of cDNA template, primers (200nm each) and rtH_20 to an appropriate volume (usually 25µl or 50µl). The appropriate cycling program was performed using a PCR express machine (Thermo Hybaid). Cycling programs consisted of an initial denaturation step of 95°C for 2 minutes, then 25-35 cycles of 95°C denaturation, a primer-specific annealing temperature and then 2 minutes of 72°C extension. A terminal extension step of 5 minutes at 72°C was used in most protocols. Non template controls were included in all reactions.

In a variation of the normal PCR protocol some enzymes require a 95°C denaturation step to activate the polymerase enzyme. When setting up a PCR reaction at low temperatures, non-specific annealing can lead to the generation of non-specific constructs which will be reamplified throughout the remainder of the PCR reaction. A system requiring hot start activation of the polymerase allows premixing of the reagents at room temperature without extension of non-specific annealed primers. This increases reaction fidelity and reduces the incidence of mis-primed PCR products and primer oligomers, particularly valuable when performing real time PCR quantification of low level expression transcripts.

The Ampiltaq Gold (Applied Biosystems) hot start PCR system was used for all real time PCR reactions performed in this study. The reaction was performed in 1x GeneAmp PCR Gold Buffer and 6.25mM magnesium chloride solution (both Applied Biosystems) with 0.15 units Amplitaq Gold polymerase per reaction. Also included were 200µmol/L dNTP's, 300nmol/L of each primer, 200nmol/L of probe and the reference dye rox (60pmol/L). The thermal profile consisted of an initial 10 minute 95°C denaturation then 40 cycles of 95°C for 15 seconds denaturation with a 60°C extension step. Thermal cycling and flouresence detection was performed by a GeneAmp 5700 system (Applied Biosystems, Cheshire, UK). A further description of real time PCR is given in Chapter 3.

2.5 Gel electrophoresis

This involves the separation of DNA or RNA products on the basis of size and electric charge. The DNA molecule is negatively charged and will migrate through the gel matrix towards a positive electrode. Product size was electrophoretically resolved by comparison with DNA size standards. The loading buffer added to the DNA sample contains both glycerol to retain the DNA within the wells and bromophenol blue to allow estimation of sample migration. Two types of gel matrix are used in this study, agarose and polyacrylamide.

Agarose gels were 1% unless otherwise stated, prepared in 0.5x TBE buffer, heated to induce dissolution of the agarose, as being cooled, ethidium bromide was added (1µl ethidium to 100mls agarose gel) to permit visualisation of the DNA product under uv light. A voltage difference of 10V/cm was applied to the gel and run for as long as required to induce separation of the DNA fragments.

Polyacrylamide gels (PAGE) are efficient at separating lower molecular weight products with better resolution than can be achieved with agarose gels. 12% PAGE gels were routinely used for separation of the PCR and restriction enzyme digest products involved in SAGE. To generate a 12% gel, 10.5ml 40% Acrylamide/Bis Solution, 19:1 (Bio-Rad Laboratories, CA) was combined with 23.5ml water, 700µl of 50x TAE, 350µl ammonium persulphate (Sigma Chemicals, UK) and 30µl TEMED (Sigma Chemicals, UK), mixed and allowed to set as a 1mm thick gel.

Following electrophoresis the gel was removed from the mould and washed in 100mls 1xTAE buffer containing 6µl ethidium bromide for 15 minutes followed by rinsing in 100mls clean 1xTAE for another 15 minutes and then visualised under UV-A light.

2.6 cDNA Cloning using TOPO vector

The TOPO (Invitrogen, UK) range of cloning vectors utilise a bound topoisomerase enzyme attached to each end of the open vector to ligate the desired insert. Selection of cloned insert over empty vector is performed by means of a suicide gene. The pCR 4-TOPO vector (Invitrogen, UK) is supplied open with an A base overhang to permit cloning of PCR products. This system was used repeatedly to clone cDNA for sequencing analysis.

The cloning reactions were set up using 4µl PCR product, 1µl salt solution (1.2M NaCl, 0.06M $MgCl_2$) and 1µl TOPO vector. The mixture was gently mixed by pipetting and incubated at room temperature for 5 minutes then placed on ice.

Ethanol precipitation was used prior to electroporation to reduce salt concentration and prevent arcing. For precipitation 3µl glycogen was added to the supernatant followed by 50µl sodium acetate 3M, pH5.2, and 1300µl 100%

ethanol and the mix was stored at -70°C for 2 hours followed by centrifugation at 18,000g for 15 minutes. The supernatant was removed and discarded, the pellet washed in 500µl 70% ethanol and centrifuged for 5 minutes at 18,000g. Again the supernatant was removed and discarded and the pellet washed in 500µl 70% ethanol before centrifugation for 5 minutes at 18,000g. The supernatant was discarded and the pellet dried before resuspension in 10µl LoTE.
Amplification of the insert in recombinant plasmids was carried out using the vector based primers M13R (5'-TCACACAGGAAACAGCTCTGA-3') and M13F (5'-TGT AAA ACG ACG GCC AGT-3'), sequencing was performed using M13R primer.

2.7 Electroporation of E.coli

The DH10B *E.coli* strain (Invitrogen, Paisley, UK) was used as the host cell for transformation with plasmid DNA. 1µl of a 10µl ligation reaction was added to an aliquot of 40µl of cells which had been stored at –70°C and then defrosted on ice. This mix was transferred to a chilled 0.1cm cuvette (Bio-Rad) and electroporation at 150kV and 200Ω using a Bio-Rad Gene Pulser carried out. Cuvettes where maintained on ice for 2-3 minutes following electroporation then cells resuspended in 1ml SOC (Invitrogen, Paisley, UK) at room temperature and incubated for 1 hour at 37°C shaking at 220 rpm. This suspension was aliquoted onto normal or low salt LB agar plates with the appropriate selective antibiotic and incubated at 37°C overnight. Analysis of growing colonies was carried out by PCR utilising plasmid based primers.

2.8 DNA Sequencing

Sequencing of PCR products or plasmid purified DNA was carried out with the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems).
Amplification reactions were performed in a 10µl reaction volume containing 3µl Big Dye buffer, 1µl Big Dye (both Applied Biosystems), 0.5µl sequence

specific primer (3.2pmol^{-1} concentration, sourced from MWG Biotech) and 2µl of PCR product as template. Thermal cycling was performed at 96°C for 30 seconds, 50°C for 15 seconds and then 55°C for 4 minutes for 25 cycles.

The amplified product was purified by ethanol precipitation. To the 10µl reaction volume was added 8µl water and 32µl 95% ethanol and this was left to stand at room temperature for 15-30 minutes then centrifuged for 30 minutes at 4000g at 4°C. The supernatant was removed and the pellet washed in 150µl 70% ethanol. Following further centrifugation at 4000g at 4°C for 10 minutes, the supernatant was removed, the pellet dried and then reuspended in 15µl formamide.

Sequencing was performed on the ABI Prism 3100 Genetic Analyser (ABI Biosystems). The sequences were viewed and text files created using Chromas 2.3 (Technelysium Pty Ltd, AUS).

2.9 Collection of experimental tissue for SAGE libraries

The experimental tissue for this study was obtained by treating 30 normal d20 female mice, bred on the C3H/Hehx101h strain, with 5IU PMSG intraperitoneally (i/p). Isolation of granulosa cells from mature antral follicles was carried out by puncturing follicles of ovaries collected 48 hours following injection. Further granulosa tissue was collected by the same method from preovulatory follicles 12 hours after the sequential administration of 5IU PMSG and 15IU hCG intraperitoneally to 30 female mice 48 hours apart. Cells were collected by follicle puncture and aspiration in Dulbecco's ME medium (Invitrogen, Paisley, UK) and stored at –70°C until use.

Chapter 3

Serial analysis of gene expression

Introduction, protocol, some methodological considerations and results.

Chapter 3: Serial Analysis of Gene Expression

3.1 Overview of Gene expression analysis

Changes in gene expression can have a major effect on ovarian function. Small shifts in the expression levels of hormones, their transporters and receptors can have wide implications for cell function. The description of gene expression patterns provides clues about the regulatory mechanisms, biochemical pathways, and broader cellular functions. Therefore identification of physiological variation in expression patterns will add to the understanding of the pathways affected. Messenger RNA expression studies are complementary to these goals. The advantage of mRNA analysis is that it is generally less difficult and more sensitive than protein based methods. The differences found in mRNA levels are often, but not always, correlated with differences in protein levels. For example, study of the C.*cresentus* bacterium cell cycle using two dimensional gel method for proteins and DNA microarrays for RNA showed similar expression profiles between protein and RNA (Grunenfelder et al 2001). The main disadvantages of measuring mRNA are associated with these molecules not being the final expression product and an inability to account for protein modification, interactions with other proteins, and to identify the location of the final product.
The extent of the genomic DNA that encodes genes varies greatly between organisms, the yeast genome, for example, is approximately 70% coding, while the human genome contains only about 3% coding sequence, equating to a surprisingly low number of genes (Lander et al 2001). It is estimated that around 10000 genes are active at a given time in a mammalian cell (Yanamoto et al 2001) and of these a proportion can be useful in identifying differences between cell types by representing cell specific functions. Gene expression studies aim to identify and measure the relative copy number of each transcript, creating an expression profile that can be used to look specifically at or for genes with altered expression levels in response to certain stimuli. It is, however, important to bear in mind that transcript and protein profiles do not always agree (Griffin et al 2002). It is also important to note that a large proportion of transcribed

sequences represent non-coding RNA. This may include ribosomal RNA, transfer RNA and other small RNA's (Mattick 2001, Eddy 2001). The separation of mRNA from other RNA species is aided by the transcriptional modification of coding RNA by the addition of a 3' polyadenylated tail, although some noncoding RNA strands also have poly A tails. Hybridisation of this tail to a complementary polyT oligonucleotide anchored on a solid support is the feature on which a variety of global expression techniques are based. By reverse transcription of the RNA into complementary DNA (cDNA) and cloning of this cDNA, a library is created with the same sequence distribution as the original mRNA.

3.1.1 Gene profiling techniques

Several approaches are possible in studying the differential expression of genes within tissue samples. The hypothesis driven selective study of specific genes and proteins can be expanded by techniques which allow the open-ended study of all genes expressed within a tissue or cell type in the hope that knowledge of complete gene expression patterns will provide insight into the physiological control of function of that tissue or cell. All techniques require the extraction of mRNA and subsequent reverse transcription to cDNA. Large-scale arrays using cDNA or oligonucleotides and tagging techniques such as serial analysis of gene expression (SAGE) and GeneCalling rely on databases of EST and gene sequences to identify expressed genes.

Older techniques frequently relied on the use of cDNA libraries in the form of a bacterial population each harbouring cDNA clones synthesized from a single mRNA molecule. These libraries may be non-normalised, preserving the relative abundance of mRNAs in the starting tissue and allowing assessment of the gene expression levels within the study sample, or normalised with relative similar levels of abundant and rare genes in the final product. These normalised libaraies are consequently useful for identification of genes with low level expression. The various methods of gene expression analysis are briefly outlined below.

EST sequencing and electronic databases

The use of cDNA technology to generate EST libraries allows sequencing of most of the expressed genes from any particular tissue. Comparison of ESTs from libraries constructed from different tissues allows identification of tissue specific gene expression patterns. Most EST libraries are constructed using directional cloning of cDNAs and then sequencing from the 5' or 3' end of the transcript. The 5' sequencing strategy is useful for identifying coding regions within genes, primarily because the majority of cloned cDNAs are truncated (Williamson 1999). In contrast 3' sequences span the 3' untranslated region (UTR), which is more transcript specific due to less evolutionary conservation in such non-coding regions. These databases allow for selection of genes associated with a particular function or cell type. The ESTs representing these genes can also be used as a starting point for synthesis of PCR primers or hybridisation probes for further investigation.

Initially EST sequencing was one of the most important methods for discovery of novel genes, currently this data is useful in predicting gene coding regions and splice variants from genomic data and for showing tissue expression profiles for particular genes. The drawback is that in order to represent all transcripts within a cell, many thousands of clones must be sequenced. EST sequencing is therefore an expensive and laborious procedure and, in the genomic era, has been superseded to an extent by more efficient methods.

Differential hybridisation and subtractive cloning

Probing a single cDNA library with two or more radioactive cDNA samples can allow the identification of variation in gene expression in different physiological settings. Comparison of the hybridisation patterns allows clones with different signal intensities to be identified. This comparative hybridisation method requires a good cDNA library and tends to be biased towards abundantly expressed transcripts. As a technique it foreshadows the later development of identifying differentially expressed genes through the use of cDNA arrays.

Subtractive hybridisation takes a different approach by hybridising single stranded cDNA from one sample (tester sample) to an excess of RNA from

another sample (driver sample) followed by the isolation of the unhybridised tester strands. These strands, following purification and amplification, could then be used to identify cDNAs that were more abundant in the tester than in the driver.

Differential Display

This technique involves two basic steps; reverse transcription of a subset of total cellular mRNA using a set of 3' anchored primers; and then PCR amplification using the same 3' primers and an arbitrary set of 5' primers. Approximately 30 upstream randomly selected 5' primers are thought to be sufficient to amplify all of the mRNA species in any one tissue. Multiple samples can then be amplified in parallel, resolved by electrophoresis and visualised. Differences in the pattern of PCR products will reflect differences in the gene expression profile of the samples.

GeneCalling

This is a modification of the differential display technique requiring cDNA generation from ploy A (+) mRNA of the tissue samples, digestion with restriction enzymes, PCR amplification with specific linkers, and identification of differentially expressed fragments by polyacrylamide gel electrophoresis. Fragments can be tentatively identified by comparison against sequence databases.

DNA Arrays and microarrays

DNA arrays and microarrays simply consist of thousands of cDNA clones or oligonucleotides, usually of known identity, attached to filters or slides which can be hybridised with flourescently labelled cDNA reverse transcribed from the tissue of interest. The fluorescent signal intensity represents the abundance of mRNA molecules in the tissue, which are hybridised to the array as cDNA. Unlike SAGE, differential display, or subtractive hybridisation, hybridisation to a specific spot on the array is immediately interpretable if the identity of the sequence was known when the array was constructed. If the clone was an EST,

bio informatics and sequence extension software can often identify the gene of interest without further cloning or sequencing. While the chief limitation of this method is that it is only able to identify those genes present on the array, it holds the advantage of being less technically challenging, and less expensive, and can thus be used to analyse more samples in less time than SAGE (as discussed below).

The use of microarrays as genome wide tools to elucidate the expression profiles of genes is increasing (for example Chuaqui et al 2002, Yengi 2005, Liew 2005). However technical difficulties involve the requirement for highly concentrated RNA samples with 50-200μg or 2-5μg polyA+ RNA required (Duggan et al 1999). Additional problems are cross hybridisation (Chuaqui et al 2002), the reproducibility of assays, and the development of efficient procedures to handle the multitude of data points produced in each individual experiment (Yang et al 2002).

The comparibility of SAGE and microarray data has been assessed (Evans et al 2002) and results showed that detectability on arrays improved with increasing tag abundance (as discussed below a SAGE tag is effectively the same as an EST clone) with 90% of those genes in the most abundant range in a rat hippocampal SAGE library being detected efficiently by array. When the data was extended to cover the full complement of over 28,000 unique SAGE tags present within that library, this figure dropped to less than 30%, as many of the genes expressed were of low abundance. This emphasises the value of using SAGE to identify transcripts present in low abundance. SAGE does not depend on a knowledge of gene sequence and will detect all genes expressed in a tissue, including many genes which are not currently characterised and not present on microarrays. The value of SAGE is immeasurable in its use to generate an initial picture of the cell transcriptome in a particular tissue or phenotypic state, with the subsequent use of microarray technology utilising this data to design tissue- or state-specific arrays for more efficient evaluation of large numbers of samples being perhaps the most efficient approach, and one that has already been utilised in the analysis of the expression of 516 genes in malignant ovarian cancer (Sawiris et al 2002)

3.1.2 Serial Analysis of Gene Expression

Developed by Velculescu et al 1995 this technique differed from others in its ability to quantatively and simultaneously provide analysis of the transcriptome of a particular cell type at any given time without restriction to known genes. The application of this technique has provided valuable information about the normal and or diseased physiological state in many tissues or organisms including yeast (Velculescu et al 1997), pancreas, colon (Zhang et al 1997), lung (Hibi et al 1998), monocytes, macrophages (Hasimoto et al 1999b), kidney (Virlon et al 1999), oocyte (Neilson et al 2000), liver (Yamashita et al 2000), thyroid (Pauws et al 2000) and testes (O'Shaughnessy et al 2003).

SAGE strategy involves the collection of short 10-14bp tags of mRNA transcripts, which have undergone reverse transcription and serial analysis (figure 3.1). Each tag contains sufficient information to identify a unique transcript provided it has been isolated from a defined position within the transcript. Each SAGE tag is prefixed by the anchoring enzyme restriction site and corresponds to a 10-11bp extension of the 3'most site in the cognate transcript. This technique has the potential to both identify (by comparison with cDNA databases) and quantify expressed genes. Tags can be matched to characterised cDNA sequences and EST sequences, or they may have no match as in the case of novel genes. In theory the variety provided by the 4^{10} possible combinations of these 10bp sequences is sufficient to identify all expected transcripts from the mouse genome. The major advantage offered by this method is the collection of tags from unknown as well as characterised sequences and its ability to detect unknown genes meaning there is no restriction to knowledge-driven analysis. The technique also provides quantitative information about the relative expression of such genes and permits comparison of gene expression under different conditions

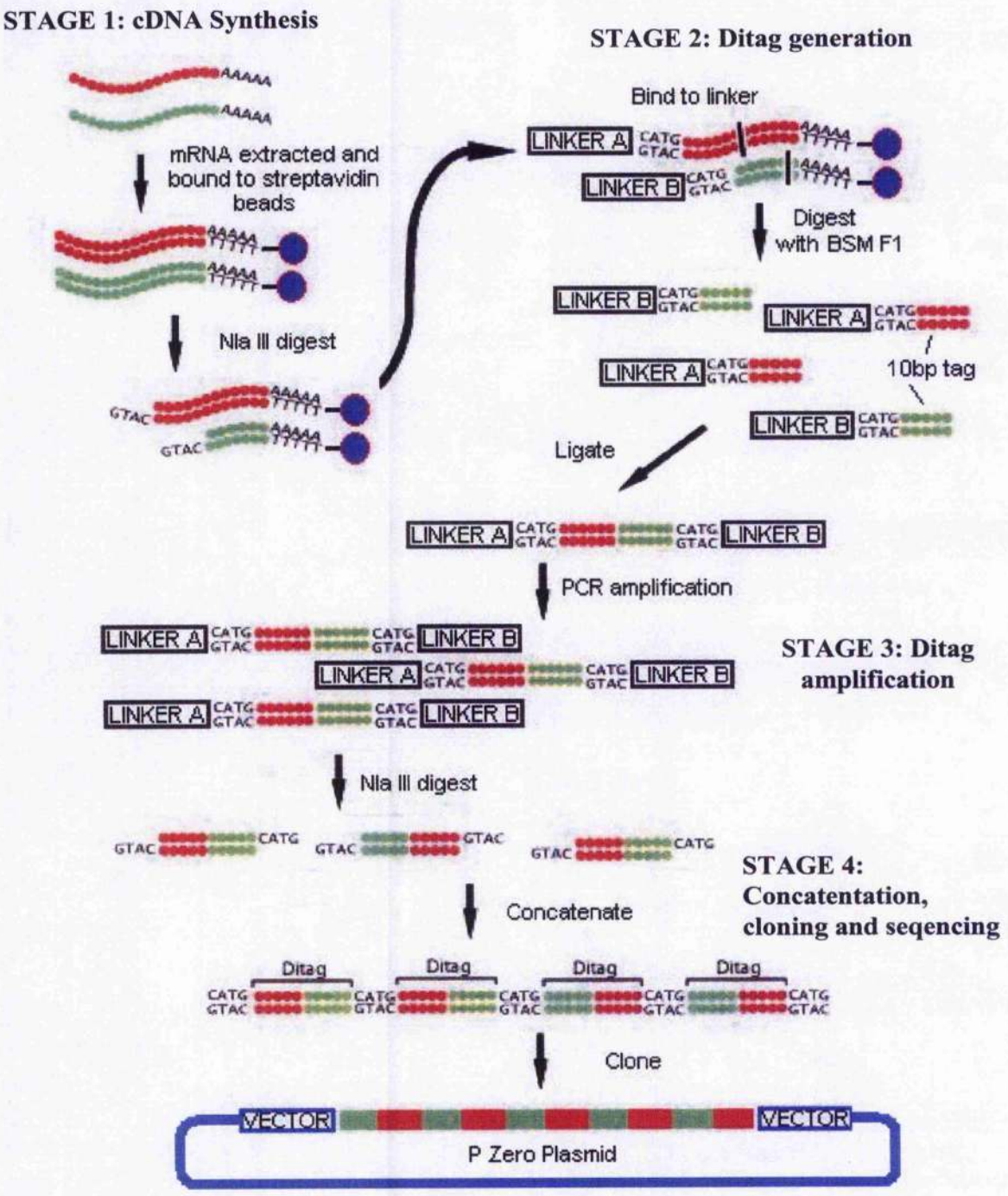


Figure 3.1 Schematic representation of Serial Analysis of Gene Expression (SAGE). Messenger RNA is extracted, reverse transcribed with a biotinylated d(T) primer, converted to double stranded cDNA then digested using NlaIII. 3' fragments of this digestion are recovered using strepavidin beads, linkers annealed to cut ends, then tags released by BSM F1 digestion. Tags are ligated to from ditags flanked by linkers and amplified using PCR. Tags are then excised by NlaIII digestion, gel purified, concatenated and cloned into plasmid vector (Pzreo) for later insert selection and sequencing.

The initial step in SAGE analysis is the extraction of mRNA and synthesis using biotinylated oligo (dt) primer of double stranded cDNA. The cDNA is then cleaved using a restriction endonuclease, in this case Nla III with a 4 bp recognition site -CATG- cleaving on average every 256bp. Binding to streptavidin beads then isolates the cleaved cDNA (figure 3.1). This isolates a unique site on the transcript corresponding to the closest Nla III restriction site to the polyadenylate tail. The cDNA sample is then split and each sample bound to one of two linkers containing a restriction enzyme site, which allows cleavage at a defined distance away from the recognition site. Cleavage with this tagging enzyme releases linkers with short attached pieces of cDNA (figure 3.1). These two pools of released tags can then be ligated to each other and then serve as templates for PCR amplification using linker-specific primers. The PCR products are ditags flanked by sites for the anchoring enzyme. Cleavage with this enzyme (Nla III) releases ditags, which can then be concatenated by ligation, cloned and sequenced (figure 3.1).

Several authors have developed variations on SAGE to resolve some of the technical problems of the procedure. The requirement for a relatively high amount of starting mRNA has been bypassed by MicroSAGE (Datson et al 1999), a simplified one tube procedure allowing analysis of microdissected samples, and SAGE-Lite, (Peters et al 1999, Virlon et al 1999). Minimising the contamination of linkers by use of biotinylated PCR primers and their subsequent removal with streptavidin beads (Powell 1998) and increasing the efficiency of the final concatamerisation by the introduction of a heating step (Kenzelmann and Muhlemann 1999) have both improved the tag yield. The newer LongSAGE method (Saha et al 2002) can distinguish 4^{17} different tags, a number sufficient to be virtually unique even within the whole genome.

There are now 209 mouse SAGE libraries containing over 16 million (corresponding to 1536012 million unique transcripts) SAGE tags of mouse origin in the SAGE database (http://www.ncbi.nlm.nih.gov/SAGE/index.cgi?cmd = printstats).

Among the negative aspects of the SAGE technique are the limitations due to the short tag length generated and a failure of a tag to match and uniquely identify

sequences in SAGE reference databases especially if it is situated in a conserved region (Ishii et al 2000, Kannbley et al 2003). Further analysis of unknown tags can be carried out using an RT-PCR method utilising the identified tag sequences and oligo-dT as PCR primers, the main disadvantage of this is the shortness of the 5' tag-derived specific primer and the common nature of the 3' primer to all mRNAs.

The identification of novel transcripts and genes from unknown SAGE tags has been elegantly demonstrated by Chen et al (2002), who showed that 67% of the unmatched SAGE tags examined in their study originated from novel transcripts that did not match existing ESTs. The extension of 17 of these tags to full-length cDNAs confirmed that these tags were derived from authentic transcripts and not genomic contamination or sequencing error. Importantly the majority of these sequences did not match any existing ESTs or predicted exons suggesting they may be indicative of novel genes.

3.1.3 Using Gene Expression Data.

The above techniques do little more than associate specific genes with specific cell types or physiological situations. They cannot suggest functional roles for the encoded proteins, and neither do they address the multiple levels of regulation that lie downstream of transcription. For the identification of genes of known function in another tissue extrapolation from previous work can be useful in determining function, while for novel genes the only basis for analysis is often the degree of homology to known genes. The sequence and number of tags can be archived electronically, and matched against several available databases to determine the significance of the genes expressed therein. Because SAGE data represents absolute expression levels of a gene, where each transcript is individually counted, cumulative data can be generated and comparisons between libraries remain valid over time. In this way SAGE data is sometimes described as 'digital' data.

The archive of SAGE data is maintained by the National Centre for Bioinformatics (NCBI) who have assigned unigene clusters to SAGE tags and

have numerous SAGE libraries available for study at http:www.ncbi.nih.gov/SAGE. The libraries reported in this thesis are available as GSM 30721 and GSM 30722. Libraries can be compared and significant differences between them re-evaluated and confirmed experimentally, allowing determination of those genes which have tissue-specific and, therefore, function-specific expression patterns.

3.2 Current detailed SAGE protocol

All work required during construction and analysis of the SAGE libraries described here was performed by myself with the exception of the generation to ditag stage of the first (PMSG-treated) SAGE library (carried out by Prof. P. O'Shaughnessy). The concatention, cloning and sequencing of the PMSG library was performed by myself as was all work relating to the hCG library. In addition to the 2 SAGE libraries reported here I have subsequently generated 3 other libraries to sequencing stage.

Extraction of mRNA and generation of cDNA (Figure 3.1, Stage 1)

Tissue collection

Granulosa cells were collected from stimulated follicles (PMSG or PMSG/hCG) by follicle puncture in Dulbecco's MEM (Invitrogen, UK), as previously described (Chapter 2). Cells were spun at 350g for 2minutes, the supernatant removed and the cell pellet stored at –70°C until use.

RNA Extraction

The cell pellet was resuspended in 400µl Trizol (Invitrogen, UK) and incubated at room temperature for 5 minutes. 80µl chloroform was added to the sample, which was then vortexed for 15 seconds and incubated at room temperature for a further 3 minutes. This was centrifuged at 12,000g for 5 minutes at 4^0C. The aqueous supernatant was removed and retained. 1µl 2% glycogen and 250µl isopropanol was added to the aqueous phase and then this mixture incubated for 20 minutes at room temperature. Centrifugation for 10 minutes at 12,000g at 4°C was subsequently performed. The supernatant was discarded and the pellet washed in 600µl 75% ethanol, vortexed and recentrifuged at 14,000g for 15 minutes at 4°C. The supernatant was again discarded and the pellet dried and resuspended in 50µl rtH_2O.

mRNA Isolation

This technique was performed using the Oligotex mRNA Spin-Columns (Qiagen, UK). The 50µl sample prepared above was made up to 250µl in rtH_2O and 250µl buffer QQB (20mM Tris-Cl pH 7.5, 1M NaCl, 2mM EDTA, 0.2% SDS) and 15µl Oligotex suspension (10% suspension Oligotex particles, 10mM Tris-Cl pH 7.5, 500mM NaCl, 1mM EDTA, 0.1% SDS, 0.1% NaN_2) and incubated at 70°C for 3 minutes to disrupt the secondary structure of the RNA. This was then allowed to cool at room temperature for 10 minutes, permitting hybridisation of the oligo dT30 of the Oligotex particle and the poly-A tail of the mRNA. Centrifugation for 2minutes at 14,000g was performed and the supernatant removed and discarded. The pellet was resuspended in 400µl buffer OW2 (10mM Tris-Cl pH 7.5, 150mM NaCl, 1mM EDTA) by pipetting, added to a spin column and centrifuged at 14,000g for 1 minute. The flow-through was discarded, the spin column transfered to a new tube and 400µl buffer OW2 added before centrifugation for 1 minute at 14,000g and then discarding the flow-through. The spin column was transferred to a new tube and 100µl buffer OEB (5mM Tris-Cl, pH 7.5), prewarmed to 70°C, added and pipetted to resuspend the resin before centrifugation for 1 minute at 14,000g. Another 100µl of prewarmed buffer OEB was added to the spin column and the procedure repeated.

The 200µl volume of mRNA was precipitated in 1ml 100% ice-cold ethanol by addition of 50µl 5M ammonium acetate and 3µl RNAase free glycogen (Invitrogen, UK) and incubation overnight at -70^{0}C.

This was centrifuged at 18,000g for 15 minutes at 4^{0}C. The aqueous supernatant was removed and discarded and the pellet washed in 500µl 70% ethanol, vortexed and recentrifuged at 14,000g for 5 minutes at 4°C. The pellet was again washed in 500µl 70% ethanol, vortexed and recentrifuged at 14,000g for 5 minutes at 4°C. The supernatant was then discarded and the pellet dried and resuspended in 25µl rtH_2O.

Reverse transcription and double stranded cDNA synthesis.

This was performed using the Superscript Double Stranded cDNA Synthesis Kit (Invitrogen, UK) according to the following protocol.

5µl Oligo dT primer (biotin $[dT]_{18}$) was added to the 25µl mRNA sample prepared above and heated to 70°C for 10 minutes. 10µl 5x First strand buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 5µl 0.1M DTT and 2.5µl 10mM dNTPs were then added to the reaction mix, this was vortexed and pulse centrifuged before heating to 45°C for 2 minutes. 2.5µl of Superscript II reverse transcriptase was added and then the reaction vessel placed in a 45°C waterbath for 30 minutes. Another 2.5µl of Superscript II reverse transcriptase was added and reaction temperature maintained at 45°C for a further 30 minutes before placing on ice.

For second strand synthesis 227.5µl DEPC water was added straight to the reaction followed by 75µl 5x second strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl; 15 mM $MgCl_2$), 7.5µl 10mM dNTPs, 2.5µl E.coli DNA ligase (10iu/µl), 10µl E.coli DNA polymerase I (10iu/µl) and 2.5µl E.coli Rnase H (2iu/µl) to produce a total volume of 375µl. This was vortexed gently and incubated for 2 hours at 16°C. Following this 5µl (5iu/µl) T4 DNA polymerase was added and the incubation continued at 16°C for a further 15 minutes. The reaction tube was placed on ice and 10µl of EDTA 0.5M added to the reaction mix. Phenol:chloroform:isoamyl alcohol (25:24:1) (375µl) (Invitrogen, UK)was added to the reaction which was then vortexed and centrifuged at 18,000g for 20 minutes and the supernatant then removed and retained. 100µl LoTE was added to the reaction which was again vortexed and centrifuged at 18,000g for 5 minutes. The supernatant was collected and combined with the previous batch.

To the combined supernatant 3µl 2% glycogen, 100µl 5M ammonium acetate, and 1ml 100% ethanol was added and the reaction stored at -70°C for 24 hours to precipitate the cDNA. This was centrifuged at 18,000g for 15 minutes at 4^0C. The aqueous supernatant was removed and discarded and the pellet washed in 500µl 70% ethanol, vortexed and recentrifuged at 14,000g for 5 minutes at 4°C. The pellet was again washed in 500µl 70% ethanol, vortexed and recentrifuged

at 14,000g for 5 minutes at 4°C. The supernatant was then discarded and the pellet dried and resuspended in 173µl LoTE.

Nla III digestion

The 173µl sample prepared above was combined with 20µl 10x buffer IV (20 mM Tris-acetate, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, pH 7.9), 2µl 100x bovine serum albumin (BSA 10mg/ml), and 5µl Nla III restriction enzyme (all New England Biolabs) and incubated for 1 hour at 37°C. 200µl of phenol:chloroform:isoamyl alcohol (25:24:1) (Invitrogen, UK) was then added and the mixture vortexed and centrifuged at 18,000g for 20 minutes. The supernatant was removed and retained. 100µl LoTE was added to the phenol:chloroform:isoamyl alcohol which was again vortexed and centrifuged at 18,000g for 5 minutes. The supernatant was collected and combined with the previous batch.

Approximately 280µl of supernatant was recovered and precipitated with 3µl glycogen, 75µl sodium acetate 3M, pH5.2, and 360µl isopropanol. The mixture was left on ice for 15 minutes then centrifuged at 18,000g for 15 minutes. The aqueous supernatant was removed and discarded and the pellet washed in 500µl 70% ethanol, vortexed and recentrifuged at 14,000g for 5 minutes at 4°C. The pellet was again washed in 500µl 70% ethanol, vortexed and recentrifuged at 14,000g for 5 minutes at 4°C. The supernatant was then discarded and the pellet dried and resuspended in 20µl LoTE.

Binding to magnetic beads

100µl of magnetic beads (Dynabeads, Dynal) was added to 2 separate tubes, the beads were washed with 200µl 1x binding and washing (B+W) buffer (5mM Tris-Hcl pH 7.5, 0.5mM EDTA, 1mM NaCl), and the tubes then placed in magnetic holders to retain the beads while removing and discarding the buffer. 100µl 2x B+W buffer, 90µl rtH_2O, and 10µl of the cDNA sample prepared above were added to each tube, the beads resuspended and the reaction kept at room temperature for 30 minutes with intermittent mixing. The beads were then washed 3 times with 200µl 1x B+W buffer and once with 200µl LoTE.

Ditag Generation (Figure 3.1 Stage 2)

Ligation of linkers to cDNA

29µl of LoTE was added to each tube to resuspend the beads and then 1µl of linker A was added to tube A and 1µl of linker B to tube B (linkers at 200pmol/µl concentration). 8µl 5x ligase HC buffer(250 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000; Invitrogen, UK) was then added to each tube. The tubes were warmed to 50°C for 2 minutes and then placed at room temperature for 15 minutes. 2µl of T4 Ligase HC (Invitrogen, UK) was added to each tube and both tubes incubated for 2 hours at 16°C while mixing the reaction solution and beads intermittently.

Linker Sequences

Linker A

TTTGGATTTGCTGGTGCAGTACAACTAGGCAATATAGGGACATG
CCTAAACGACCACGTCATGT TGATCCGT TATATCCCT

Linker B

TTTCTGCTCGAATTCAAGCTTCTAACGATGATCGGGGACATG
GACGAGCTTAAGTTCGAAGATTGCTACTAGCCCCT

The beads were washed with 1x B+W buffer eight times, and twice with 1x buffer IV (New England Biolabs).

Release of cDNA using tagging enzyme (BSM F1)

The beads were resuspended in 97µl 1x buffer IV, 1µl 100x BSA and 2µl BSM F1 (all from New England Biolabs) added and incubated at 65°C for 1 hour and both reactions mixed regularly. The magnetic holders were used to retain the beads to allow the removal and retention of the supernatant.

100µl of phenol:chloroform:isoamyl alcohol (25:24:1) (Invitrogen, UK) was added to the superantant which was then vortexed and centrifuged at 18,000g for 10 minutes. The supernatant was removed and retained. 100µl LoTE was added to the phenol:chloroform:isoamyl alcohol which was again vortexed and centrifuged at 18,000g for 5 minutes. The supernatant was collected and

combined with the previous batch and precipitated using 3µl glycogen, 100µl sodium acetate 3M, pH5.2, and 900µl cold 100% ethanol at -70°C for 3 hours and then centrifugation at 18,000g for 15 minutes. The aqueous supernatant was removed and discarded and the pellet washed in 500µl 70% ethanol, vortexed and recentrifuged at 14,000g for 5 minutes at 4°C. The pellet was again washed in 500µl 70% ethanol, vortexed and recentrifuged at 14,000g for 5 minutes at 4°C. The supernatant was then discarded and the pellet dried and resuspended in 8µl LoTE.

Blunt ending and ligation

This was carried out using the Takara blunt ending and ligation kit (Takara Mirus Bio, Madison, USA) according to following protocol.

To the 8µl sample 1µl 10x blunt ending buffer was added and the mix warmed to 37°C for 5 minutes before cooling at room temperature for a further 5 minutes. 1µl T4 DNA polymerase was added to the solution which was left at room temperature for 10 minutes before being placed on ice. 40µl of ligase solution A and 10µl of ligase solution B were added and the reaction incubated overnight at 16°C. Following this the total reaction volume was increased to 100µl using LoTE.

Ditag Amplificaiton (Figure 3.1, Stage 3)

Polymerase chain reaction of SAGE template

	µl	Cycling parameters	
$MgCl_2$ [25mM]	1.5		
10x buffer*	3	95°C	for 1 minute
dUTP [20pmol]	0.3	Then 24 cycles of:	
Primer A [100pmol]	1	95°C	for 20 seconds
Primer B [100pmol]	1	60°C	for 20 seconds
Taq (5iu/µl)*	0.3	72°C	for 2 minutes
H_2O	21.9		
Template	1	Then	4°C hold

(*Abgene, UK. 10x buffer composition 750mM Tris-HCl pH 8.8, 200mM $(NH_4)_2SO_4$ 0.1% (v/v) TWEEN 20)

This 100µl sample was used as a PCR template to bulk up the ditags. Samples were prepared at dilutions of 1/10, 1/50 and 1/200 and used as templates for the above PCR protocol, one non template control was included with each reaction

Primer A 5'-TTTGCTGGTGCAGTACAACTAGGCAAT-3'
Primer B 5'-GCTCGAATTCAAGCTTCTAACGATGAT-3'

After completion of the PCR reaction the products were run on a 12% PAGE gel which was stained by washing in ethidium bromide solution (6µl ethidium in 250µl 1x TAE) for 15 mins and then rinsing in 1x TAE for 15mins. The cDNA could then be visualised under uv light. The sample with the most well demarcated 100bp band was selected for bulk amplification.

100bp Bulk amplification

Using the protocol below 95 PCR amplifications and 1 non template control were prepared

	µl	Cycling parameters	
$MgCl_2$ [25mM]	2.5		
10x buffer*	5	95°C	for 1 minute
dUTP [20pmol]	0.5	Then 24 cycles of :	
Primer A [100pmol]	0.6	95°C	for 20 seconds
Primer B [100pmol]	0.6	60°C	for 20 seconds
Taq (5iu/µl)*	0.5	72°C	for 2 minutes
H_2O	40.1		
Template	0.2	Then	4°C hold

(*Abgene, UK. 10x buffer composition 750mM Tris-HCl pH 8.8, 200mM $(NH_4)_2SO_4$ 0.1% (v/v) TWEEN 20)

The PCR product was run on a 12% PAGE gel alongside 100bp ladder at 120V for 3 hours, stained by washing in ethidium bromide solution (6µl ethidium in 250µl 1x TAE) for 15 mins and then rinsed in 1x TAE for 15mins, The 100bp band was visualised cDNA under uv light and excised from the gel and the eluted using the electrolavage protocol (discussed later).

70bp Bulk amplification

Using the protocol below 95 PCR amplifications and 1 non template control were prepared

	µl	Cycling parameters	
$MgCl_2$ [25mM]	2.5		
10x buffer*	5	95°C	for 1 minute
dNTP [20pmol]	0.5	Then 13 cycles of :	
Primer 1 [100pmol]	0.875	95°C	for 20 seconds
Primer 2 [100pmol]	0.875	53°C	for 20 seconds
Taq (5iu/µl)*	0.3	72°C	for 2 minutes
H_2O	39.45		
Template	0.5	Then	4°C hold

(*Abgene, UK. 10x buffer composition 750mM Tris-HCl pH 8.8, 200mM $(NH_4)_2SO_4$ 0.1% (v/v) TWEEN 20)

Primer 1 5'-AACTAGGCAATATAGGGA-3'

Primer 2 5'-TCTAACGATGATCGGGGA-3'

The PCR product was run on a 12% PAGE gel alongside 100bp ladder at 120V for 3 hours, stained by washing in ethidium bromide solution (6µl ethidium in 250µl 1x TAE) for 15 mins and then rinsed in 1x TAE for 15mins, The 70bp band was visualised cDNA under uv light and excised from the gel and then eluted using the electrolavage protocol (discussed later).

Nla III digestion of 70bp product.

To 100µl sample in LoTE 13µl 10x Buffer IV, 2µl 100x BSA, and 13µl NlaIII (New England Biolabs) were added and the reaction incubated at 37°C for 3 hours. 30µl of loading buffer was combined with the reaction mixture and the sample run on a 12% PAGE gel alongside 100bp ladder at 120V for 3 hours. The gel was stained by washing in ethidium bromide solution (6µl ethidium in 250µl 1x TAE) for 15 mins and then rinsed in 1x TAE for 15mins, The 26bp cDNA band was visualised under uv light and excised from the gel and then eluted using the electrolavage protocol (discussed later).

Removal of residual linker with streptavidin beads

The linker sequence can erroneously contribute to the SAGE library by contributing tags based on linker cDNA rather than sample mRNA. Although

knowledge of the linker sequence means these tags can be easily recognised they may mask genuine SAGE tags which coincidentally possess the same or similar sequence. They may also reduce the efficiency of library sequencing since large proportions of linker based tags will be sequenced alongside genuine SAGE tags. Removal can be performed by using strepavidin coated magnetic beads with poly A oligonucleotides to adhere to the short poly T tail present on the linkers. In order to carry this out 100µl of magnetic beads (Dynabeads, Dynal) were placed in a 1.5ml eppendorf tube, washed with 200µl 1x binding and washing (B+W) buffer, and then the tube placed in a magnetic holder to retain the beads and allow buffer to be removed and discarded. The cDNA sample was added to the tube and left at room temperature for 15 minutes while mixing intermittently. Using the magnetic holder the suspension was removed and retained, and the beads washed with a further 100µl LoTE. The wash was removed and combined with the suspension, made up to 450µl and precipitated in 1400µl 100% cold ethanol, 75µl sodium acetate 3M, pH 5.2, and 5µl glycogen at -70°C for 2 hours. The sample was centrifuged at 18,000g for 15 minutes. The aqueous supernatant was removed and discarded and the pellet washed in 500µl 70% ethanol, vortexed and recentrifuged at 14,000g for 5 minutes at 4°C. The pellet was again washed in 500µl 70% ethanol, vortexed and recentrifuged at 14,000g for 5 minutes at 4°C. The supernatant was then discarded and the pellet dried and resuspended in 7µl rtH_2O.

Concatamerisation, cloning and sequencing (Figure 3.1 Stage 4)

Concatamerisation

To the ditag suspension 2µl 5x ligase buffer was added along with 1µl of DNA ligase HC (Invitrogen, UK). This was incubated at 16°C for 1 hour, a further 1µl of DNA ligase HC was added and the reaction incubated for 1 hour, a further 1µl of DNA ligase HC was again added and the reaction incubated for a final hour. The sample volume was increased to 25µl with LoTE, and warmed to 65°C for 15 minutes and then cooled on ice for 10 minutes. 5µl loading buffer was added and the sample run on a 1% agarose gel at 150V for 35-40 minutes alongside

100bp ladder. The gel was visualised under uv light and the concatamer cDNA excised in two sections, from 200-800bp and above 800bp. Extraction was performed by electrolavage elution (described later) and the sample resuspended in 6µl rtH_2O.

Preparation of pZERO vector

7µl rtH_2O was added to 1µl 10x reaction buffer 6 (50 mM Tris-HCl (pH 7.4), 6 mM $MgCl_2$, 50 mM NaCl, 50 mM KCl), 1µl pZERO vector (1µg/µl) and 1µl SpH 1 (10U/µl) restriction enzyme (All Invitrogen, UK). The sample was incubated at 37°C for 30 minutes, 20µl LoTE added and then warmed to 70°C for a further 20 minutes. Samples of cut and uncut vector were run on a 1% agarose gel to ensure complete cutting.

Ligation of concatamers into vector

The 6µl concatenation product was combined with 2µl 5x ligase buffer, 1µl DNA ligase HC and 1µl opened pZERO vector. This was incubated at 16°C for 3 hours then its volume raised to 200µl with LoTE and 200µl of phenol:chloroform:isoamyl alcohol (25:24:1) (Invitrogen, UK) added. This was then vortexed and centrifuged at 18,000g for 10 minutes. The supernatant was removed and retained. Another 200µl of LoTE was added to the phenol:chloroform:isoamyl alcohol which was again vortexed and centrifuged at 18,000g for 5 minutes. The supernatant was collected and combined with the previous batch and precipitated using 3µl glycogen, 50µl sodium acetate 3M, pH5.2, and 1300µl cold 100% ethanol at -70°C for 2 hours and then centrifuged at 18,000g for 15 minutes. The aqueous supernatant was removed and discarded and the pellet washed in 500µl 70% ethanol, vortexed and recentrifuged at 14,000g for 5 minutes at 4°C. The pellet was again washed in 500µl 70% ethanol, vortexed and recentrifuged at 14,000g for 5 minutes at 4°C. The supernatant was then discarded and the pellet dried and resuspended in 10µl LoTE.

Transformation of cells

The vector was inserted into DH10B E.*coli* cells (Invitrogen, UK) by electroporation at 2.5V in a electroporation cuvette (Invitrogen, UK). 1µl DNA was added to 40µl cells defrosted on ice, electroporated, and cells transferred to 1ml SOC medium (Invitrogen, UK). The SOC/cell suspension was incubated at 37°C for 1 hour, shaking at 220rpm. Cells were then plated out on low salt agar plates (10g/L tryptone, 5g/L NaCl, 5g/L yeast extract, 5g/L agar; all Sigma-aldrich, UK) containing Zeocin (Invitrogen, UK) at 50µg/ml and cultured overnight at 37°C.

Colony PCR and insert selection

PCR was performed using the M13R (5'-GAATTGTAATACGACTCACT-3') and pZERO (5'-TCACACAGGAAACAGCTATGA-3') primers as described previously. PCR product was run on a 1% agarose gel and PCR products greater than 400 bp selected for sequencing. Empty vector was 217 bp in size meaning that insert sizes of approximately 200 bp and above were chosen to maximise sequencing efficiency.

Sequencing of concatamers

This was performed using the P zero primer as previously described.

3.3 Methodological alterations required for successful SAGE

During work on this project four main problem areas developed with the original protocol and required consideration to improve efficient production of the SAGE libraries. Firstly, linker release by Nla III cutting of the genuine 70bp PCR product was inefficient and unreliable. Secondly, during production of the second library we experienced a problem with preferential amplification of a spurious 70bp product without the CATG cutting sites. Thirdly, extraction of any of the DNA products from PAGE gels, but especially the 26bp band, using standard techniques proved problematic and inefficient. Finally, concatamerisation and cloning was subject to an element of chance, with a frequent occurrence of high background levels of empty vector. The approaches used to address these issues shall now be described.

3.3.1 Failure of restriction enzyme digestion

Inefficient Nla III digestion of amplified PCR product (figure 3.1, stage 3) created a substantial loss of amplified ditags due to complete or partial failure of linker release and subsequent discarding of poorly digested product during gel purification. This problem arose due to two factors, the first was solved with the use of PAGE gel purification of the cDNA substrate, the second by PCR selection, through specific primer design, of sequences containing the Nla III recognition site

3.3.1.1 PAGE gel purification

Inefficient Nla III digestion was encountered during construction of the first SAGE library (figure 3.1, stage 3). This is a problem previously reported by others (Angelastro et al 2000). The ditag yield from this reaction was frequently so poor as to preclude successful attempts at concatmerisation. The original protocol (Velculescu et al 1995, Angelastro et al 2000) required the NlaIII digestion of PAGE gel purified 102bp ditag and linker cDNAs. Angelastro et al (2000) suggested that the inhibition of Nla III action was the result of soluble

contaminants carried over from the PAGE gel purification of the 102 bp cDNA, and developed two protocols which solved this problem. Firstly by binding the cDNA to a fused silica membrane, followed by washing and subsequent elution (Qiaquick Kit, Qiagen), secondly by removal of contaminants with centrifugation gel filtration (Clontech spe10 spin gel filtration columns) in both cases using commercially available apparatus.

Interestingly this was at odds with our own experience. No PAGE gel separation or purification was initially required in our technique since we utilised a nested 70bp PCR reaction to further amplify the ditag pool prior to Nla III digestion. Our own protocol incorporating this nested PCR reaction required phenol/chloroform/isoamyl alcohol purification of the PCR reaction, followed by ethanol precipitation and resuspension in 1x buffer IV prior to NlaIII cutting. Consequently PAGE gel interference with restriction enzyme function should not have arisen. Despite this we also encountered unreliable NlaIII digestion. Similarily, our attempts to improve NlaIII performance by using commercial purification kits were equally unsuccessful. The Nucleotrap Gel Extraction Kit (BD Biosciences, UK) allows extraction of DNA molecules greater than 20bp in size from gels and aqueous solutions via reversible matrix binding allowing elution of nucleic acid in low volumes of low salt buffer. This kit was used according to manufacturers instructions to attempt purification of the 70 bp PCR product to improve subsequent restriction enzyme activity. No consistently improved results were noted. Typical Nla III performance after using these methods is demonstrated by the PAGE gels in figure 3.3

We eventually found that, in complete contrast to Angelastro et al, purification of the 70bp band by running in and extracting from a 12% PAGE gel consistently resulted in successful cleavage of the band in question. This step was therefore adopted as an additional stage routinely performed on the PCR product prior to attempting Nla III cleavage. To date near complete Nla III digestions have been successfully performed on all sequences containing the CATG recognition site using this method.

Figure 3.4 demonstrates Nla III digestion products from PAGE gel purified PCR products.

3.1.1.2 Spurious 70bp sequence.

The PMSG/hCG treated SAGE library also experienced problems with Nla III digestion (figure 3.1, stage 3) due to the preferential amplification of a spurious 70bp band containing linker sequence at either end but no CATG recognition sites. This band was cloned into TOPO TA vector (Invitrogen, UK) and sequenced, all clones yielding the same sequence.

The sequence contained between the linker sequences (underlined) does not contain the CATG cutting site and does not match any of the tag sequences produced in the SAGE libraries. Elimination of this sequence was achieved by extending the PCR primers to incorporate the first C base of the CATG sequence. The failure of the last base to hybridise prevents primer extension by the polymerase and thereby stopped replication of this particular sequence and allowed unhindered amplification of the ditag containing sequences.

Interestingly others (Du et al 2003) have proposed using primers containing the full CATG recognition sequence for ditag amplification. While this undoubtedly improves the efficiency of subsequent NlaIII digestion by eliminating polymerase errors within the recognition site, it does mean that any spurious sequence carried through into the PCR reaction will have recognition sites artificially inserted and can thus contribute erroneous tag sequence to the final analysis.

Figure 3.2 Spurious 70bp band sequence, primer sequences are underlined

AACTAGGCAATATAGGGATTGGATTTGCTGGTGCAGTACAACTAA
GGTCCCGATCATAGTTAGA

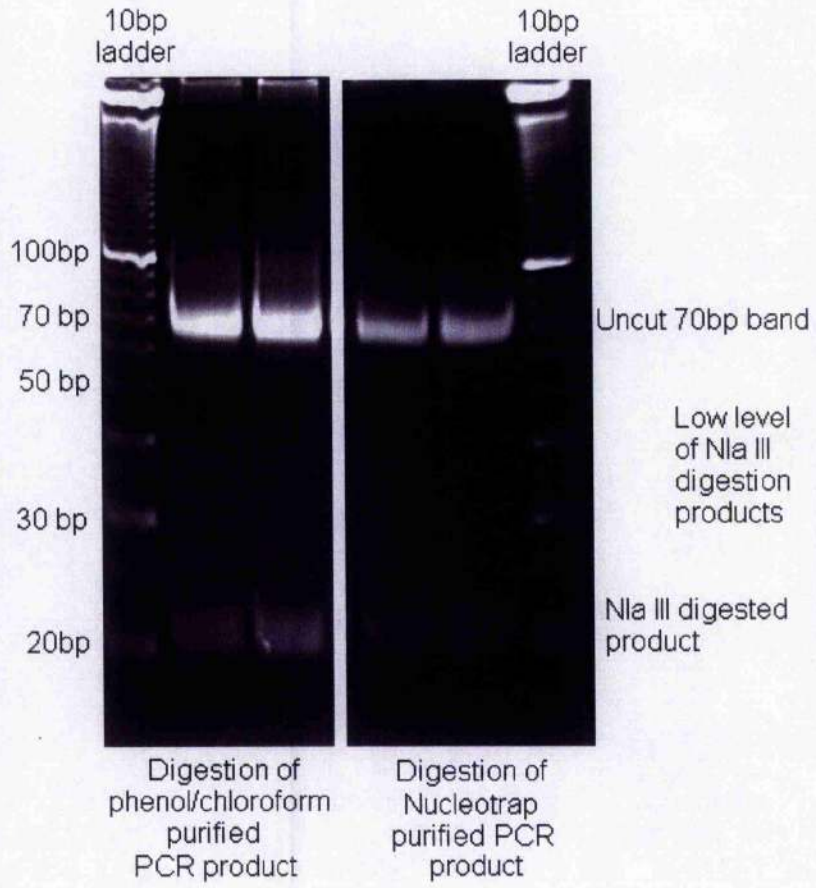


Figure 3.3 Efficiency of Nla III digestion following differing substrate purification methods, namely Nucleotrap (BD Biosciences, UK) and phenol:chloroform extraction.

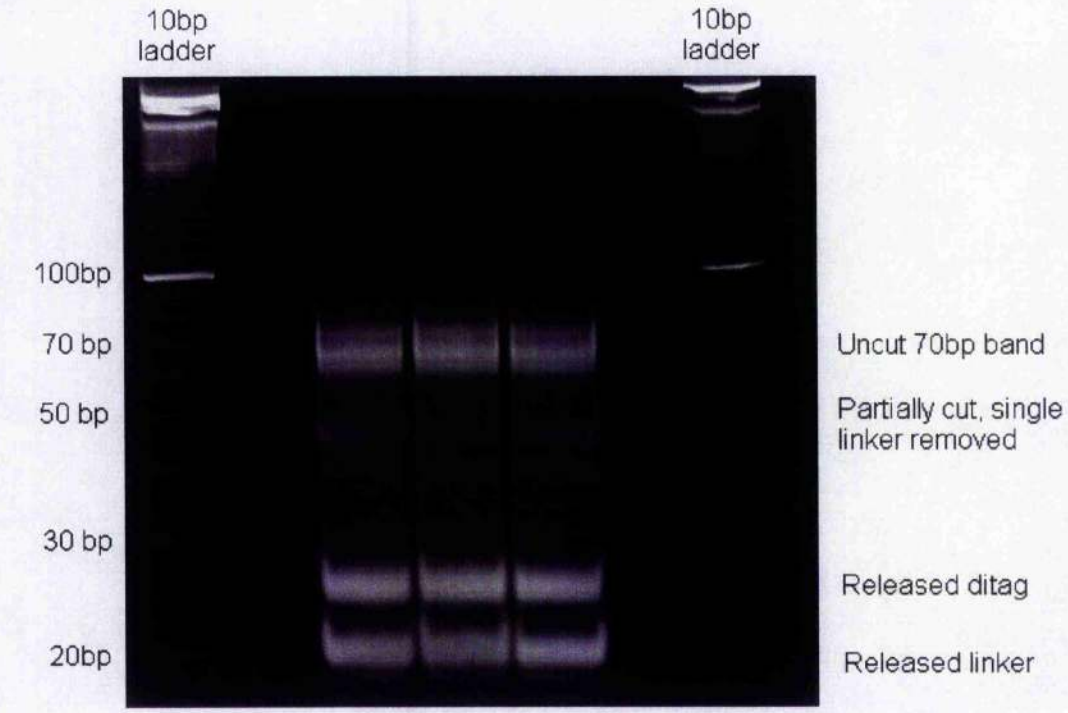


Figure 3.4 Nla III digestion following purification and extraction of 70bp cDNA substrate in PAGE gel

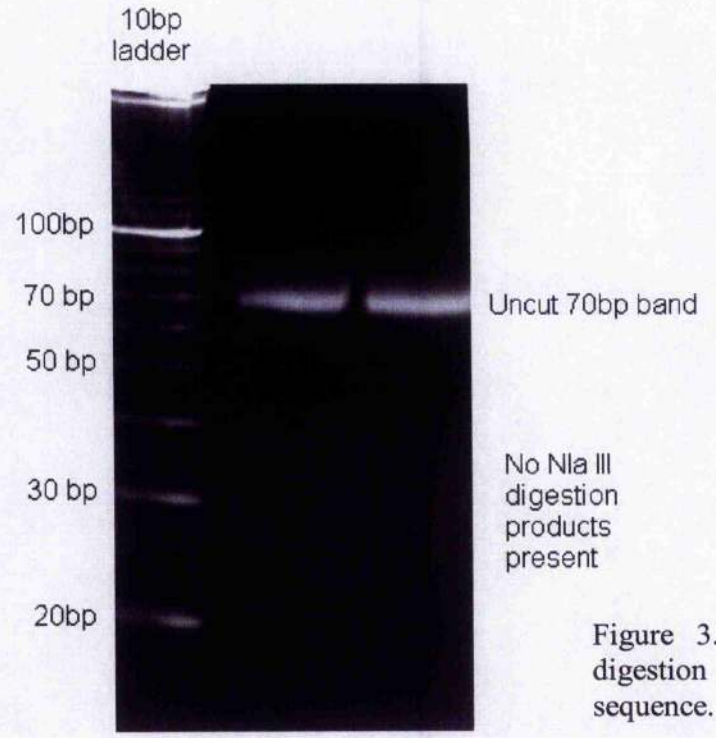


Figure 3.5 Unsuccessful Nla III digestion of spurious 70bp sequence.

3.3.2 PAGE gel extraction

Our SAGE protocol requires 4 PAGE gel extraction steps. The first to purify the 100bp band from the initial PCR reaction, the second to purify the 70bp band prior to NlaIII digestion, the third to purify the ditags following Nla III digestion, and the fourth to allow size selection following the concatamerisation reaction. Three of these steps occur after the final PCR amplification. Our experience is that the relative recovery rates from acrylamide gels using the maceration and incubation steps as described in the original SAGE protocol are 40% for incubations carried out at 65oC and less than 25% for incubations carried out at around room temperature (room temperature incubations being required to prevent dissociation of small double stranded cDNA products). The consequence of this is that following the final 70bp PCR amplification only 40% recovery can be expected from the 70bp 12% PAGE gel, only 25% retrieval of this can be expected from the 26bp ditag purification (12% PAGE gel), and only 40% of this will survive the size separation of the concatamers (8% PAGE gel). If we are to optimistically assume 100% efficiency of the Nla III cut, and at least 50% of the ditags being incorporated into suitably sized concatamers, with no loss whatsoever in the various intervening phenol/chloroform/isoamyl alcohol extractions and ethanol precipitations, this still means that only 2% of the ditags produced by the final 70bp PCR amplification will survive to the cloning stage. This is clearly grossly inefficient and a major flaw in the SAGE procedure. Other separation and extraction methods were tried in order to improve this situation.

3.3.2.1 Agarose gel separation

Use of agarose gel as an alternative to PAGE to try to separate and purify the 70bp and 26bp band from the PCR reaction and Nla III digestion respectively was, perhaps predictably, unsuccessful. Extraction can be easily performed using several commercially available kits but poor band separation and cross contamination of sequences between bands lead to the carry over of linker sequence into the subsequent stages, the importance of avoiding this carryover

has already been explained. Use of 1% agarose was however adopted for the separation of concatamers due to the ease of DNA extraction using either the Nucleotrap (Clontech) or electroelution and lavage methods (as discussed below).

3.3.2.2 Electroelution extraction

The extraction of DNA from all gel types by electroelution is a well known method which has previously been employed in the SAGE protocol with success (Hayday laboratory, personal communication). Our own attempts at electroelution met with reasonable success with extraction of the 70bp PCR product, but very little success with the extraction of the 26 bp ditags. Two methods were employed, the first as recommended by the Hayday laboratory (personal communication) using dialysis tubing, the second using GeBA flex tubes (GeBA Ltd, USA).

The Hayday laboratory used the apparatus shown below to elute ditags from polyacrylamide gel. Electrophoresis was performed at 100V for 30 minutes in 300µl 1x TAE buffer. The buffer was then removed and replaced before twice repeating the procedure. The 900µl of buffer was collected and precipitated.

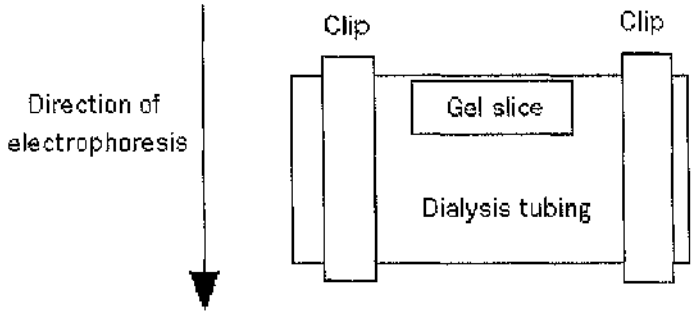


Figure 3.6 Hayday apparatus for ditag elution

Our own attempts with this model were moderately successful for elution of the 102 bp and 70 bp band from acrylamide gel but failed completed to elute the 26 bp ditags with total loss of ditags from the gel and the buffer, presumably due to penetration of the tags through, or into, the dialysis membrane.

The GeBA flex system relied on the same electroelution principle. Using the apparatus shown (figure 3.7) the gel slice was exposed to 100V for 15-30 minutes. The polarity of the current was reversed for the last 60 seconds to release any nucleic acid that may have become embedded in the dialysis membrane.

Again this system was reasonably successful for the elution of 102 bp and 70 bp sized products but it failed to recover any of the 26 bp ditags.

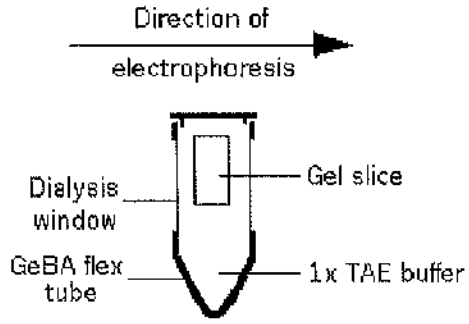


Figure 3.7 GeBA flex apparatus for eluting nucleic acids

3.3.2.3 Extraction by electroelution and lavage.

This method which I developed was based on the principle that the smaller 26bp DNA fragments are managing to penetrate or become embedded into the dialysis membrane during the electroelution. If these fragments could be continuously washed off the membrane surface by fluid currents within the elution buffer they should be retained within the buffer rather than lost through or into the tubing. The continuous drawing off and replacement of buffer would result in an excessive elution volume and so necessitated the constant recirculation of the elution buffer by means of a syringe attached to the dialysis tubing. The rather simple apparatus shown below was employed to this end.

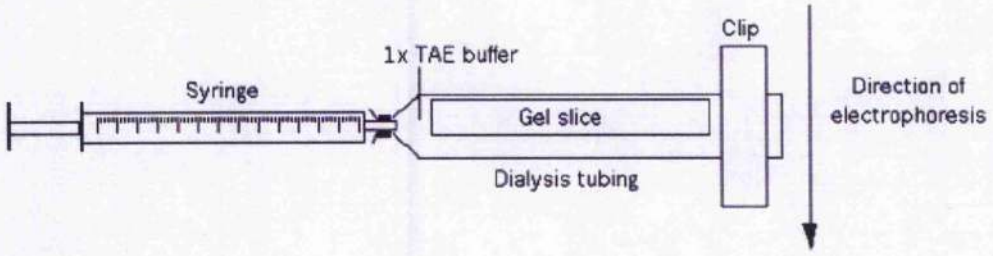


Figure 3.8 Apparatus for elution of nucleic acids from polyacrylamide gels.

The comparative yields of a control cDNA sample 217bp in size achieved by using the above methods is demonstrated visually by the PAGE gel shown in figure 3.9. A typical example of Nla III digestion of the 70 bp PCR product following electroelution and lavage is shown in Figure 3.10. This improvement in our ability to elute cDNA from PAGE gels was the principle factor in improving the ease and efficiency with which we were able to perform SAGE.

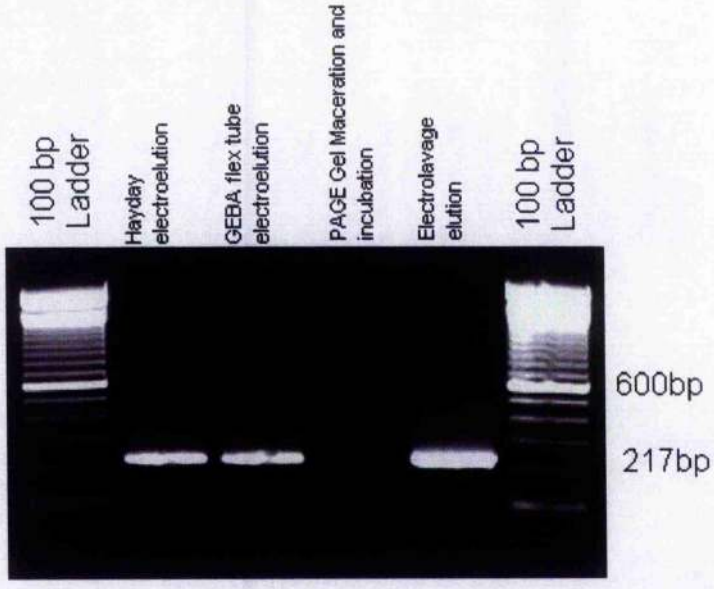


Figure 3.9 Comparative yields of a 217 bp cDNA recovered from PAGE gel using incubation or electroelution methods.

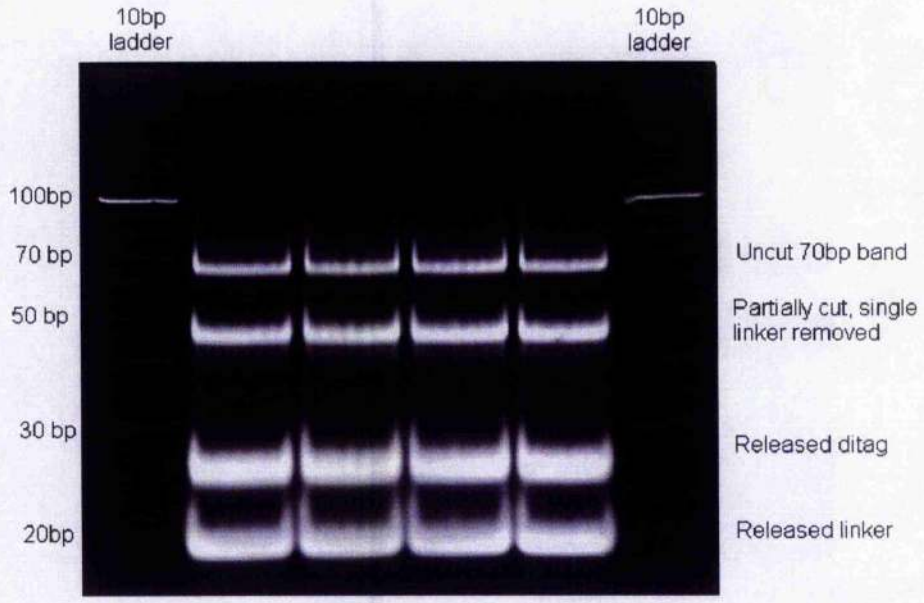


Figure 3.10 Typical Nla III digestion after using the electroelution and lavage technique for extraction of substrate from PAGE gels.

3.3.3 Formation and selection of concatamers

The successful production of concatamers by ligating ditags end to end (figure 3.1 stage 4) is more complex than might be expected. The reaction set up is straight forward but judging the correct reaction time to ensure the majority of concatamers are in the correct size range is difficult. The obvious solution of removing samples from the reaction mixture to check concatamer formation on an agarose gel substantially reduces the final volume of concatamers available for cloning. Equally the formation of secondary structure by the DNA molecules means the gels often run as smears rather than clear bands making size determination awkward. The possibility of capping of concatamers by ditags or short sequences (such as carried over linkers) with only one correct CATG binding site is also a real problem. The likelihood of this happening is increased by the intrinsic exonuclease activity of cDNA ligases. A final possibility is the circularisation of concatamers formed by the ligation of complementary CATG sites at either end of the ditag chain, in this event the product will not size correctly on gels and neither is it possible to clone it into vector. In order to minimise the occurrence of either truncated cDNA ends or circularisation the reaction time was kept to a maximum of 3-4 hours.

One possible solution to the problem is to set up a cloning reaction with the vector and ditags without a separate concatamerisation step. This was the solution adopted for the first PMSG stimulated SAGE library which on that occasion provided an acceptable result with approximately 50% of vector inserts being large enough to countenance sequencing. Unfortunately this method proved unreliable with the majority of the inserts usually being low size and after repeated attempts we reverted to performing concatamerisation for the second library.

3.3.3.1 Size selection for cloning

In order to maximise the efficiency of the final sequencing stage of SAGE, important since this is the most labour intensive and expensive part of the

protocol, it is desirable that the final cloned insert should be within a suitable size region, ideally 400bp or greater. Our own efforts to separate concatamers where not as successful as we would have liked. Several commercially available kits claim the ability to efficiently separate and purify DNA on the basis on molecular weight such as Nucleotrap (BD Biosciences) and CHROMA SPIN (BD Biosciences). The use of such apparatus to size select concatamers should in theory improve the cloning and sequencing efficiency of SAGE. We attempted this with CHROMA SPIN-400 columns (BD Biosciences) which claim to purify and size select nucleic acids >600bp with a recovery rate of up to 90%. The columns were used according to the manufacturers protocol which involved prespinning at 700g for 5 minutes to remove buffer, loading the sample into the column and then centrifugation at 700g for 5 mins and collecting the eluted size selected cDNA. Smaller molecules should be retained within the matrix pores. Subsequent cloning into pZERO (Invitrogen, UK) vector yielded an average insert length of 550bp, equivalent to 40 SAGE tags per insert. This is an excellent ratio but had to be offset against a disappointingly low yield of concatamers leading to only 23% of colonies containing inserts. Consequently although a viable method of size selection low insert yield from the subsequent cloning reaction rendered this an inefficient method.

Gel electrophoresis separation has been shown to yield an average concatamer size of only 137bp within the 700-1000bp fraction of the gel and the average concatamer size within a 700-2500bp fraction being only 287 bp (Kenzelmann and Muhlemann 1999). This anomaly can be explained by the aggregation of smaller fragments by hydrogen bonding leading to a communal migration as a larger unit. Equally the presence of inactivated enzyme molecules binding to cDNA fragments may cause slower migration. Although some of this effect can be alleviated by heating the concatamer sample prior to electrophoresis to disrupt secondary structure (Kenzelmann and Muhlemann 1999) it still remains a source of disruption of size selection. Gel separation does however have the considerable advantage that it at least retains the entire product within the gel and consequently a high proportion of the concatamers can be recovered

following electrophoresis. It remains the preferred method for concatamer selection.

3.3.3.2 Cloning concatamers

Cloning inserts into vector presented a problem with a high incidence of apparently empty vector despite the use of suicide gene or blue/white selection. Different vectors and cloning/selection methods were investigated to attempt to minimise this problem.

Suicide Selection

pZERO (Invitrogen UK) is the vector of choice due to ease of cloning, presence of a SpH 1 restriction endonuclease site allowing insertion of sequence containing a CATG overlap and the suicide selection method for eliminating empty vector. On numerous occasions using this vector we obtained a high proportion of empty vector containing colonies, indicating a high background of nonrecombinant plasmids and a failure of suicide gene activation. Attempts to reduce this background by using calf intestinal alkaline phosphatase (Promega, USA) to dephosphorylate cut vector ends (carried out according to manufacturers instructions) did not produce any improvement in background levels of empty vector. Equally the length of the cloning reaction was varied from 2 hours to 24 hours without marked difference in the level of recombinant product. The occurrence of high levels of nonrecombinant vector did appear to be associated with both individual batches of vector and the increasing age of the vector batch. Examples of PCR amplification of vector inserts using the SAGE vector based primers on colonies produced from successful and unsuccessful cloning reactions are shown in figure 3.11.

Optimisation of the cloning reaction with regard to vector insert ratio is not easily achievable due to the highly variable sizes of the concatamers being cloned. Attempts were made to increase this ratio by carrying out a short 30 second digestion of the concatamers with the SpH 1 restriction enzyme (8µl concatamers added to 1µl 10x reaction buffer 6 and 1µl SpH 1 restriction

enzyme (All Invitrogen, UK). The reaction mix was incubated at 37°C for 30 seconds, and then 20µl LoTE was added and the mix heated to 70°C for a further 20 minutes.) This enzyme has the 6 base recognition site GCATGC which should digest 1 in every 16 CATG sites, thus shortening the concatmers and increasing the number of cDNA ends available to ligate into vector. No appreciable difference was produced in relation to reducing the number of nonrecombinant colonies using this method.

We did however investigate other cloning methods in an attempt to improve cloning efficiency.

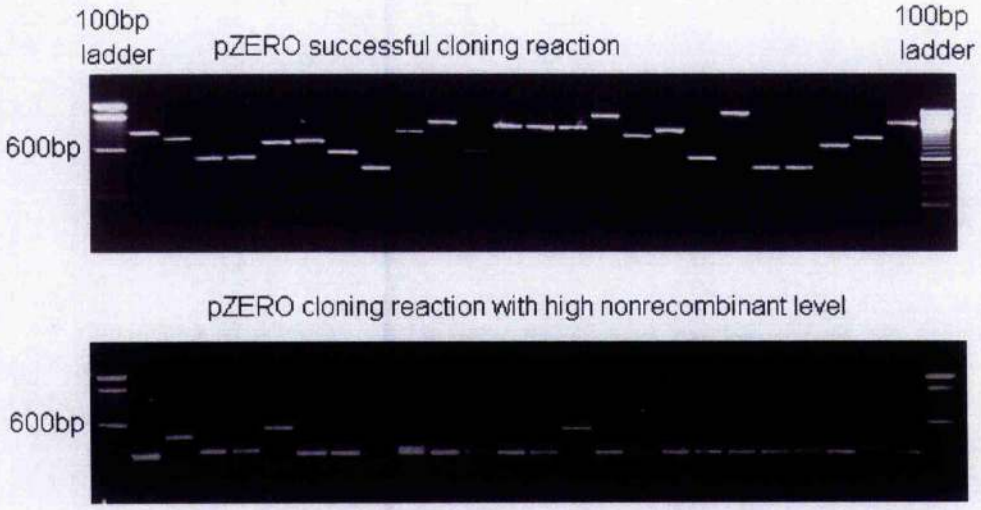


Figure 3.11 Successful and unsuccessful pZERO cloning attempts under identical reaction conditions

Blue/white Selection

The apparent failure of the pZERO selection strategy to eliminate empty vector led us to attempt another method of selection, namely blue/white selection. Increased efficiency with this method has been reported by Angelastro et al (2002). pGEM vector (Promega, USA) makes use of the lacZ blue/white selection to determine the presence of inserts. Unfortunately this proved no more successful than the pZERO system, with equal levels of empty and recombinant

vector in both blue and white colonies, with the largest proportion of colonies containing nonrecombinant vector.

TOPO TA Cloning System

The TOPO (Invitrogen, UK) range of cloning vectors utilise the bound topoisomerase attached to each end of the open vector to ligate the desired insert. Selection of cloned insert over empty vector is performed by means of a suicide gene.

The pCR 4-TOPO vector (Invitrogen, UK) is supplied open with an A base overhang to permit cloning of PCR product. In order to use this vector the concatamers were added to a 30µl reaction volume containing taq polymerase (0.5U) and dNTPs (0.2mmol) (both Abgene, UK) before incubation at 72°C to fill the CATG site and leave only an adenosine base overhang. Cloning was then performed as per manufacturers instructions. This method was successful in eliminating the carryover of empty vector but unfortunately was not sufficiently productive for SAGE. Each cloning reaction yielded only 350-400 colonies, far short of the 5000 or more colonies required (based on an average yield of 10 tags/insert) to produce a SAGE library of around 50,000 tags.

In conclusion successful pZERO concatamer cloning reactions are considerably more efficient than alternative vectors, yielding a far greater number of recombinants than TOPO and having a higher selection efficiency of recombinants than blue/white screening. The absence of the high number of non recombinants occurring with the pGEM and on occasions with the pZERO vectors when using the TOPO cloning system suggests that the incidence of failure of suicide gene selection may be related to the use of either SpH 1 to cleave the plasmid prior to cloning, or the subsequent use of DNA ligase to religate the plasmid.

The improvements to Nla III digestion and polyacrylamide gel extraction meant that it became easier to generate larger volumes of concatamers in a consistently repeatable manner. This means that generating concatamers for repeated cloning attempts on those occasions when pZERO cloning produced high levels of

recombinant vector in the end proved not to be a significant problem. Consequently pZERO cloning was retained as the method of choice for the SAGE protocol, although this is not a particularly satisfactory resolution and refinement is still required.

3.4 SAGE Results

The total number of tags sequenced in the PMSG library (treatment with PMSG alone) was 51,528 while the total number sequenced in the PMSG/hCG library (treatment with PMSG followed by hCG) was 53,696. The combined total of 105,224 tags corresponded to 40,248 unique transcripts of which 9,877 were represented by 2 or more tags. Of the transcripts represented by more than one tag, 5689 were shared between both libraries, 1806 were unique to the PMSG library and 2382 were unique to the PMSG/hCG library (figure 3.12a). Using the chi-squared test to detect significant differences in tag abundance between those tags with greater than 5 transcripts present in the combined libraries, 499 tags were significantly up-regulated by hCG treatment while 216 tags were significantly down-regulated.

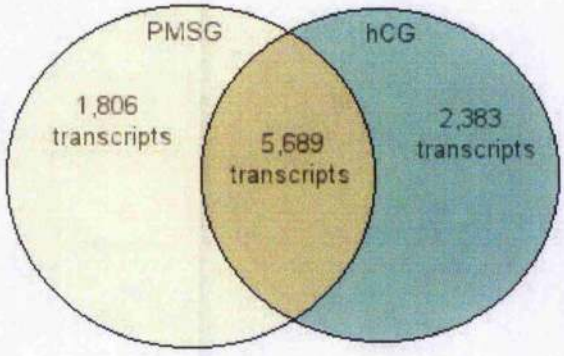


Figure 3.12a. Venn diagram illustrating the distribution of the 9,877 transcripts sequenced at least twice between both libraries

Comparison of the SAGE libraries was carried out using Microsoft Access and statistical calculations performed with Microsoft Excel. Statistical analysis using the Chi Squared test enabled identification of tags over-expressed within one particular library. In cases where tags matched to multiple sequences the identity of the 11^{th} base was used where possible to differentiate between real and spurious matches.

Tables of abundant and differentially expressed tags were generated and tables of granulosa cell specific tags created by comparison with other available murine SAGE libraries (http://www.ncbi.nlm.nih.gov/projects/SAGE/). Tag

identification was achieved using the SAGEmap ftp site (ftp://ftp.ncbi.nlm.nih.gov/pub/sage/) and tags of interest manually curated. Matches were checked manually to ensure reliability.

Matched transcripts have been categorised, and are presented here, either based on abundance of expression in either or both libraries (table 3.1), significant differential expression (table 3.2), current knowledge of transcript function (table 3.3), and uniqueness of expression to this cell type (tables 3.4 and 3.5).

3.4.1 Abundant Tags

The 30 most abundant tags in the combined libraries are shown in Table 3.1. Three of this list are from the mitochondrial genome, 4 encode ribosomal proteins and a further 4 match to more than a single gene. Two tags have no match to known genes within the NCBI database and both of these tags show significant differential expression between the two libraries. This list contains a number of genes known to be highly expressed in granulosa cells or induced by gonadotrophic stimulation including 3β hydroxysteroid dehydrogenase, scavenger receptor 1B (Ghersevich et al 1994, Rajapaksha et al 1997), connexin 43 (Itahana et al 1996) and hyaluronidase 1 (Eppig 1979). If absolute abundance is to be taken as a measure of transcript importance for cellular function those genes identified will necessarily include a number of housekeeping genes such as those encoding metabolic enzymes and the protein synthesis apparatus, i.e. ribosomal components. In addition since this is a remodelling tissue we would expect transcripts responsible for products such as extracellular matrix components and for the maintenance of cytoskeletal structure. This is indeed the case although we also find within this group genes not previously associated with this cell type such as secreted phosphoprotein 1, and translationally controlled tumor protein 1.

Table 3.1 Top 30 most abundantly expressed transcripts present in the combined libraries

Tag Sequence	PMSG	hCG	Total	Unigene	Gene
GTGGCTCACA	878	564	1442		Multiple matches
GCTGCCCTCC	566	409	975	104368	Ribosomal protein L32
ATACTGACAT	321	391	712		Mitochondrial
TAATGTAGAC	405	116	521	4504	Gap junction membrane channel protein alpha 1
CCTTTAATCC	397	61	458	10305	Hyaluronidase 1
TTGCTGCCTT	39	410	449		Multiple matches
TCGCTGCTTT	13	418	431	28327	RIKEN cDNA 2510049I19 gene
TGGGTTGTCT	156	253	409	254	Tumor protein, translationally-controlled 1
TTGTTGCTAC	8	387	395	268000	Vimentin
ATAATACATA	154	231	385	200362	Cytochrome b-245, beta polypeptide
TTGCTACTTT	6	367	373		Multiple matches
AACTGAGGGG	49	306	355	233010	Prosaposin
ATGACTGATA	105	215	320		Mitochondrial
CAAACACCGT	2	278	280	285918	Secreted phosphoprotein 1
GGTTAAATGT	56	206	262	930	Cathepsin L
AGCAAGAATT	28	226	254	1061	Ferredoxin 1
CAGTCAATAC	212	30	242		Unknown
CAGGACTCCG	105	136	241	193096	Stearoyl-Coenzyme A desaturase 2
AGGCAGACAG	94	131	225	335315	Eukaryotic translation elongation factor 1 alpha 1
TGGTTGCTGG	149	70	219		Multiple matches
GCTCTGGGAG	152	56	208	140811	Hydroxysteroid dehydrogenase-1, delta<5>-3-beta
AGCAGTCCCC	94	101	195		Mitochondrial
ACTGAAGCAA	46	146	192	4603	Scavenger receptor class B, member 1
TGACCCCGGG	112	72	184	43005	Ubiquitin A-52 residue ribosomal protein fusion product 1
GGCTTTGGTC	119	60	179	3158	Ribosomal protein, large, P1
TGGTGTAGGA	83	93	176	918	RIKEN cDNAD730039F16 gene
AACAGGGCCA	13	159	172	324864	Mus musculus transcribed sequences
CTAGTCTTTG	94	68	162	154915	Ribosomal protein S29
GAAAATGAGA	133	28	161	315259	Mus musculus transcribed sequences
GGATTTGGCT	107	51	158	14245	Ribosomal protein, large P2

3.4.2 Abundant, differentially expressed SAGE tags

A total of 715 tags showed a significant difference in abundance between the two libraries of which 216 were significantly down-regulated by hCG and 499 were significantly up-regulated. Table 3.2 lists the 35 most abundant tags which were shown to be differentially expressed between SAGE libraries by the Chi-squared test. Most of these tags match unambiguously to known genes and the majority of them are known to be expressed in granulosa cells during development such as the peptide hormones inhibin $ß_B$ and follistatin (O'Shaughnessy and Gray 1995, Jorgez et al 2004), or to show a change in expression after luteinization for example the steroidogenic components P450 11a1, 17ß-hydroxysteroid dehydrogenase type 1 and scavenger receptor class B type 1 (Oonk et al 1989, Ghersevich et al 1994, Rajapaksha et al 1997). This list also contains 13 tags which have no match, are linked to sequence of unknown function or have multiple assignments. In addition there are also a number of transcripts whose expression patterns have not been described previously during luteinisation.

Among those highly upregulated by hCG are vimentin, an intermediate filament protein, developmentally regulated and found in cells of mesenchymal origin, it has been implicated in steroid synthesis via its function as a storage and transport network for lipid droplets containing cholesterol. (Runembert et al 2002, Evans 1998). Leprecan, a basement membrane associated proteoglycan shown to have growth suppressor activity (Kaul et al 2000). Secreted acidic cysteine rich glycoprotein (SPARC) is an upregulated transcript known to inhibit cell cycle progression, influence extracellular matrix interactions and to be strongly angiogenic (Sweetwyne et al 2004). Secreted phosphoprotein 1 can act as a cytokine and has multiple functions associated with cell growth, survival, proliferation and repair (Standal et al 2004). Prosaposin is involved in the lysosomal degradation of sphingolipids (Vaccaro et al 1999) and ferredoxin in electron transfers from NADPH to cytochrome P450 in mitochondria (Liu and Chen 2002). Vanin 1 is a cell surface molecule thought to be involved in the migration of mesenchymal cells, particularly during thymic and gonadal

development, and may be involved in follicular remodelling during and after ovulation (Bowles et al 2000, Aurrand-Lions et al 1996).

The functional distribution of the abundantly upregulated tags reflects accurately what is already known with regard to the changing functions of the differentiating granulosa cell with metabolic, steroidogenic, endocrine and paracine signalling, cytoskeletal and extracelluar matrix proteins all represented within this group.

The complete list of significantly differentially expressed tags that can be unambiguously matched to a single gene can be found in Appendix 1 with annotations describing gene function (functions described on the basis of information contained in either the EMBL Harvester website (http://harvester.embl.de/) or the Information Hyperlinked Over Proteins (IHOP) website (http://www.pdg.cnb.uam.es/UniPub/iHOP/). Of the 715 differentially expressed tags 18% are unmatched, 11.6% match to uncharacterised transcripts, and a further 8.5% match to multiple genes (figure 3.12). The remainder match to characterised named sequences. The functional distribution of these significantly differentially expressed tags is shown in figure 3.13. Many genes have previously been described as having multiple or uncertain functions and consequently may appear in more than one category.

Table 3.2 Top 35 significantly differentially expressed tags present in the combined libraries.

Tag Sequence	PMSG	hCG	Unigene	Gene	P Value
TCGCTGCTTT	13	418	28327	RIKEN cDNA	$4.04x10^{-81}$
TTGTTGCTAC	8	387	268000	Vimentin	$1.29x10^{-77}$
TTGCTACTTT	6	367		Multiple match	$1.22x10^{-74}$
TTGCTGCCTT	39	410		Multiple match	$2.71x10^{-65}$
CCTTTAATCC	397	61	10305	Hyaluronidase 1	$1.49x10^{-58}$
CAAACACCGT	2	278	285918	Spp1 secreted phosphoprotein 1	$2.01x10^{-58}$
AACTGAGGGG	49	306	233010	Prosaposin	$6.37x10^{-40}$
TAATGTAGAC	405	116	4504	Gap junction membrane channel protein alpha 1 (Connexin 43)	$2.42x10^{-39}$
AGCAAGAATT	28	226	1061	Ferredoxin 1	$1.94x10^{-33}$
CAGTCAATAC	212	30		Unmatched	$4.98x10^{-33}$
AGGCAATAAA	3	143	27154	Vanin 1	$1.95x10^{-29}$
AAAGCACACA	0	128		Unmatched	$3.76x10^{-28}$
AACAGGGCCA	13	159	324864	Mus musculus transcribed sequences	$3.55x10^{-27}$
TAACTGACAA	11	140	147226	Metallothionein 2	$2.62x10^{-24}$
CCTCCCCTTG	1	98		Unknown	$3.39x10^{-21}$
TTGCTGCTTT	0	89	250254	RIKEN cDNA	$6.27x10^{-20}$
AGAATGTTAT	3	97		Multiple match	$9.01x10^{-20}$
GTGGCTCACA	878	564		Multiple match	$9.96x10^{-20}$
GGTTAAATGT	56	206	930	Cathepsin L	$6.40x10^{-19}$
ATACTAACGT	6	99	34102	Ornithine decarboxylase, structural	$1.73x10^{-18}$
TACAGTATAA	98	9	3092	Inhibin beta-B	$2.62x10^{-18}$
GGGCATTTGA	11	108	108678	Cytochrome P450, family 11, subfamily a, polypeptide 1	$9.30x10^{-18}$
GAAAATGAGA	133	28	315259	Mus Musculus transcribed sequences	$2.53x10^{-17}$
CAAACTCTCA	16	116	35439	Secreted acidic cysteine rich glycoprotein	$4.97x10^{-17}$
GATACTTGGA	3	73	297	Actin, beta, cytoplasmic	$9.96x10^{-15}$
TGTCATCTAG	1	67	4071	Laminin receptor 1 (ribosomal protein SA)	$1.19x10^{-14}$
GCGAAGCTCA	5	74	265	Ribosomal protein S25	$7.90x10^{-14}$
AAAACAGTGG	91	16	21529	Ribosomal protein L37a	$1.67x10^{-13}$
ACAGTTAATT	0	55		Unmatched	$9.89x10^{-13}$
TACTACATAG	0	54		Unmatched	$1.61x10^{-12}$
GCTCTGGGAG	152	56	140811	Hydroxysteroid dehydrogenase-1, delta<5>-3-beta	$5.48x10^{-12}$
ACTGAAGCAA	46	146	4603	Scavenger receptor class B, member 1	$6.55x10^{-12}$
GACTCAGGGC	0	47	2580	Syndecan 1	$4.98x10^{-11}$
GAAAAGTGGA	9	69	15295	Epoxide hydrolase 2, cytoplasmic	$7.91x10^{-11}$
GGATGGGGAG	3	54	204705	Pending type I transmembrane receptor (seizure-related protein)	$9.78x10^{-11}$

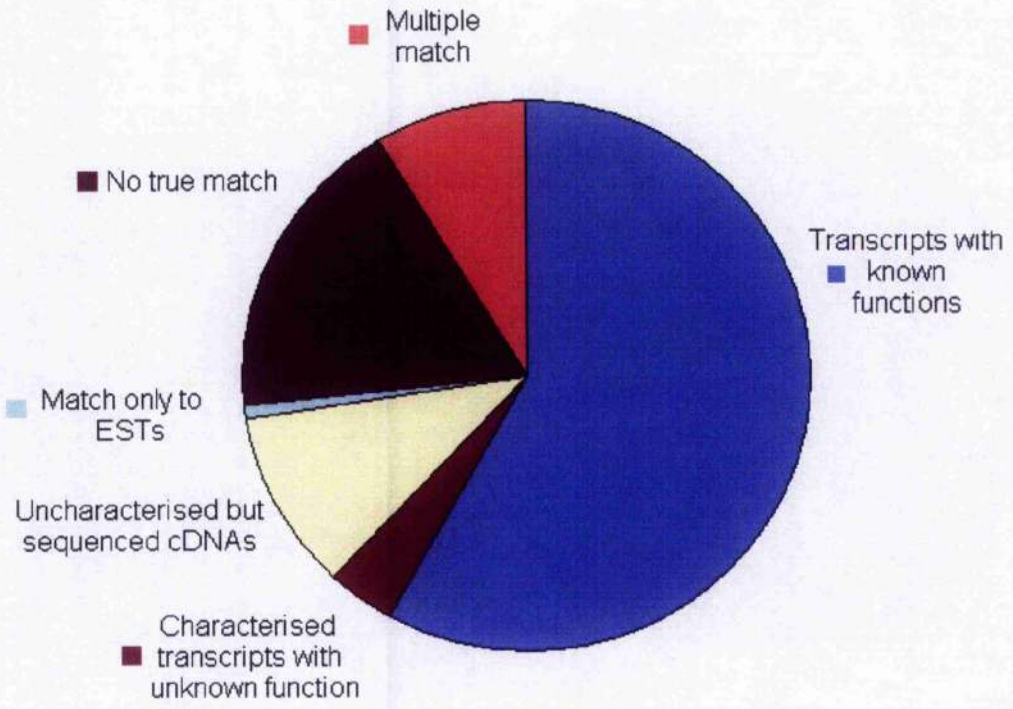
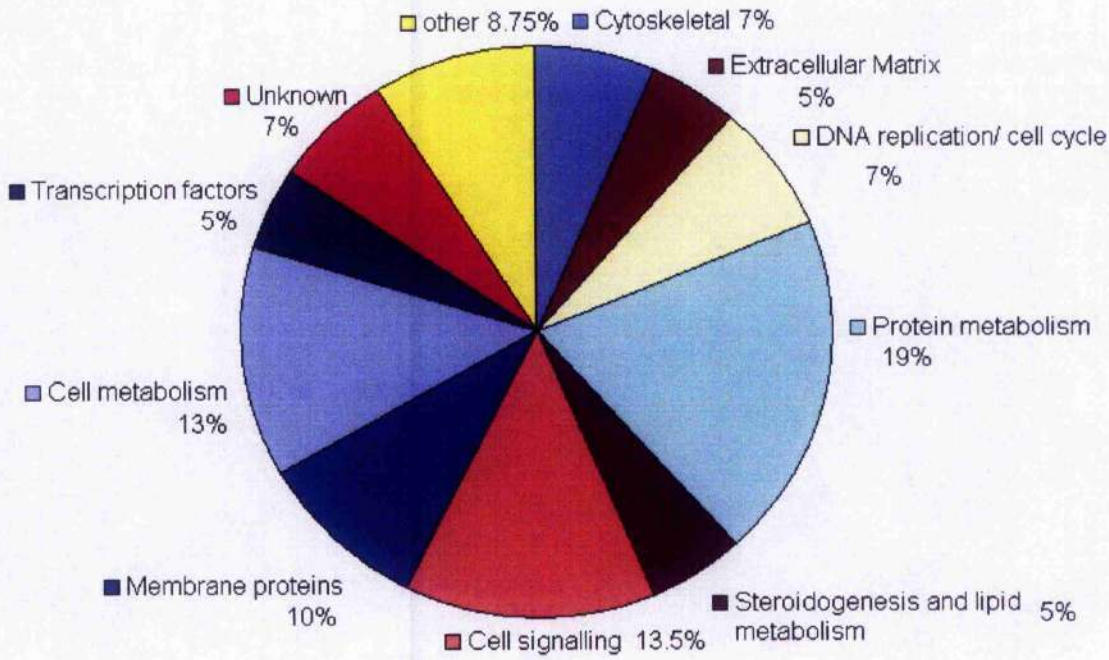


Figure 3.12b Distribution of significantly differentially expressed transcripts.

Total number of significantly differentially expressed transcripts	715
Transcripts with known functions	416
Characterised transcripts of unknown function	26
Uncharacterised but sequenced cDNAs	77
Tags matching clusters containing only ESTs	6
No true match in Unigene database	129
Multiple match in Unigene databse	61

Figure 3.13 Functional distribution of the 416 identifiable significantly differentially expressed transcripts present in the combined SAGE libraries



3.4.3 Functional Groups

Table 3.3 shows the expression of a number of tags in the libraries that represent genes linked with established functional groups. These tables include all identifiable significantly differentially expressed genes and the top 100 most abundant tags in the combined libraries which can be assigned, or hypothesised to have, a specific function within the granulosa cell. Many of these are genes which have previously been shown to be associated with follicular growth such as extracellular matrix components (Smith et al 1999), genes involved in cytoskeletal remodelling (Gricshaber et al 2003) and the rearrangement of steroidogenic machinery reflecting the shift from oestradiol to progesterone dominance (Rodgers et al 1987). This is demonstrated by the decline in expression of aromatase, a change mirrored by the rise in P450scc, StAR, ferredoxin, scavenger receptor B1 and LDLR during the same phase. Cellular filaments such as actin, profilin, cofilin, tropomysoin and tubulin show expression within one or both libraries with significant differential expression of different actin and tropomyosin isoforms. A number of signalling and receptor molecules are expressed in one or both libraries including FSH receptor, LH receptor, IGFBP4, inhibin, follistatin, and kit ligand, again reinforcing expression patterns established by earlier observations. Gap junction membrane channel proteins (connexins) show fluctuations in expression level around the ovulatory period. We now report the expression of connexins 26, 29, 30.3 and 43 within the pre-ovulatory follicle. Cx29 has not previously been identified as having involvement in folliculogenesis while Cx 26 and Cx 30.3 have not previously been recorded as having a role in folliculogenesis in the mouse.

Many of the identified genes also have diverse functions of a 'housekeeping' nature including metabolic enzymes, genes involved in protein synthesis and degradation, metabolite/ion transporters and energy metabolism These genes only indirectly relate to granulosa cell function by maintaining cell health and providing the framework and metabolites for more 'granulosa specific' processes.

Within all these groupings are genes with multiple functions and, consequently, some have been placed in more than one category. Also present are a large number of transcripts not previously associated with granulosa differentiation, particularly with relation to genes influencing cell cycle control and apoptosis, cellular differentiation and a number of transcription factors. Clearly it is not feasible to discuss changes in every gene listed in this table, although general trends can be established in many cases. For example, there is an increase in the majority of angiogenesis related genes, and in a large proportion of the cytoskeletal transcripts. These changes will be discussed at more length later in this thesis.

Table 3.3 Functional distribution of SAGE transcripts

Tag Sequence	Tag number		Unigene	Gene
	PMSG	hCG		
Angiogenesis				
TACTTTATAA	0	39	1421	ADAMTS 1
TCCATATATT	7	21	259667	RNA binding motif, single stranded interacting protein 1
GGAATTTAGA	0	12	332793	Blood vessel epicardial substance
GTGCTGTTGT	4	26	196189	Angiopoietin like 4
CTTGCTCTGT	6	35	263396	Integrin 1 beta
GAAAGCCTCT	1	24	8245	TIMP 1
CCCTTCTTCT	44	10	196110	Hemoglobin alpha adult chain 1
TGCTGTGCAT	14	60	289662	FGF inducible 14
TCTCACCTCA	0	2	15607	Vascular endothelial growth factor B
Apoptosis				
TTAGAAGTGA	1	40	373563	Salvador homolog 1 (Drosphilia)
TGAATGAGCG	0	6	1401	Chemokine (C-X-C motif) receptor 4
CTCTCCAGAA	0	6	274810	HRAS like suppressor 3
TATCTATACA	0	7	258475	Zinc finger and BTB domain containing 24
TATGCAGATG	9	1	2662	Glutathione S-transferase, alpha 4
GTGCTATTCA	2	12	1639	Myeloid cell leukemia sequence 1
CAAAATACAT	21	8	27578	Maged1 Melanoma antigen, family D, 1
CTGTAGAGTG	0	9	196508	Mortality factor 4 like 1
TTAGAACGTG	0	11	27218	Mortality factor 4 like 2
AGGAAGATCA	14	1	4078	Antigen identified by monoclonal antibody Ki 67
GGTTATAATA	5	34	28405	Serum/glucocorticoid regulated kinase
TCTCGTAATG	1	29	42095	Secreted frizzled related sequence protein 4
TGCTGTGCAT	14	60	289662	FGF inducible 14
Cell Cycle				
TACTGCTGAT	9	11	250419	Cyclin I
TCCCCCCCCT	0	33	51116	Mastermind like 1 (Drosophila)
TTTAATACAA	4	10	13725	Cyclin L2
TCGCTGCTGC	3	20	27921	Cyclin G associated kinase
AATGACACAA	2	10	219645	Cyclin dependant kinase 8
TAGTTGCAAA	5	9	2823	B cell translocation gene 3
TTAGAAGTGA	1	40	373563	Salvador homolog 1 (Drosophilia)
TGCTGTGCAT	14	60	289662	FGF inducible 14
GGGAGCGAAA	2	28	34871	Inhibitor of DNA binding 2
TCTTTAATCC	23	2	196638	CDC 23 (Cell division cycle 23, yeast homolog)
TGCACCACCT	5	30	182470	Ribonuclease H2 large subunit.
AGGAATCCAC	0	14	22701	Growth arrest specific 1
ATGAGAACAG	0	13	236123	Splicing factor 3b, subunit 3
GAAACTGAAC	12	0	42196	Ubiquitin-like, containing PHD and RING finger domains, 1
CTTAAATCTT	0	12	239605	B-cell translocation gene 2, anti-proliferative
AAGCAGAAGG	4	16	1	S100 calcium binding protein A10 (calpactin)
TGCAGGAGCT	0	12	333388	Chromodomain helicase DNA binding protein 4
AAAATGTACT	11	0	3752	RAN binding protein 1
GGAGTAAGAA	18	4	371563	H3 histone, family 3B
TCCATATATT	7	21	259667	RNA binding motif, single stranded interacting protein 1
CCCTCTGGAT	0	8	100144	S100 calcium binding protein A6 (calcyclin)
AATGCTTGAT	7	24	270186	Retinoblastoma binding protein 7
AACTTTTAAA	7	0	354643	Heterochromatin protein 1, binding protein 3
AATGTTTCTG	10	2	7141	Proliferating cell nuclear antigen
AAGAGAAAAG	6	0	240066	Proteasome (prosome, macropain) activator subunit 4
GAGTCTCTTC	0	6	184021	Protein tyrosine phosphatase, receptor type, D
TACTATAGTC	0	6	123211	Polymerase (DNA directed), beta
TGGAGCGTTG	0	6	6839	Cyclin-dependent kinase 4
TATTGTGGCT	0	9	195663	Cyclin-dependent kinase inhibitor 1A (P21)

Tag Sequence	Tag number		Unigene	Gene
	PMSG	hCG		
TTCATTATAA	17	6	19187	Prothymosin alpha
CAGACCTCAA	2	11	250605	Sel1 (suppressor of lin-12) 1 homolog (C. elegans)
Cell Differentiation				
GGATGGGGAG	3	54	283926	Seizure related 6 homolog (mouse)-like 2
TATCCCACGC	8	23	280038	S100 calcium binding protein A11
TCTTCATCAA	3	12	229151	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1
AGCCAAACAA	0	7	172897	Mitogen activated protein kinase kinase kinase 12
CCCTGATTTT	13	3	185453	Eukaryotic translation initiation factor 4, gamma 2
TCCCGATATC	2	13	263414	Poliovirus receptor-related 4
TAAGTGGAAT	12	26	3360	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide
TGTTGGTTGA	1	12	246990	Reticulon 3
CCTCTAGCTG	1	10	29346	Guanosine monophosphate reductase 2
GTCTGCTTGT	10	0	4375	Fatso
TAAATTCAGG	0	11	275909	Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4B
TCCCCCCCCT	0	33	51116	Mastermind like 1 (Drosophila)
Cytoskeletal Related Transcripts				
GATACTTGGA	73	3	297	Actin, beta, cytoplasmic
TTGTTGCTAC	8	387	395	Vimentin
TGGCTCGGTC	4	53	196173	Actin, gamma, cytoplasmic
AAGATCAAGA	6	44	214950	Actin, alpha 1, skeletal muscle
GGCTGGGGGC	18	18	2647	Profilin
GGATGGGGAG	3	54	4024	Cofilin 1 non muscle
CCCTCACCCA	4	22	246377	Tubulin beta 2
GCAGGCACTC	21	24	1703	Tubulin beta 5
ATGTCTCAAA	48	30	231463	Tubulin alpha 2
AAGGAAGAGA	2	5	7	Vimentin
TTCAGGTGGT	6	9	240839	Tropomyosin 3, gamma
CCCGTAGCCC	1	9	121878	Tropomyosin 1, alpha
GACTGTGCCA	8	6	256858	Dynein, cytoplasmic, light chain 1
TCCCCAATCA	2	9	21109	Gelsolin
TCTCGGGGGC	7	1	219663	Fibulin 1
ATCAGTGTGA	0	6	275555	Calponin 3 acidic
ATTTGACTGG	1	30	29677	Myosin heavy chain IX
AGGATCAATG	6	28	205601	Cortactin
TTTCATTGCC	5	25	308452	Transforming, acidic coiled-coil containing protein 1
CCCTCACCCA	4	24	371591	Tubulin, alpha 1
ACTCCTTAGT	0	11	336400	Syntrophin, gamma 1
ATAGTAAGCT	11	1	289707	Fascin homolog 1, actin bundling protein
GTGCTGCCTT	0	9	295565	Echinoderm microtubule associated protein like 4
GTGTCTGATA	0	9	738	Procollagen, type IV, alpha 1
TAACCGAGAC	0	9	277812	Villin 2
TGTTCATCTT	2	13	249555	Procollagen, type III, alpha 1
TCATTTGGTG	1	10	172	Lysyl oxidase
AGATGTACTG	0	7	21767	Cadherin 5
AACTGCTTCA	3	13	30010	Actin related protein 2/3 complex, subunit 1B
CTCTGGGGTT	3	13	271711	Transgelin 2
ACCACTGATA	1	9	50424	Tax1 (human T-cell leukemia virus type I) binding protein 1
ATCAAAGTTC	1	9	10299	Procollagen, type V, alpha 2
ATTTCCCGAG	1	9	288974	Actin related protein 2/3 complex, subunit 5
GGGAACAACT	2	11	8687	CAP, adenylate cyclase-associated protein 1 (yeast)
CAGAACTTTG	0	6	4352	Procollagen, type XVIII, alpha 1
GTAATCACGT	0	6	2654	WD repeat domain 1
GGAAATGACT	0	9	46497	Ras homolog gene family, member E
GCTCCCCCAC	7	6	2509	Procollagen type IV

Tag Sequence	Tag number		Unigene	Gene
	PMSG	hCG		
TGTGCCAAGT	36	30	190641	Collagen type XXV alpha 1
GCTCTAGCCA	6	10	181021	Procollagen type IV alpha 2
TGTTCATCTT	2	13	234850	Procollagen type III alpha 2
Extracellular Matrix Related Transcripts				
CCTTTAATCC	397	61	10305	Hyaluronidase 1
GTGGCGCACG	54	10	214645	Hyaluronidase 3
TTGCTACTTT	6	367	27961	Leprecan 1
CCAACGCTTT	2	10	193099	Fibronectin 1
AAGATCAAGA	6	44	44176	Epidermal GF, containing fibulin, like ECM protein 1
GGTTAAATGT	56	206	930	Cathepsin L
CCTCAGCCTG	24	20	231395	Cathepsin D
GTTTGCTGTG	12	9	22753	Cathepsin B
GGAGGGGGGA	10	7	156919	Cathepsin Z
GACCACCTCT	2	7	7386	Microfibrillar associated protein 2
GAAAGCCTCT	1	24	8245	Tissue inhibitor of metalloproteinase 1
GTGGCTCACG	11	9	217116	Matrix metalloproteinase 15
TGTGGTACGC	4	8	29373	Matrix metalloproteinase 23
CTTGCTCTGT	6	35	4712	Integrin beta 1
GGAGGGATCA	14	20	8131	Integrin linked kinase
CAAACACCGT	2	278	288474	Secreted phosphoprotein 1
CAAACTCTCA	16	116	291442	Secreted acidic cysteine rich glycoprotein (SPARC)
GACTCAGGGC	0	47	2580	Syndecan 1
TTGGCTGGAT	0	7	3815	Syndecan 4
TACTTTATAA	0	39	1421	ADAMTS 1
TGCCAATAAT	0	7	23156	ADAMTS 4
TATGAATGCT	7	48	158700	Chondroitin sulphate proteglycan 2
TACAAAATTA	8	1	355306	ADAM 17
TGGAACAATG	1	18	338790	Proteoglycan secretory granule
GTTTGTACAA	4	24	182396	Latent TGF beta binding protein
CGTGGTGGCC	8	0	45071	Cartilage oligomeric matrix protein
CTGAGGAAGT	3	14	29027	SPARC-like 1
AAATGCACTA	0	7	57734	LIM and senescent cell antigen-like domains 1
TTTACTGTGT	8	1	28897	Pyrophosphatase
Membrane Proteins				
GAAGAGAGCA	12	9	243	Laminin alpha 1
TAATGTAGAC	415	116	4504	Gap junction membrane channel protein alpha 1
TCCCTATCCT	3	4	102422	Gap junction membrane channel protein epsilon 1
AACTGGGGAT	2	3	56906	Gap junction membrane channel protein beta 4
GTGTGTAACC	2	3	34118	Gap junction membrane channel protein beta 2
TCTCCAGGCG	55	31	200608	Clusterin
AGACACTTCC	48	6	584	Annexin A2
TTGTTACTGC	1	11	20794	Annexin A7
AAGGGTGCTG	1	10	1620	Annexin A5
CAAGAATTAA	0	6	294083	Annexin A11
AAGGGTGCTG	1	10	265347	Annexin A6
GTGTTTTGTC	44	25	201455	Secretory carrier membrane protein 1
GGTGGGACAC	6	16	276326	TMP 21 transmembrane trafficking protein
CCTTTTCCTT	5	7	182912	Growth hormone inducible transmembrane protein
TGTGTCCCGC	0	6	287810	Importin 13
AGGCAATAAA	3	143	27154	Vanin 1
GATATGGTCT	1	23	2863	Integral membrane protein 1
TTAAATGCAG	1	27	273188	Coagulation factor III
CTTGCTCTGT	6	35	263396	Integrin beta 1
GGGTTTGGAG	8	27	274463	Endothelin converting enzyme 1
AATCCAGCCC	11	0	20206	Aquaporin 2
CTTTAGAAAA	0	9	268798	Solute carrier organic anion transporter family, member 3a1
TTCATCTGTC	1	16	272675	Solute carrier family 20, member 1

Tag Sequence	Tag number		Unigene	Gene
	PMSG	hCG		
TTTCAAGGCA	2	19	46067	Solute carrier family 25, member 30
AAAAGAAAAT	1	20	276831	Solute carrier family 7, member 8
TGATGTGTGA	0	14	260988	Solute carrier family 7, member 11
AGAAGGACCT	0	13	21002	Solute carrier family 2 member 1
CTCCTGCAGA	3	12	275489	Solute carrier family 7, member 1
TTGCTAAGAA	0	7	272675	Solute carrier family 20, member 1
CACTGTCTTC	0	9	4114	Solute carrier family 3, member 2
ATTAATCAGT	11	32	46754	Solute carrier family 38, member 2
TCTAGCCAGA	1	10	35253	Solute carrier family 12, member 8
TGACATCCAT	0	8	233889	Solute carrier family 39, member 10
TGAACTGTAA	1	9	270647	Solute carrier family 39, member 14
GACTGAATCT	23	33	298	Solute carrier family 25 member 3
GTGGGCGTGT	24	27	5353	Solute carrier family 29 member 3
ACAATAATGA	2	38	658	Solute carrier family 25 member 5
GTGTCCGTAC	1	12	294882	F11 receptor
TCACATAAAT	1	10	31752	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)
TGTACAAATG	1	10	15622	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1
ATGCACAGAT	1	9	2551	SEC22 vesicle trafficking protein-like 1 (S. cerevisiae)
AATCACTGTG	0	44	33240	Epithelial V like antigen
TCCCGGATCA	35	2	18962	Catenin alpha 1
CAGAACAATG	1	15	282084	Adhesion regulating molecule 1

Cell Metabolism Related Transcripts

GCCTCCAAGG	23	24	5289	Glyceraldehyde 3-phosphate dehydrogenase
CCAAATAAAA	38	31	29324	Lactate dehydrogenase 1, A chain
TGATATGAGC	18	15	9745	Lactate dehydrogenase 2, B chain
GCCCCGGAAT	61	33	196605	Hexokinase 1
GCAATCTGAT	25	23	188	Phosphoglycerate kinase 1
CCTACCAATA	16	9	29182	Transaldolase 1
CCTACTAACC	20	25	16763	Aldolase 1 A isoform
CAATAGAGAC	1	5	216135	Pyruvate kinase, muscle
TAAGGGAAAT	24	12	4222	Triosephosphate isomerase
CTTTGTTAGT	5	12	3879	Hypoxia inducible factor 1, alpha subunit
ATACTAACGT	99	6	34102	Ornithine decarboxylase
AGGGTGCAGT	2	4	28146	Mevalonate (diphospho) decarboxylase
TCCTGTGGGA	21	7	4533	Apolipoprotein A-IV
CTGGAGACGC	0	11	26743	Apolipoprotein A-I
TGCTGCATCA	2	4	168157	Apolipoprotein E
AGCCAAGAGA	5	1	38901	Fatty acid desaturase 2
CAGGCCACAC	46	30	103838	ATP Synthase Mit. F1 complex, beta subunit
GAAAAGTGGA	9	69	15295	Epoxide hydrolase 2 cytoplasmic
CCCTTCTTCT	44	10	196110	Hemoglobin alpha, adult chain 1
GGTTATAATA	5	34	28405	Serum/glucocorticoid related kinase
CAACCATCAT	3	26	30071	Lysomsomal associated protein transmembrane 4A
TACAATATAC	8	35	31403	TNF alpha induced protein 9
GTTGTTAACA	2	22	143768	F-box only protein 3
TGTATCCAGT	32	8	373561	Nucleosome assembly protein 1-like 5
AACCTCGCTG	4	26	30221	Insulin induced gene 1
GTCTATGTTG	7	0	4952	Insulin receptor substrate 1
CTATCCTCTC	7	29	200916	Glutathione peroxidase 3
TTCTCTCCCT	14	1	306954	Carbonic anhydrase 14
TGATGTTAAC	1	12	255848	Hexokinase 2
TTCTGTGTCA	0	10	19669	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
CAAAAATAAA	32	18	70666	Enolase 1, alpha non-neuron
TGTACCCAGG	7	23	3196	Alpha glucosidase 2 alpha neutral subunit
TGTGAAGTAG	16	37	371546	ADP-ribosylation factor 1
GGGGGGAAGA	0	8	27308	ADP-ribosylation factor 6
GTAGCGCTCA	0	8	5121	Peptidylglycine alpha-amidating monooxygenase
TGTGCTGTTG	0	7	32700	Sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog (S.

Tag Sequence	Tag number		Unigene	Gene
	PMSG	hCG		
				cerevisae)
TGTGAACGAA	4	15	18522	Carnitine palmitoyltransferase 1a, liver
GCAGCTCACA	12	3	7236	Dolichyl-di-phosphooligosaccharide-protein glycotransferase
TGAGTTCCCT	2	11	38390	Copine III
TCTGGCAGTC	6	0	221452	RAN binding protein 5
CGTCTGTGGA	7	19	30155	ATPase, H+ transporting, V0 subunit C
GTTTAGTGGA	16	6	252255	Proteasome (prosome, macropain) subunit, alpha type 2
CAGTCTTGAG	0	6	22560	Diaphorase 1 (NADH)
AGCAAGATGG	11	40	290578	Aminolevulinic acid synthase 1
ATGAGTGAGC	2	11	27183	Phenylalkylamine Ca2+ antagonist (emopamil) binding protein
CAGATTGTGA	2	11	317701	Thioredoxin domain containing 4 (endoplasmic reticulum)
AACAAATTCT	8	1	35628	Fucosyltransferase 8
CAGGGCTCCG	8	1	193096	Stearoyl-Coenzyme A desaturase 2
TCATTCTCCA	1	13	257837	ATPase, class VI, type 11A
AGCAAGAATT	28	226	1061	Ferredoxin 1
TCCCCCCCTT	0	8	4946	Insulin II
CAGGCAAAAC	25	3	171378	Uncoupling protein 2 mitochondrial
TTGTCAGGTA	20	68	148155	Malic enzyme supernatant
GGGAAGTCTG	5	33	347009	Peroxiredoxin 2
AATTAGTTGT	34	8	353	ATP synthetase H+ transporting, mitochondrial F0 complex, subunit F
ATAATACATA	154	231	200362	Cytochrome b-245 beta polypeptide
GTGATGTTTC	55	26	31018	Cytochrome b-5
CAAGGTGACA	3	20	328846	Phosphodiesterase 6A, cGMP-specific, rod, alpha
ACCCTGCTTA	10	0	206417	Cystathionine beta-synthase
GGCAATAATG	25	53	9925	Isocitrate dehydrogenase 1 (NADP+), soluble
TCATTCTCCA	1	13	257837	ATPase, class VI, type 11A
AATGGCTAGC	18	5	35389	Cytochrome c, somatic
GAAATATATG	27	11	2966	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 3
GGAGCCATTG	10	1	275780	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5
AACTGCACAC	0	6	246965	Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)
CCAATGAACT	7	0	235123	Inner membrane protein, mitochondrial
Oocyte specific genes				
TCAGAGTGCT	11	3	9714	GDF 9
AGCTACCTGG	1	0	18213	Transforming growth factor, beta 2
GAATACCCCC	2	4	42160	Bone morphogenetic protein 15
AAAATGTCAA	9	1	208554	Oogenesin 1
Protein synthesis and degradation				
Ribosomal proteins L32, S17, large P2, S15a, L3, S3a, L21, S4 (X-linked), L11, L17, L15, S3, L41, S24, L36a, L10, L38, L24, S29, P1, S23, L5, L13, S11, L36, L40 and mitochondrial ribosomal proteins S21, S27, L53, L5				
AGGCGTGGCT	0	6	30849	Prenylcysteine oxidase 1
TACATTCCAA	1	45	3401	Proprotein convertase subtilisin/kexin type 5
ATCACACACT	0	21	62886	UDP-N-acetyl-alpha-D-galactosamine polypeptide N-acetylgalactosaminyltransferase 7
TGCAATATGG	0	9	260084	Eukaryotic translation initiation factor 4A2
TGAACACTGA	0	6	330731	Transglutaminase 2, C polypeptide
TAGACAAAGG	0	9	276815	Adenosine deaminase, RNA-specific, B1
GAAATGTTGT	11	1	22117	Polymerase (RNA) II (DNA directed) polypeptide G
TGTATAAAAA	30	12	87773	Tumor rejection antigen gp96
ATTGCTTAGA	19	4	371574	RNA binding motif protein 3
GCTATACAGA	11	0	286830	Leucine aminopeptidase 3
GAGCGTTTTG	57	28	5246	Peptidylprolyl isomerase A
AGCCAAATAC	2	19	261831	Basic leucine zipper and W2 domains 1
GGCAGCACAA	8	24	9043	Heterogeneous nuclear ribonucleoprotein F
TGTGGATGGC	0	8	30602	Ubiquitin specific protease 22
AATAAACACG	6	20	257629	Protease, serine, 35

Tag Sequence	Tag number		Unigene	Gene
	PMSG	hCG		
CAAATGCTGT	10	1	20943	FK506 binding protein 9
TTGATGTACA	3	15	223946	Splicing factor, arginine/serine-rich 11
GGCAGCACAA	8	24	9043	Heterogeneous nuclear ribonucleoprotein L
GCATCCAGCT	7	0	277091	Poly(A)-binding protein, cytoplasmic pseudogene
AATTGTATTT	7	0	10651	GTP cyclohydrolase 1
CCTGATCTTT	1	10	4071	Laminin receptor 1 (ribosomal protein SA)
CTGAATATCT	0	6	371545	Acidic ribosomal phosphoprotein P0
AGGTGTACAG	0	6	29397	Splicing factor, arginine/serine-rich 15
GCTCACAACC	7	0	272930	Component of oligomeric golgi complex 4
CATTGCGTGG	9	22	27955	Williams-Beuren syndrome chromosome region 1 homolog (human)
ATTATACAGT	3	13	29192	Asparaginyl-tRNA synthetase
ATGCTTCTCA	0	6	248313	RAB12, member RAS oncogene family
ATTAGGATGT	0	6	22347	Pinin
TGAGGCCTCG	0	13	21671	Eukaryotic translation initiation factor 3, subunit 9 (eta)
GATGTGGCTG	41	15	2718	Eukaryotic translation elongation factor 1 beta 2
TGGGCAAAGC	23	8	371625	Eukaryotic translation elongation factor 1 gamma
ATCCGGCGCC	0	8	153758	Transcription elongation factor B (SIII), polypeptide 2
AGGCAGACAG	94	131	335315	Eukaryotic translation elongation factor 1 alpha 1
AAGAAAACAT	6	0	218851	Eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked
CCTTTAATTC	14	3	154306	Ubiquitin specific protease 45
TATAGTATGT	8	30	210745	Glutamine synthetase
CGAAAGAACA	0	7	23335	YME1-like 1 (S. cerevisiae)
AGACAAGCTG	0	7	43331	Splicing factor, arginine/serine-rich 5 (SRp40, HRS)
TAACAGTTGT	18	38	248827	Calnexin
TGTGGATGGC	0	8	30602	Ubiquitin specific protease 22

Cytokines, growth factors and signalling and receptor molecules

TACAGTATAA	89	9	3092	Inhibin beta B
AGGTCCCTAC	39	27	1100	Inhibin alpha
TCTTAATGAA	22	11	4235	Kit ligand
TGCTGTGCAT	14	60	18459	FGF inducible 14
GTTTGTACAA	4	24	182396	TGF beta BP3
ACAGTCGACT	3	2	209571	Bone morphogenic protein 3
CAGCATTAGA	2	0	27757	Bone morphogenic protein 1
TGAGCATCAA	2	0	140965	Bone morphogenic protein receptor 1a
ATCACAGGTG	1	4	22248	IGFBP 4
TTTGCACCTT	7	8	1810	Connective tissue growth factor
TAGCTTTAAA	5	3	233470	IGFBP 7
AAAGCACCAT	7	1	3904	Fibroblast growth factor 15
TAAATGTGCA	45	8	4913	Follistatin
CTTGTATTTA	10	10	4132	Suppressor of cytokine signalling 2
GTTTGTACAA	4	24	182396	Latent transforming growth factor binding protein 3
TACTTGTGTT	3	18	15125	Stromal cell derived factor receptor 1
ACTCTAAGTT	0	7	347919	B-cell stimulating factor 3
TACCTTGACA	0	45	4791	Epiregulin
TCCCCCCCCC	0	28	35088	Cholinergic receptor, nicotinic, beta polypeptide 2 (neuronal)
CTTAGATGTT	0	19	309193	Rapporin 1-like
AACAAAATCT	13	1	3903	RAS, dexamethasone-induced 1
AAGTAATGTG	1	12	10516	Prolactin receptor
TTACCACATA	5	20	28262	Regulator of G-protein signaling 2
ACAGCCAGGG	11	1	242413	G protein-coupled receptor, family C, group 5, member C
TCCTTATATT	42	73	290285	RAB39, member RAS oncogene family
ATGTTCGTGG	3	16	1791	Dual specificity phosphatase 6
GTCTTGGGCG	15	36	30156	Protease, serine, 11 (Igf binding)
TCAGTTTAAT	0	9	275266	Rho guanine nucleotide exchange factor (GEF) 12
TTCCTATATT	32	59	179011	Vav2 oncogene
ATCACTCCAA	1	11	273142	Membrane interacting protein of RGS16
GGAGATCTTT	10	1	167625	G protein-coupled receptor 85

Tag Sequence	Tag number		Unigene	Gene
	PMSG	hCG		
CGCTCTAACG	6	0	25594	Protein kinase, cAMP dependent regulatory, type II beta
AGATCATCTA	0	8	246003	Frizzled homolog 1 (Drosophila)
GCACAACTTG	27	12	329243	Calmodulin 2
ACAACAGAGG	1	10	24807	Prostaglandin F2 receptor negative regulator
GCCACTTCCT	1	10	371598	Nuclear receptor coactivator 4
AGAAAGGATA	0	7	296814	Phosphodiesterase 7A
AGTGTGACGT	0	7	43081	Mitogen-activated protein kinase 8 interacting protein 3
TGTAACTGGT	4	15	233009	RAS related protein 1b
ACTCCTCCCT	1	9	333868	RAS-related protein-1a
TACAAAATTA	8	1	355306	ADAM 17
GGCTTTTTCG	9	1	27832	V-ral simian leukemia viral oncogene homolog B (ras related)
TGATGCTAAA	1	9	304976	Down-regulated by Ctnnb1, a
GTCTTTGTGA	6	0	200775	Transforming growth factor, beta receptor III
TACAATAAAC	0	6	40321	Progesterone receptor membrane component 2
TTTGTAATAA	0	30	284855	Endothelin 2
CTCCCACCCA	0	18	249318	Frequenin homolog (Drosphilia)
ACTCGGAGCC	34	9	285993	Calmodulin 1
AGGTGGCATT	0	6	2442	Calcium binding protein intestinal
ATGACATAGA	5	28	235182	Calcium/calmodulin-dependant protein kinase II gamma
CAAACAATGT	1	19	277351	G protein coupled receptor 48
AGAGGACTAG	0	15	358930	G protein coupled receptor associated sorting protein 2
TTCTTGTATT	3	22	276405	FK506 binding protein 5I
ATGATGGTAG	54	24	353171	EpH receptor A6
CCCCTATATT	14	37	288639	RAB guanine nucleotide exchange factor (GEF) 1
ACAGTTAAGC	0	10	209813	Ephrin-B class 2
TGTTAGCTCC	0	6	21667	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 12
TAAGTGGAAT	12	26	3360	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide
GCACAATTG	27	12	35677	Epidermal GF receptor pathway substrate 15
CACCACCACA	11	16	2924	Platelet derived growth factor receptor
AGGGCACTGG	13	6	43760	Nerve GF assoc. receptor protein
TGTAAGGTGT	1	4	254496	FGF receptor
TTGCCATCTC	8	16	1644	LH Receptor
GGGTAGATAT	6	13	142929	Anh type 2 receptor
GCTGTTTTCA	15	11	35009	G protein coupled receptor 27
TGACTCATCT	4	3	57155	FSH receptor
GGATGGGGAG	3	54	204705	Psk 1 transmembrane receptor
TGTCATCTAG	1	67	4071	Laminin receptor 1
GGCCCTCTTT	1	3	197552	TGF beta receptor 1
CTTGCTCTGT	1	35	4712	Integrin beta 1 (fibronectin receptor beta)
TCCCCCCCCC	28	0	35088	Cholinergic receptor, nicotinic, beta polypeptide 2 (neuronal)
TTCTTGGTTT	4	13	40636	G protein coupled receptor 48
GCACTAGCTG	6	9	9052	Progesterone receptor membrane component 1
TTTAGGGGAG	12	5	22440	Thyroid hormone receptor associated protein 3
TTTGACCCCC	2	0	4839	Activin A receptor type II-like 1
CAACACCACC	1	3	689	Activin A receptor type 1
ATAGCTGGGC	8	6	248907	Mitogen activating protein kinase kinase 1
GAAATGTAAG	19	15	4358	Mitogen activating protein kinase kinase kinase 12
CAAAAAAAAA	6	7	44193	Mitogen activating protein kinase kinase kinase 7 interacting protein 1
CATTTGAAAA	5	3	196584	Mitogen activating protein kinase kinase 2
CCTGCTCTGT	3	4	4437	Mitogen activating protein kinase 14
CCAGACATCT	5	1	205152	Mitogen activating protein kinase associated protein 1
AACTGTGTGA	2	3	18856	Mitogen activating protein kinase 6
GTTTGGAGCT	2	3	18494	Mitogen activating protein kinase kinase 3
TCCAATTCCT	3	0	68993	Mitogen activating protein kinase 9
TTTTTGATAA	3	32	10504	3-phosphoinositide dependant protein kinase 1
ATGACATAGA	5	28	235182	Calcium/calmodulin-dependant protein kinase II gamma
TTTTCTATTT	23	3	25594	Protein kinase, cAMP dependant regulatory, type II beta
TAGGAGACTG	6	15	2314	Protein kinase C delta
ACCGGGCTGG	9	7	9334	Protein kinase, cAMP dependant regulatory, type I beta

Tag Sequence	Tag number		Unigene	Gene
	PMSG	hCG		
TTCCATTAAA	3	2	30234	IK cytokine
TGCAGTCAGT	0	4	37204	Suppressor of cytokine signalling 5
GAGTCAGCAA	0	4	227274	Protien regulator of cytokinesis 1
GTTGTTGTTA	7	4	78106	Serum/glucocorticoid regulated kinase 3
TTAATTACAG	0	19	28405	Serum/glucocorticoid regulated kinase

Steroidogenesis and Lipid metabolism related transcripts

GGTCAAGATA	46	4	188939	17β HSD 1
CTAAAAAAAA	9	7	12882	17β HSD 7
CACCACCACC	5	1	8877	17β HSD 2
GTGCATTTCA	13	3	5199	Cytochrome P450 19 (aromatase)
GGGCATTTGA	11	108	108678	Cytochrome P450 11a (cholesterol side chain cleavage)
GAAGCTGTAT	10	0	5079	11β HSD 2
TGTGCCGGCC	1	18	142364	StAR
CAAACTGTAT	6	0	196405	3β HSD 2
GCTCTGGGAG	152	56	140811	3β HSD 1
ACTGAAGCAA	46	146	4603	Scavenger receptor class B member 1
GGTAACCTAA	1	9	3213	Low density lipoprotein receptor
TGTCCACACA	0	8	196675	LDLR related protein 8
GAAAATGAGA	133	28	30012	HDL binding protein
AGCAAGAATT	28	226	1061	Ferredoxin
GAGTGGATTC	7	20	277857	Hydroxysteroid (17-beta) dehydrogenase 4
CACATTATCA	0	25	28099	Sterol-O-acyltransferase (cholesterol metabolism)
CTGGAGACGC	0	11	26743	Apolipoprotein A-I
GAACTTGCAA	10	24	39472	Farnesyl diphosphate synthetase
TCCTGTGGGA	21	7	4533	Apolipoprotein A-IV precursor
TGGCTCCATC	0	6	316652	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
AACTGAGGGG	49	306	277498	Prosaposin
TGCAGTGTTA	1	11	249342	7-dehydrocholesterol reductase
TGTTATGTAA	1	10	277376	ATP-binding cassette, sub-family A (ABC1), member 1
GTGTACTTTC	0	10	20615	Peroxisomal biogenesis factor 11a
TTGCTGCCCT	0	8	247764	Peroxisome biogenesis factor 19

Transcription factors and related genes

GGGAGCGAAA	2	28	34871	Inhibitor of DNA binding 2
GGTGTTCCTT	3	0	31123	Desert hedgehog
GGAGAATTTT	7	1	2543	Indian hedgehog
TAGCCAACTT	0	13	4509	Runt related transcription factor 2
TAGCTTTAGG	0	13	22480	Cyclin D binding myb-like transcription factor 1
TCTCGTAATG	1	29	42095	Secreted frizzled related sequence protein 4.
AAAGACACTA	0	6	360445	Interferon regulatory factor 2 binding protein 2
TGCACTGCTG	7	19	44151	Cofactor required for Sp1 transcriptional activation, subunit 6
TGAAATGGCC	6	0	28020	Cofactor required for Sp1 transcriptional activation, subunit 3
GGTTTTCAAG	7	0	259278	SMT3 suppressor of mif two 3 homolog 1 (yeast)
TCCTTATTGC	2	12	14547	Chromobox homolog 2 (Drosophila Pc class)
GTTGTTTGTT	1	10	20827	Homeodomain interacting protein kinase 1
AACGTGAGGT	0	8	258773	Zinc finger, MYND domain containing 11
AAGCTCCGAC	0	8	12917	Multiple endocrine neoplasia 1
GTATGTATGG	2	13	17977	Transcription factor Dp 2
TGCTACTTTA	0	9	153415	E2F transcription factor 5
TATTGTGGCT	0	9	195663	Cyclin-dependent kinase inhibitor 1A (P21)
GAGGAGGAGG	0	10	136604	Nuclear factor, interleukin 3, regulated
GGTATCAGTC	4	19	21281	Ring finger protein 4
GAGTGATTAT	0	12	256422	Zinc finger protein 162
GGATATGTGG	19	42	181959	Early growth response 1
CTAGATGTCG	9	10	34537	CCAAT/enhancer binding protein (C/EBP), alpha
TCTGAACACA	10	2	287795	General transcription factor II E, polypeptide 1 (alpha subunit)
ACCAAGAGTC	2	12	641	Activating transcription factor 4

Tag Sequence	Tag number		Unigene	Gene
	PMSG	hCG		
Unknown function-significantly differentially expressed named genes				
TGGGTTGTCT	156	253	296922	Tumour protein translationally controlled 1
ACCGGGTCAT	1	30	206919	Male sterility domain containing 2
TTGAAATTAC	0	45	362063	Proline rich protein MP5
AGAAGACAGA	0	15	16769	Testes enhanced gene transcript
CTGCTCTGAC	2	17	153566	BC019776 Meteorin, glial cell differentiation regulator-like
CCTGTGTATG	0	11	293605	Tumor protein p53 inducible nuclear protein 2
GTTGAGGTTT	13	32	331964	DNA segment, Chr 8 ERATO Doi 531, expressed
GCTCAGCACC	20	3	218957	Gene regulated by oestrogen in breast cancer protein
CTTCCCCGGG	0	10	34903	FSH primary response 1
TACTGCTAAG	0	10	273915	Gene rich cluster, C3f gene
AGCAGTGCTT	9	0	274715	Coiled-coil domain containing 3
GTTCTGACAG	4	14	261025	Carnitine deficiency-associated gene expressed in ventricle 3
TACCCCACAA	0	6	41849	Brain expressed, associated with Nedd4
TTTGAGGATT	0	6	333893	Non imprinted in Prader-Willi/Angelman syndrome 2 homolog (human)
CACTGACGAG	10	2	2871	DnaJ (Hsp40) homolog, subfamily C, member 9
ATAAACTGCA	0	6	230654	Testis derived transcript
TTCAGGCACT	1	9	38436	Tetratricopeptide repeat domain 13
GTACTTGTCT	0	7	29658	Chemokine-like factor super family 3
ATCAGTACTA	0	7	46401	Son cell proliferation protein
CCTTTAATGC	7	0	219648	THO complex 1
TCAACTTGGG	2	12	156727	Hyperparathyroidism 2 homolog (human)
TGCCGTATGC	1	10	6442	Polycystic kidney disease 2
GTTGAGGTTT	13	32	331964	DNA segment, Chr 4, Wayne State University 53, expressed
TAACATTGTA	0	9	22225	Zinc finger protein 313
CTAATAAAGC	51	28	329631	Fau Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed (fox derived)
GTACTTGTCT	0	7	29658	Chemokine-like factor super family 4

3.4.4 Genes showing selective expression within granulosa cells

In order to select transcripts which have a direct luteinisaton-related function the SAGE libraries were compared to the other 81 murine libraries available on SAGEMAP. These included libraries constructed from embryonic stem cells, cardiac muscle, neural tissue, spleen, kidney, liver, adipose tissue and testis in a variety of physiological and pathological states. It is reasonable to presume that transcripts unique to granulosa cells when compared to other libraries may be considered more likely to possess a cellular specific function.

Table 3.4 lists the 20 highest expressed genes within the combined libraries which show no more than a single tag in any other non-gonadal murine SAGE library. Tags found in SAGE libraries sourced from testes tissue have been included in this list in order to include genes expressed within the Sertoli cell in the male since it has an analogous function to the granulosa cell in the female. These genes, while not necessarily unique to granulosa cells, are nonetheless showing highly selective expression patterns. Eight of these genes are unmatched, have multiple matches or are linked to uncharacterised sequences. The possibility that such narrow expression across tissue types is linked to a luteinisation-specific function is enhanced by the fact that 17 of the 20 genes listed show significant differential expression. This is borne out by the presence of the steroidogenic gene 17β HSD 1 and the luteinisating hormone receptor within this group. Also of interest are leukocyte cell derived chemotaxin 1 and mastermind like 1 (Drosophilia) as genes likely to have influence on cell proliferation, survival and/or differentiation.

Table 3.5 lists the 20 most abundant tags which are unique to the granulosa cell, excluding those tags also found in the testis libraries. Six of these genes are unmatched, have multiple matches or are linked to uncharacterised sequences and 19 of the 20 genes are significantly differentially expressed. Likely to stimulate interest are 4 transcription factors found only within the granulosa cell namely transcription factor Dp 2, cyclin D binding myb-like transcription factor 1, runt related transcription factor 2 and general transcription factor II E, polypeptide 1 (alpha subunit), all upregulated by hCG. It should be noted that

these genes are, of course, expressed in other tissues it is just that they have not been detected in SAGE libraries.

Table 3.4 Top 20 most abundant transcripts found specifically in gonadal tissue

Gonadal Tag_Sequence	PMSG	hCG	Unigene	Gene
CCATCGTCCT	13	66		Unmatched
GTGTTTTGTG	44	25	201455	Secretory carrier membrane protein 1
TACTACATAG	0	54		Unmatched
GGTCAAGATA	46	4	188939	Hydroxysteroid (17-beta) dehydrogenase 1
TACCTTGACA	0	45	4791	Epiregulin
TTGAAATTAC	0	45	4491	Proline-rich protein MP5
ACAATAATGA	2	38	261182	RIKEN cDNA 2310061B02 gene
ACAACTCCAC	0	39		Unmatched
CGAAGCACAA	2	35	264680	Cysteine-rich secretory protein LCCL domain containing 2
TTTTTGATAA	3	32	10504	3-phosphoinositide dependent protein kinase-1
TCCCCCCCCT	0	33	51116	Mastermind like 1 (Drosophila)
TTTGTAATAA	0	30	1366	Endothelin 2
AATTTCTCAA	24	3	24295	Mus musculus transcribed sequences
TATATACTTC	3	22	33062	CDNA sequence BC010552
CCAAGAGACC	20	4	46561	Leukocyte cell derived chemotaxin 1
TTGCCATCTC	8	16	1644	Luteinizing hormone/choriogonadotropin receptor
TTACTGCTAC	0	21	347368	Expressed sequence AI987712
AACAAACGCA	0	19		Unmatched
GGGTAGATAT	6	13	142929	Anti-Mullerian hormone type 2 receptor
TTAGATATTG	5	13	1664	Luteinizing hormone/choriogonadotropin receptor

Table 3.5 Top 20 most abundant transcripts found specifically in granulosa cells

Granulosa Tag_Sequence	PMSG	hCG	Unigene	Gene
TACTACATAG	0	54		Unmatched
TACCTTGACA	0	45	4791	Epiregulin
TTGAAATTAC	0	45	4491	Proline-rich protein MP5
ACAATAATGA	2	38	261182	RIKEN cDNA 2310061B02 gene
CGAAGCACAA	2	35	264680	Cysteine-rich secretory protein LCCL domain containing 2
TTTTTGATAA	3	32	10504	3-phosphoinositide dependent protein kinase-1
TCCCCCCCCT	0	33	51116	Mastermind like 1 (Drosophila)
TTTGTAATAA	0	30	1366	Endothelin 2
AATTTCTCAA	24	3	24295	Mus musculus transcribed sequences
CCAAGAGACC	20	4	46561	Leukocyte cell derived chemotaxin 1
CTGGAGACAT	0	18		Unmatched
CAAGTTTCAG	0	17	312623	Membrane-associated ring finger (C3HC4) 3
ATTGTAATAT	2	13	4509	Runt related transcription factor 2
GTATGTATGG	2	13	17977	Transcription factor Dp 2
TGAGGCCTCG	0	14	21671	Eukaryotic translation initiation factor 3, subunit 9 (eta)
AGTTCATAAG	1	12	284855	Endothelin 2
TAGCTTTAGG	0	13	22480	Cyclin D binding myb-like transcription factor 1
TATAACACTG	1	12	358736	RIKEN cDNA 3830408G10 gene
TCTGAACACA	10	2	287795	General transcription factor II E, polypeptide 1 (alpha subunit)
CGACCTTTAC	0	12	27154	Vanin 1

3.4.5 Source and Purity of Granulosa Cell cDNA

In order to confirm the purity of the source mRNA it is necessary to check for thecal contamination by searching for highly expressed thecal cell specific transcripts that we would not expect to find in granulsoa cell generated cDNA. Steroidogenic enzymes such as 17 alpha hydoxylase and signalling molecules such as BMP 7 and keratinocyte growth factor are known to be highly expressed in thecal cells at this time point and no detection has occurred in either SAGE library.

A number of oocyte specific genes are present since no effort was made to isolate oocytes from the aspirated follicle contents. GDF 9, BMP 15 and oogenesin are oocyte specific transcripts found in the SAGE libraries. GDF 9 and BMP 15, as paracrine effectors secreted to influence granulosa cell function, would be expected to be among the most highly expressed oocyte mRNAs. The level of transcript detection is sufficiently low that these transcripts would not be expected to skew the SAGE results. If doubt is raised about the source of a transcript of interest its expression can easily be localised using in situ hybridisation.

3.5 Investigation and Identification of Unmatched Transcripts

3.5.1 RACE analysis of unknown SAGE tags

One of the inherent problems with SAGE is that many SAGE tags do not match to any known gene in the database. This could be because they correspond to novel transcripts, or because there is alternative splicing at the 3' end of the gene. The length of a tag does not unfortunately yield sufficient cDNA sequence for further analysis. In most cases searching the mouse genome for a particular tag sequence will give many matches. In addition, a SAGE tag may match to more than one gene in the database, these genes having no homology, functional or otherwise, except for this short sequence. In order to address this problem Chen et al (2000) developed a technique known as the generation of longer cDNA sequences from SAGE tags for gene identification (GLGI). This is a technique based on the original SAGE method which helps to validate and better characterise the information generated by the SAGE library. Essentially this technique uses the SAGE tag as the sense primer and a modified anchored oligo d(T) as an anti sense primer, thus converting the 10bp SAGE tag into a much longer sequence (figure 3.14). These longer 3' cDNAs provide much higher specificity than the SAGE tag for identifying correct genes for SAGE tags with multiple matches and for further characterisation of novel genes generated from the SAGE tags with no match. A high throughput protocol (Chen et al 2002) has improved the efficiency and permits a larger scale analysis of SAGE tags. By using this approach it is possible to efficiently generate longer 3'cDNA sequences up to a few hundred bases long (256 bases being the theoretical maximum for a 4 base recognition site) for multiple SAGE tags.

3' RACE procedure

The RNA for the generation of longer cDNA fragments for gene identification (GLGI) procedure was obtained from multiple combined granulosa cell samples sourced from mice treated with PMSG and hCG and cells collected as described for the SAGE experiments. Equal aliquots from both PMSG and PMSG/hCG

treated mice were combined to give one sample. The RNA extraction, reverse transcription and 3'cDNA generation was as previously described for SAGE as was the annealing of SAGE linker A to the 5' end of the cDNA fragments (figure 3.14), the only difference was that the oligo dT primer had an additional sequence at the 5' end (figure 3.14). Following the 3'cDNA generation an additional PCR step is employed to increase the amount of cDNA for large scale GLGI analysis. Amplification is carried out using an oligo dT primer (figure 3.14) and a SAGE linker based primer (Primer 1; 5'-AACTAGGCAATATAGGGA-3'). This amplification was carried out in a 50µl reaction volume with 200nm of primer in each reaction, with other constituents as previously stated (Chapter 2). The product of this PCR reaction serves as the template for the gene specific amplification.

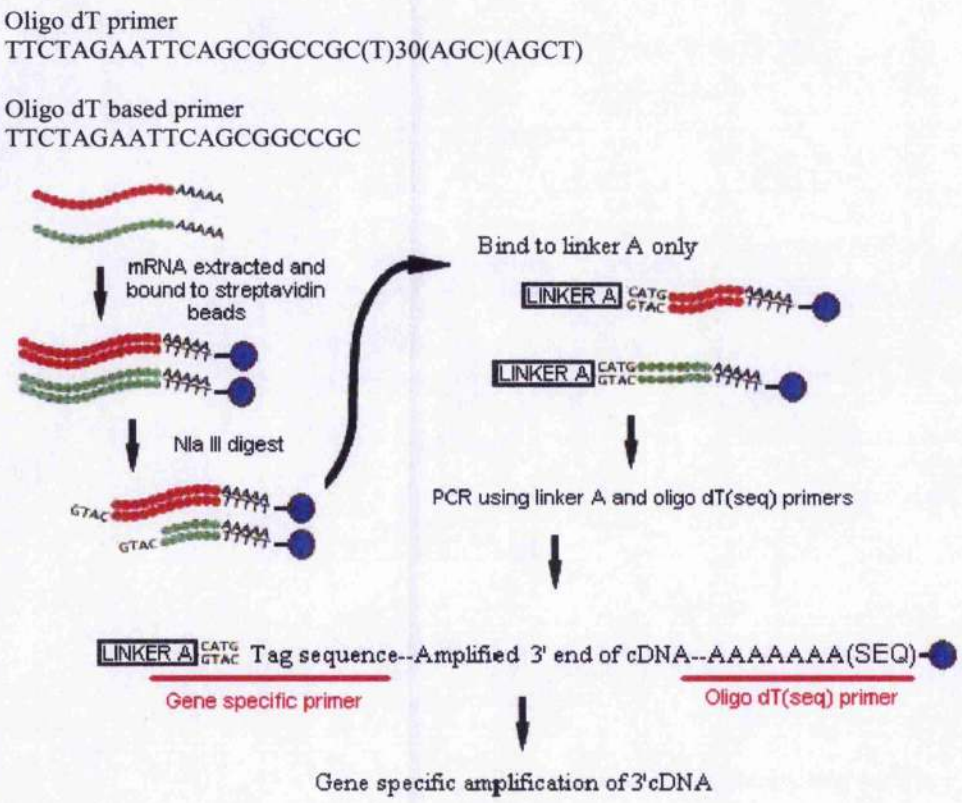


Figure 3.14 Schematic representation of the large scale GLGI procedure for sequencing 3' cDNAs from SAGE tag transcripts. Annealing a known oligonucleotide linker to the 5' end of a 3' cDNA fragment allows gene specific amplification using a linker/SAGEtag based primer.

Eleven novel SAGE tags representing unidentifiable transcripts were selected for amplification and sequencing on the basis of abundant, differential or selective expression patterns within the granulosa cell. The sense primer sequences are listed in table 3.6. The tag sequence on which the primer is based is underlined, the remainder of the primer is composed of the CATG recognition site and the last 7 bases of the SAGE linker sequence giving a total of 21 bases for each primer.

The oligo dT antisense primer was based on the oligo dT primer used for reverse transcription in the SAGE procedure.

Each PCR reaction was set up in a 50µl reaction volume containing 200nm of the gene specific primer and 200nm of the oligo dT primer. Reaction components were described for normal PCR in the basic laboratory methods section. Cycling parameters of 95°C for 90 seconds then 30 cycles at 95°C for 20 seconds, 55°C for 30 seconds and 72°C for 60 seconds were followed.

The subsequent PCR products were run on a 12% polyacrylamide gel (figure 3.15) containing ethidium bromide as described under basic laboratory procedures. The gel was visualised under u.v. light and bands excised and extracted from the gel using the electroelution and lavage method as described for SAGE.

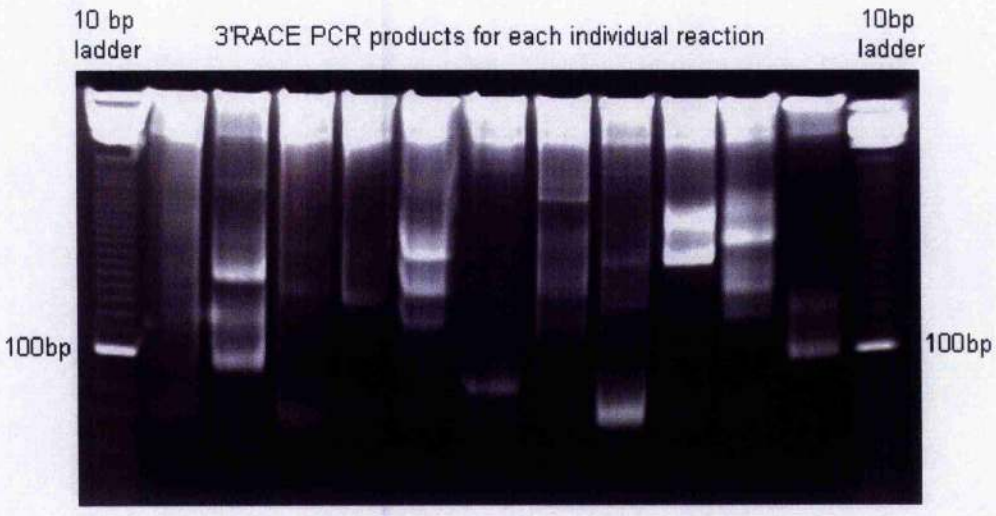


Figure 3.15 PAGE gel containing 3'RACE reaction products from each of the individual reactions using GSPs listed in table 3.6

The PCR product was cloned into TOPO vector and electroporated using E.coli DH10B cells (both products from Invitrogen, Paisley, U.K.) as previously described, cell suspensions were plated out, incubated overnight, colonies selected, plasmid inserts amplified and sequenced as previously described for SAGE using the plasmid based primers M13R (5'-GGA TAA CAA TTT CAC ACA GG-3') and M13F (5'-TGT AAA ACG ACG GCC AGT-3') (obtained from MWG Biotech, UK).

The sequences obtained are listed below (figure 3.16). These sequences were then BLAST searched to obtain matching ESTs. The best match for each transcript is listed alongside that transcript.

Table 3.6 Gene specific primer sequences for 3' race

Primer sequence (SAGE tag underlined)	Transcript number	
	PMSG	hCG
ATAGGGACATGCCTCCCCTTG	1	98
ATAGGGACATGTCCGCGATCA	58	1
ATAGGGACATGATCAGTGTGC	1	32
ATAGGGACATGACAGTTAATT	0	55
ATAGGGACATGTACTACATAG	0	54
ATAGGGACATGACAACTCCAC	0	39
ATAGGGACATGGGAGCAGACC	0	25
ATAGGGACATGAACAAACGCA	0	19
ATAGGGACATGTCGCTGCCTT	0	15
ATAGGGACATGAAAGCACACA	0	128
ATAGGGACATGCAGTCAATAC	212	30

Figure 3.16 3' RACE results for unidentified SAGE tags.

Tag-CCTCCCCTTG PMSG 1 hCG 98

CATGCCTCCCCTTGTAGTCACCAAGGAAAATTACCACTGCTCCCCCTGCCCTTCTGC
ATAAGGGTTATTTCCCCTTTGATCTTTTTGTATAAAAACTGTAAGTTTTGCTGAATAC
AACGAGACCTTGACAAGATTC-poly(A)

Matches to 442bp mRNA, Accession number AA791500, GI:2854455, Stratagene mouse Tcell 937311 Mus musculus cDNA clone.

Tag-TCCGCGATCA PMSG 58 hCG 1

CATGTCCGCGATCATCGTTAGAAGCTTGAATTCGAGCAGT-poly(A)

Matches to 66 bp mRNA, Accession number BG370289, GI:13266826, Homo sapiens cDNA clone.

Tag-ATCAGTGTGC PMSG 1 hCG 32

CATGATCAGTGTGC-poly(A)

No significant similarity

Tag-ACAGTTAATT PMSG 0 hCG 55

CATGACAGTTAATTCCTTAGATTAAGTTCAATTGGTATTGTAAATATTTTCAACTGA GTTTTTAATTGACAATAAATAAAATACCACATTATGCTG-poly(A)

Matches to 674 bp mRNA, Accession number CX567486, GI:57594515, Mus musculus cDNA clone.

Tag-TACTACATAG PMSG 0 hCG 54

CATGTACTACATAGCAATACCTTGTTAT-poly(A)

No significant similarity

Tag-ACAACTCCAC PMSG 0 hCG 39

CATGACAACTCCACTCTGGTGGAGTTCTCTCAAACTATAAGCCAAAATAAACCCTTC CTTCTT-poly(A)

509 bp mRNA, Accession number BG148701, GI:12652123, mouse_NMGB_bcell Mus musculus cDNA clone.

Tag-GGAGCAGACC PMSG 0 hCG 25

CATGGGAGCAGACCAGTAAGGGACCTTCAATTTAAAACAAAACAAAACAAAAAAA CAATAAAAAGGCTAATTAACAGTGGAG-poly(A)

Matches to 273 bp mRNA , Accession number BB421909, GI:9243264, embryo spinal cord Mus musculus cDNA clone, similar to D00472 Mus musculus mRNA for cofilin.

Tag-AACAAACGCA PMSG 0 hCG 19

CATGAACAAACGCAGGAAG-poly(A)

No significant similarity

Tag-TCGCTGCCTT PMSG 0 hCG 15

CATGTCGCTGCCTTATTAAATCCTGCCTTCTACATTGT-poly(A)

Matches to 311 bp mRNA, Accession number CX734226, GI:58061062, Mus musculus cDNA clone whole eye.

Tag-AAAGCACACA PMSG 0 hCG 128

CATGAAAGCACACAGAGCTCTGGGGTCGAAACTCATACACCTTTGCACAGGGTAGA GGAGTCTCGACGACGACTCAAATTTTTCACAAGCTTTCG-poly(A)

Matches to 483 bp mRNA, Accession number BF453690, GI:11519859, Mus musculus cDNA clone

Tag-CAGTCAATAC PMSG 212 hCG 30

CATGCAGTCAATACTTTGTACAGTTAGTAGGCAGTATTCAGCAATGCCCGATAGCTT CTTTCCGGTTATGTTAAATAAAAAGTCCTGTT-poly(A)

Matches to 457 bp mRNA, Accession number BB821750, GI:16994379, Mus musculus cDNA clone. Matches to Unigene cluster Mm 290944.

A number of sequences do not produce a significant match when BLAST searched against EST sequences. These sequences do so when the SAGE tag is immediately against or in close proximity to the poly (A) tail, leaving little additional sequence information to be recovered by 3' RACE. Further information about these can be acquired by sequencing the 5' end of the gene as described in the next chapter. Among those sequences giving clue as to function is the EST matching to the SAGEtag GGAGCAGACC which shows similarity to the mouse cofilin sequence. The EST match for the SAGEtag CAGTCAATAC is also intriguing since it matches to a Unigene cluster (Mm 290944) showing homology with a highly conserved noncoding human mRNA sequence. In addition this tag shows a selective expression pattern for the granulosa cell and has been investigated further (Chapter 4).

3.6 Validation of SAGE results using Real Time PCR

Validation and further investigation of the SAGE results requires the use of another method of mRNA quantification - several are available. Hybridisation-based methods such as microarrays, northern blot, and in situ hybridisation are restricted by their requirement for large volumes of samples, by being labour intensive (Bird et al 1998, Duggan et al 1999), and by having a smaller dynamic range (Mirnics et al 2001). The real time PCR method requires less sample volume and has previously been used for validation of larger scale gene expression studies and for more precise analysis of selected genes (Chuaqui et al 2002).

Real time PCR is used to quantify cDNA produced by reverse transcription of mRNA. The reverse transcription reaction can be carried out using gene-specific, random, or oligo d'T primers. Gene specific primers increase specificity and decrease background while random hexamers and oligo dT primers maximise the number of mRNA molecules available for analysis (Bustin 2000). The reverse transcription step is the source of most variability between different reactions in quantative RT-PCR measurements (Freeman et al 1999). In order to minimise this variability a reference value can be used to adjust the obtained quantities of cDNA, this can be an added RNA standard of known amount added prior to the reverse transcription reaction, or the use of reference genes within the RNA sample.

Real time PCR measures the PCR product as it accumulates in the exponential phase of the reaction. The quantity is characterised by the point at which the amplified PCR product climbs above an arbitrary threshold (Ct). The greater the quantity of template, the sooner the PCR product level exceeds threshold. This is preferable to an endpoint measurement because the end PCR product quantity is sensitive to the reaction conditions, whereas during the exponential phase the reaction components are not limited; consequently Ct values are reproducible for reactions starting with the same copy number.

The PCR product can be detected by amplicon specific probes where fluorescence is released by removal of a quencher during the reaction.

Alternatively, DNA intercalating dyes such as ethidium bromide or SYBR green can be used. These dyes are not sequence specific and will intercalate in unspecific amplification products and in primer dimers, although it is possible to study melting curve profiles to detect these artefacts (Bustin 2000).

This study used the Taqman 5' nuclease assay which utilises a probe, designed with a short sequence complementary to the sequence of interest, with both a fluorescent (5') (FAM) and quencher dye (3') attached (TAMRA). Prior to digestion of the probe by the PCR reaction the fluorescence is limited by the proximity of the quencher. As the reaction progresses fluorescence is released in direct proportion to the accumulation of PCR product. The higher the starting copy number of the nucleic acid target, then the sooner a significant increase in fluorescence is observed.

3.6.1 Method

The mRNA extractions, DNAase treatments and reverse transcriptions are performed as previously described. The reverse transcription in this case employed random hexamers to prime the reaction in order to maximise the number of mRNA molecules available for analysis (Bustin 2000). Although the reverse transcription step is the source of most of the variability inherent in quantitative RT-PCR the later use of an internal reference gene negates the need to analyse and adjust obtained quantities of cDNA or to include external RNA standards in the sample.

To validate differences in gene expression between SAGE libraries real-time PCR was used to measure relative mRNA levels of a number of transcripts. In eukaryotic cells the use of stably expressed housekeeping genes as standards allows for the relative quantification of gene expression (Bustin 2000). In this case the ubiquitous housekeeping gene Glyceradehyde-3-phosphate dehydrogenase (GAPDH) was used as standard. GAPDH is one of the classical normalisation genes and has been shown in microarray studies to be the most invariable housekeeping gene (Lee et al 2002). In both SAGE libraries the expression levels of GAPDH were similar. The use of mRNA is preferable to the more stable ribosomal RNA which constitutes almost 90% of the total cellular

RNA and is therefore present at a far more abundant level, complicating comparison with mRNA which constitutes only 3-5% of cellular RNA (Alberts et al 1994). At the time of this study we lacked the equipment necessary to carry out multiplex PCR which would have allowed the reference gene to be measured in the same reaction as the gene of interest, with the advantage that PCR variability would be similar for both ampilcons, but also with the risk of primer interference between reactions.

In order to calculate a relative copy number from the Ct values we created standard curves from which to the measure amplification efficiency of a diluted series of samples. The DNA concentrations in these samples are relative, not absolute, values since an arbitrary quantity is sufficient. In this study a serial five-fold dilution was employed. For each standard the concentration was plotted against the cycle number at which the fluorescence increased above the threshold value (Ct value). An efficiency of one reflects a doubling of the product in each PCR cycle.

Each mRNA sample was prepared from the granulosa cells of 2 normal female mice which had undergone superovulation at 20 days of age with either PMSG or PMSG/hCG combination and collected at 48 hours or 12 hours post PMSG or hCG injection as for SAGE library construction. Each group contained 5' RNA samples. These samples were DNAase treated (Ambion, UK) and checked for genomic contamination using the GAPDH primers and probe for real time PCR. If any amplification was detected then samples were retreated and rechecked. No sample had to be treated more than twice in order to remove genomic contamination.

The primer and probe sequences used for this analysis are as listed in table 3.7. These sequences were either as previously published, or if novel, designed using Primer Express (Applied Biosystems, Warrington, UK) with sequence information obtained from Genbank or in the case of the transcript associated with the SAGE tag CAGTCAATAC from RACE analysis of SAGE data.

Real time PCR's were carried out in a 25µl reaction volume in a 96 well plate format. The reaction was performed in 1x GeneAmp PCR Gold Buffer and 6.25mM magnesium chloride solution (both Applied Biosystems), 200µM

dNTP's, 300nM of each primer, 200nM of probe and 0.15 units of enzyme (Amplitaq Gold; Applied Biosystems, UK) per 25µl reaction volume. A reference dye, Rox, was included in these reactions at a concentration of 60nmol/L. The thermal profile consisted of an initial 10 minute 95°C denaturation then 40 cycles of 95°C for 15 seconds denaturation with a 60°C extension step. Thermal cycling and flouresence detection was performed on a Stratagene MX3100P machine and reaction efficiencies were calculated using the Stratagene MX3100P software (Stratagene, UK).

3.6.2 Results

Standard dilution series for the gene of interest and GAPDH were produced on each reaction plate and the associated standard curves and reaction efficiencies are shown in figure 3.16 for each reaction.

Each sample was run in duplicate and 6 samples from each time point (48hrs post PMSG injection and 12hrs post hCG injection) were collected, each consisting of granulosa cells from 2 normal superovulated mice. The mean values from the duplicate reactions, adjusted with reference gene GAPDH, are shown in Table 3.8 for each sample. The overall mean value between samples was used to calculate relative fold change in expression between the two time points.

The expression changes reveal genes both upregulated and downregulated within the two sample groups. The steroidogenic enzymes show an expected pattern of change, as do the gonadotrophic hormone receptors. These results show that there is good correlation between data obtained by real-time PCR and data derived from SAGE libraries (figure 3.18).

Table 3.7 Sequences of real-time PCR primers and probes used in this study.

Gene	Primer/Probe	Sequence
Aromatase	Forward	CCGAAAAAGAATGACCTGTCCTT
	Reverse	TTGTCTGAATTCCTTGGAGAGAAAA
	Probe	CACCCAAATGAGGACAGGCACCTTGT
Unmatched SAGE tag	Forward	TTCTTTAACCAATGTCTGGCTAATG
	Reverse	TCCAACCGTTATCTCTTTAAACATAT
	Probe	TGAGTGCATTTCAACTATGTCAATGGTTTCTT
Kit ligand	Forward	GCCGGCAATGCCATG
	Reverse	AGGTCCCGAGAAAGGGAAA
	Probe	CTGTCAATTGTAGGCCCGAGTCTTCA
FSH receptor	Forward	GGCGGCAAACCTCTGAACT
	Reverse	CCAGGCTGAGTCATATCATCAATATC
	Probe	CATCCAATTTGCAACAAGTCTATTTCAAGGCA
LH receptor	Forward	GACCAAAAGCTGAGGCTGAGA
	Reverse	CAATGTGGCCATCAGGGTAGA
	Probe	TGCCATCCCAATTATGCTCGGAGGA
Wbscr 1	Forward	CTGCAATGTCCACACGAAGTG
	Reverse	TCCCTGAAGGAGGCTCTGACT
	Probe	CCGGTCACCCAAGAGTGCACCG
Cholesterol side chain cleavage	Forward	CCAGTGTCCCCATGCTCAAC
	Reverse	TGCATGGTCCTTCCAGGTCT
	Probe	TGCCTCCAGACTTCTTTCGACTCCTCAGA
GAPDH	Forward	TGCCCCCATGTTTGTGATG
	Reverse	TCATGAGCCCTTCCACAA
	Probe	TTGTCAGCAATGCATCCTGCACCAC
StAR	Forward	CCGGAGCAGAGTGGTGTCA
	Reverse	CAGTGGATGAAGCACCATGC
	Probe	CAGAGCTGAACACGGCCCCACC

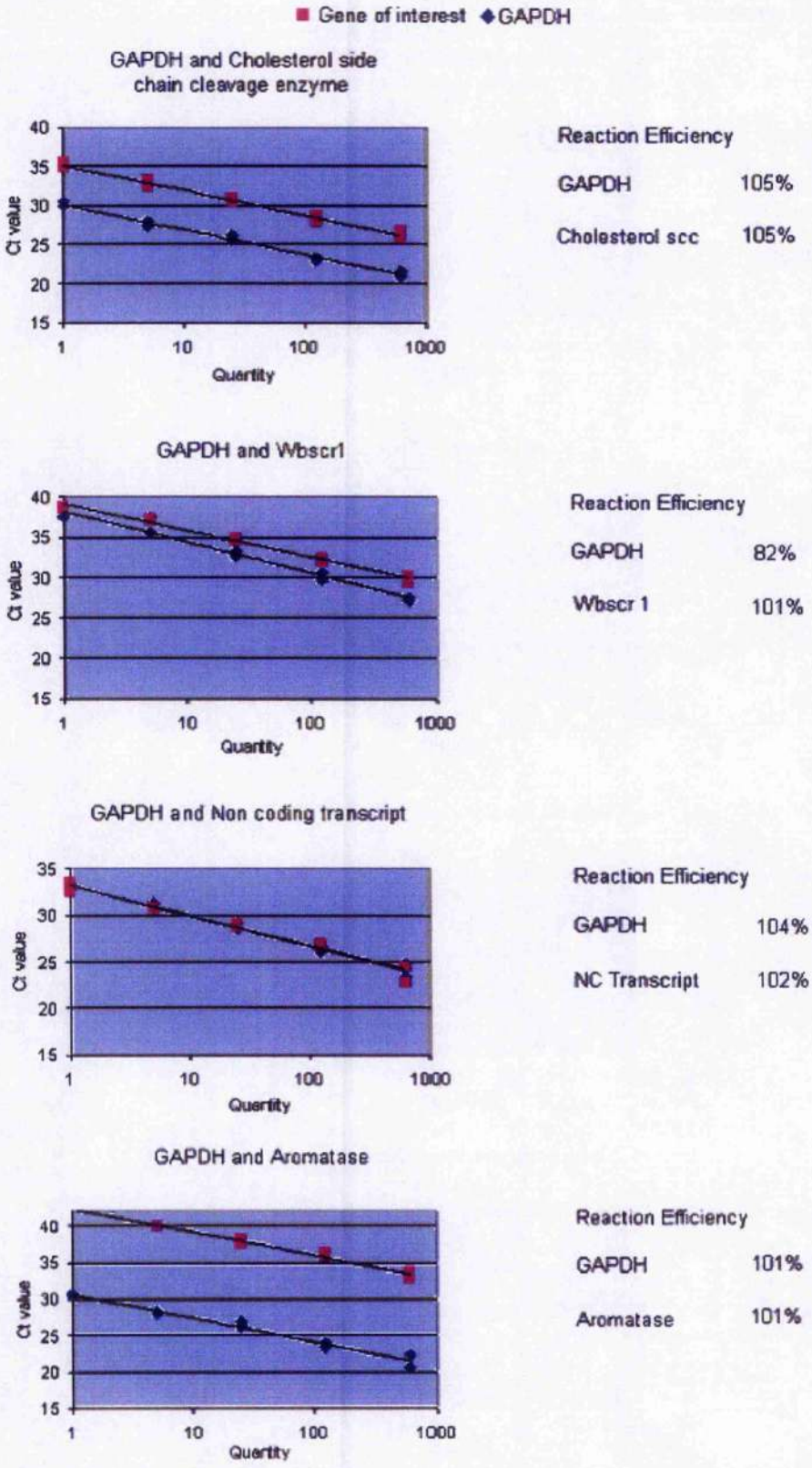


Figure 3.17 Standard curves and reaction efficiencies for real time PCR reactions

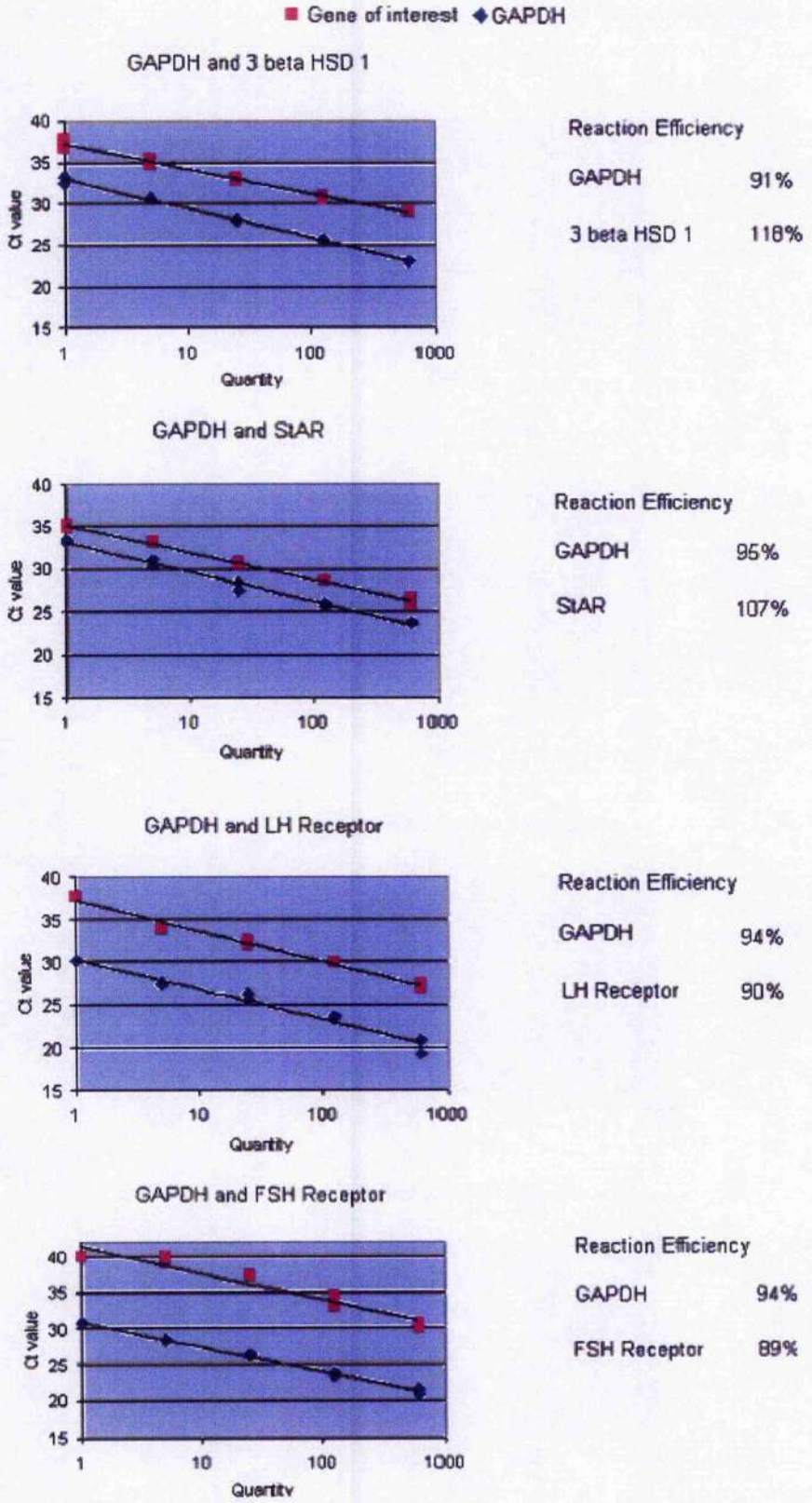


Figure 3.17 cont'd. Standard curves and reaction efficiencies for real time PCR reactions

Table 3.8 Real time PCR results for genes of interest relative to GAPDH expression

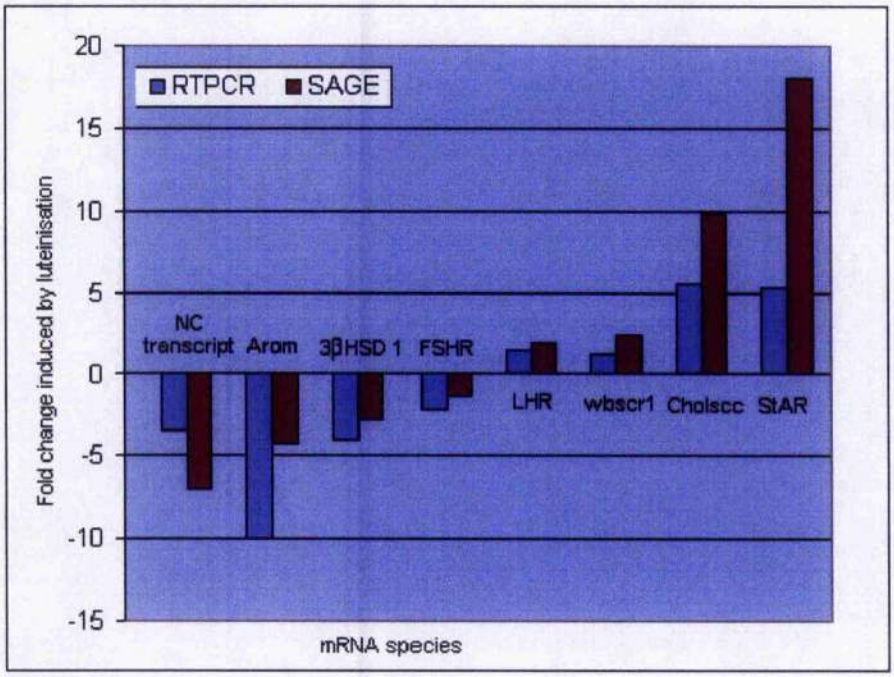
PMSG treated granulosa cell samples

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean	SEM
FSH receptor	18.9	19	19.4	35.7	14.4	40	24.6	10.5
LH receptor	64.3	98.6	196.5	416.4	271.7	332.6	230	136.3
Wbscr 1	312.1	185	140.1	387	153	119.5	216.1	108.1
StAR	17.3	6	127.2	10.2	61.6	25.7	41.3	46.5
Cholesterol P450 scc	159.8	104.4	158.8	101.9	261.5	104	148.4	61.8
Aromatase	7.9	19.1	30.5	21.7	13.4	27.2	20	8.4
3b-HSD 1	86.1	51.2	79.5	42.5	78	89.2	70.6	19.4
Non-coding transcript	102.6	530.3	271.7	519.4	356	697.4	412.9	212.4

PMSG and hCG treated granulosa cell samples

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Mean	SEM
FSH receptor	8.5	7.6	16.9	7.2	10.1	15.7	11	4.2
LH receptor	277.8	355.8	189.7	306	917	38.7	347.5	300.4
Wbscr 1	90.2	111.9	180.5	498.3	729.5	118.8	288.2	264.4
StAR	219.9	184.2	192.1	308.9	386.9	32.8	220.8	120.7
Cholesterol P450 scc	611.3	1681.8	979.4	1144.7	491.4	84.5	832.2	559.5
Aromatase	2.2	2.1	1.1	1.4	1.5	3.7	2	0.9
3b-HSD 1	21.2	11.2	25.7	22	25.9	49.8	17.3	12.8
Non-coding transcript	52.9	315.3	25.2	157.7	49.4	124.1	120.8	107.7

Figure 3.18 Comparison of SAGE and QRT-PCR expression levels



Chapter 4

Investigation of the non coding transcript associated with the SAGE tag CATGCAATAC

Chapter 4: Investigation of non coding transcript associated with the SAGE tag CAGTCAATAC

4.1 Rationale for study

This particular tag warranted further investigation not only because it was the 6th highest transcribed sequence in PMSG treated granulosa cells and significantly differentially expressed during luteinisation but also because the 3'race data matched this tag to a unigene cluster (Mm.290944) showing 95% homology with a highly conserved noncoding human mRNA sequence. In addition this tag is not highly expressed in other mouse SAGE libraries, suggesting a selective expression within the granulosa cell.

Initially this investigation required the full length sequencing of the cDNA transcripts, followed by identification of the genomic location using bioinfomatical methods (BLASTn). The analysis of temporal and tissue specific expression was carried out using real time PCR and finally cellular localisation was achieved by in situ hybridisation.

4.2 Full length sequencing of non coding transcript

The 5' RACE procedure (figure 4.1) allows the 5' extension of cDNA fragments using a single gene specific primer from the 3' end of a cDNA sequence. This procedure was performed using the SMART RACE cDNA Amplification Kit (BD Biosciences, UK).

The 5' primer utilises a oligonucleotide containing multiple G residues on the 5' end which anneal to multiple C residues left by the reverse transcritpase enzyme on the first strand cDNA and serves as an extended template for extension by the reverse transcritpase enzyme. This incorporates an extended oligonucleotide sequence at the 5' end which can subsequently be used for PCR amplification in conjunction with a 3' gene specific primer. The only requirement for performing this procedure is that enough 3' sequence information is available to enable the design of at least one but preferably two nested gene specific primers.

The SMART RACE reaction kit (Clontech, UK) allows for further amplification by providing a nested SMART oligo primer.

The 5' RACE was carried out using RNA collected from the granulosa cells of 9 female mice 48 hours after PMSG treatment as previously described for SAGE.

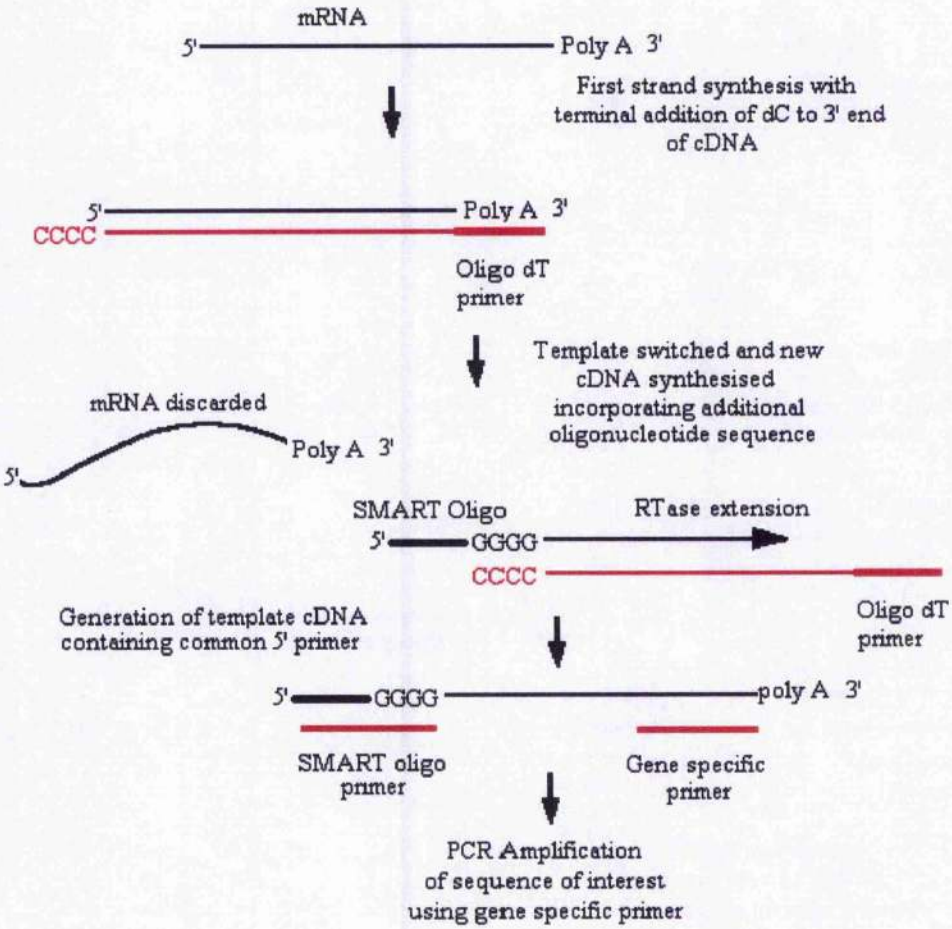


Figure 4.1 Schematic illustration of 5' RACE. Synthetic oligonucleotide (SMART) hybridises to the 5' end of reversed transcribed cDNA by means of a short poly(C) tail and is extended in the 3' direction.

Isolated RNA solution (3µl), 1µl 5' CDS primer (12µM), 1µl of the BD SMART II oligo (12µM)(sequences listed in table 4.1) were mixed by pipetting and collected by pulse spinning, heated to 70°C for 2 minutes to remove secondary structure and then cooled on ice for 2 minutes to allow annealing. Added to this

mixture were 2µl 5x first strand buffer (250mM Tris HCl, 375mM KCl, 30mM $MgCl_2$), 1µl DTT (20mM), 1µl dNTP mix (10mM) and 1µl BD Powerscript RTase to give a total reaction volume of 10µl. The reaction was gently mixed, pulsed and incubated at 42°C for 90 minutes. 20µl tricine/EDTA buffer (10mM tricine, 1mM EDTA) was then added and the reaction heated to 72°C for 7 minutes. This provided the PCR template for subsequent amplification using gene specific primers.

PCR amplification of gene specific sequence was carried out using 0.5µl of 20mM gene specific primer (table 4.1) in a 50µl reaction mix containing 34.5µl rtH_2O, 5µl BD Advantage 2 PCR Buffer, 1µl dNTP mix (10mM), 5µl Universal Primer Mix (long primer 0.4µM, short 2µM), 1µl BD Advantage 2 Polymerase Mix (All BD Biosciences, UK) and 2.5µl cDNA template from the 5' RACE amplification. Negative controls were set up without one of the two primers.

Cycling parameters were 94°C for 30 seconds followed by 72°C for 3 minutes repeated for 5 cycles, then another 5 cycles of 94°C for 30 seconds, 70°C for 30 seconds, followed by 72°C for 3 minutes, and finally 25 cycles of 94°C for 30 seconds, 68°C for 30 seconds, followed by 72°C for 3 minutes.

Table 4.1 Primer sequences for 5' RACE

Primer	Sequence (5' to 3')
BD SMART II Oligonucleotide	AAGCAGTGGTATCAACGCAGAGTACGCGGG
5' RACE CDS Primer	$(T)_{25}$ VN (N= A,C,G,T; V=A,G,C)
Universal Primer Mix	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT CTAATACGACTCACTATAGGGC
Nested UPM	CTAATACGACTCACTATAGGGC
Gene Specific Primer for CAGTCAATAC	TAACATAAGGCCAAAGAAGCTATCGGGCATTGCTGAATACTGC
Nested GSP for CAGTCAATAC	ATCGGGCATTGCTGAATACTGC

Products were visualised on a 1% agarose gel containing ethidium bromide using u.v. light. In this particular case the 5' RACE reaction gave a smear between the 300bp and 700bp markers.

The PCR product was diluted in Tricine-EDTA buffer (5µl in 245µl Tricine-EDTA) and reamplified using the Nested Universal Primer Mix (1µl, 10µM) (BD Biosciences, UK) and the nested gene specific primer (1µl, 10mM) (table 4.1). The reaction mixture was composed of 36µl rtH_2O, 5µl BD Advantage 2 PCR Buffer, 1µl dNTP mix (10mM), 1µl BD Advantage 2 Polymerase Mix (All BD Biosciences, UK) and 5µl cDNA template. Cycling parameters of 94°C for 30 seconds, 68°C for 30 seconds, followed by 72°C for 3 minutes for 20 cycles were employed. The product was visualised on 12% polyacrylamide gel to reveal a smear of multiple products ranging from 200bp to 600bp in length (figure 4.2). The PCR product was TOPO cloned, electroporated into DH10B E.coli cells (Invitrogen, Paisley, UK) and vector insert PCR amplified and sequenced as previously described using M13R and M13F primers for amplification and M13R for sequencing.

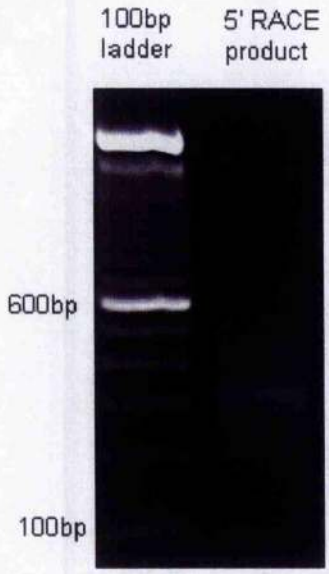


Figure 4.2 12% PAGE gel showing nested 5'RACE reaction products alongside 100bp ladder

5' RACE Results

The sequenced transcripts were compared and aligned using NCBI BLAST (web address). All 81 sequenced transcripts had a constant sequence with variable 5' lengths. The longest sequence is shown in figure 4.3 with the Nla III site and the adjacent SAGE tag highlighted in red. The sequence length of the transcript varied from 70bp to 682bp, with a mean of 264bp (the distribution is shown in figure 4.5). This sequence contains no open reading frame suggesting it may function as an untranslated mRNA.

Its genomic sequence is located on chromosome 13 (bases 15480202-15480884) approximately 3500 bp downstream of the inhibin βA subunit (figure 4.3), a component of both the inhibin and activin peptide hormones. Comparison of the rat, mouse and human genomic sequence is shown in figure 4.6

5 prime-aaatttaatttgggacatagtgactcttcgataacctccccagtctctattacttacg

tatctacgacaagtgtagaagttctcgttttcgtttagtgcacagagtcgatataataataaata

aaaatacgtatttcacttagtaaagaagacataattaaaagtcaccccaaacgggagataaattt

acgaaacttttgtcacgtaactgttaccaac**tataaaaagaaattttctttttata**ttaatact

ttcggttatattagactttagacaaaactagattttgaaaaatacaatacaccaactacaacaaa

caaacaaaaaataaaaataaaacactcaaggaaacgtatgatgtacgttaagaaattggttacag

accgattacattaatttcaacaattaaatatactcacgtaaagttgatacagttaccaaagaatt

ataaataaaacatcttcacgaccattaaaaaataaatgctatacaaatttctctattgccaacct

atacaaaagtacacaaatatcgtcttcaataaataaagataaggtaaggtcgcctataagactac

aaacgctcc**gtacgtcagttatg**aaacatgtcaatcatccgtactaagtcgttacgggctatcga

agaaaccggaatacaatttatttttctggacaaacccta-3 prime

Figure 4.3 682 bp sequence for transcript associated with the SAGEtag CAGTCAATAC. The SAGE tag is highlighted in red and the hairpin fold in black.

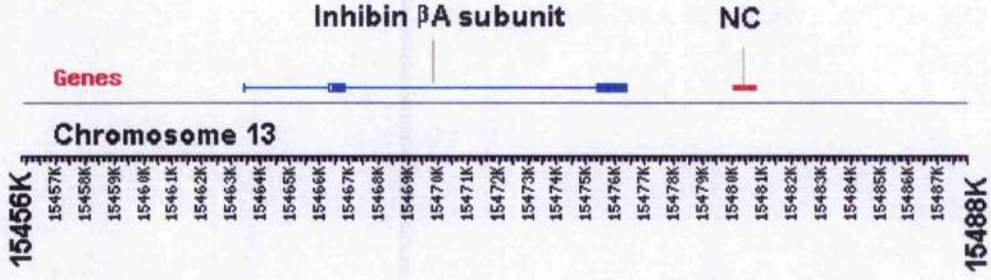
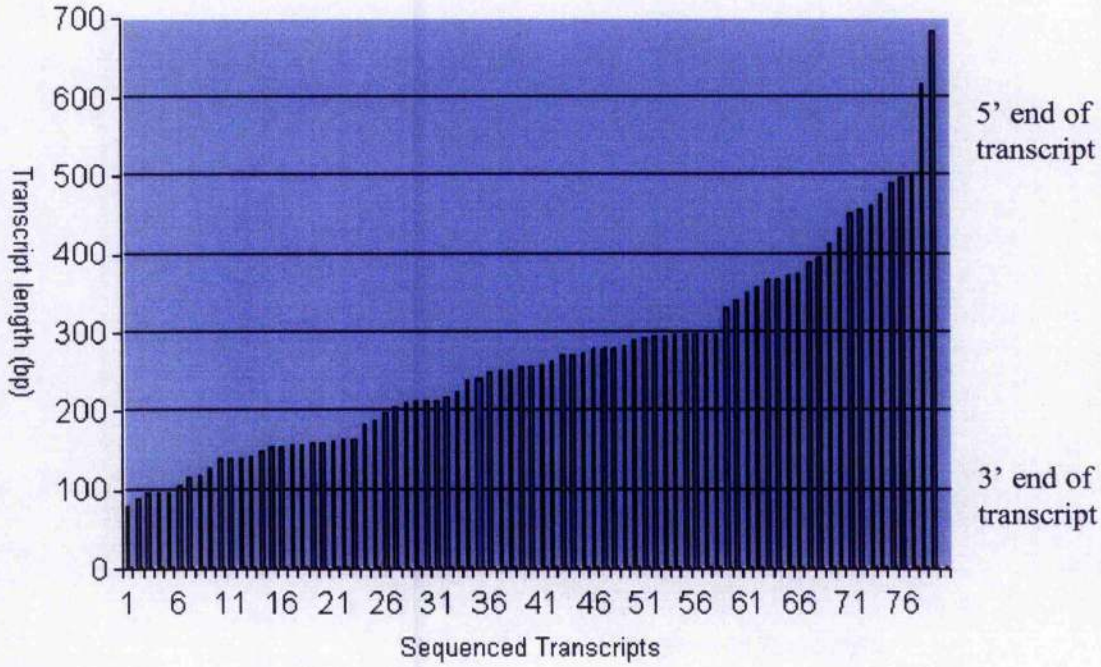


Figure 4.4 Genomic location of noncoding transcribed fragment (NC) in relation to inhibin βA subunit



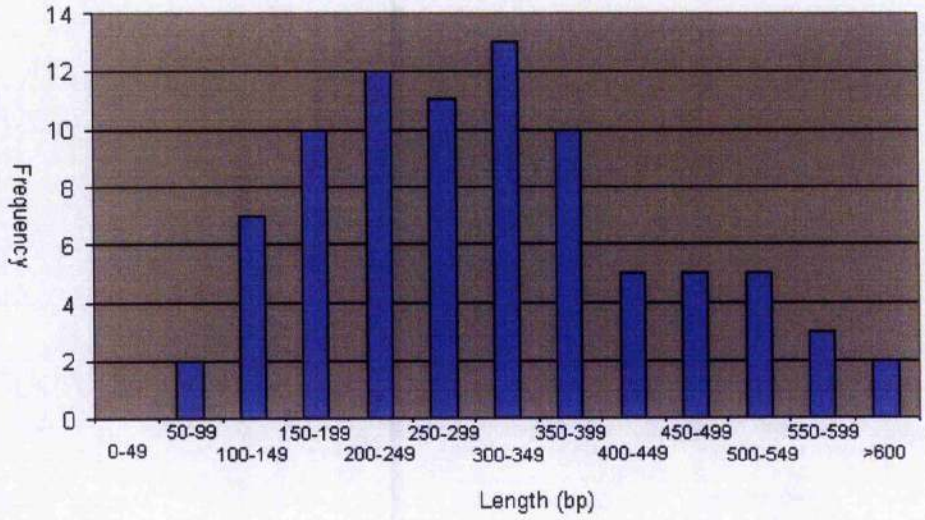


Figure 4.5 Variation in size of noncoding transcripts

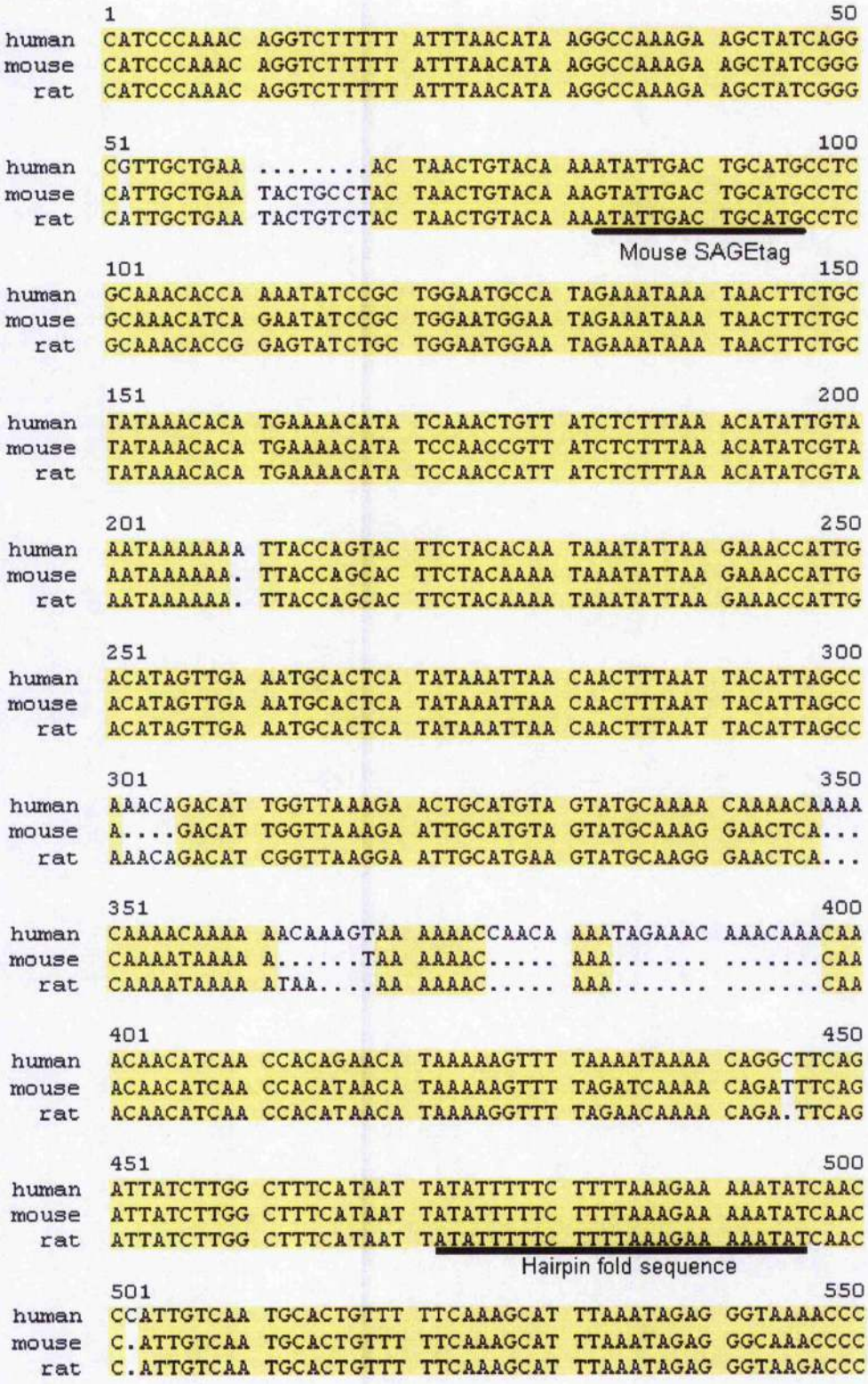


Figure 4.6 Homology between noncoding transcript in mouse, rat and human DNA. Identical regions have yellow background.

```
        551                                                         600
human   TTTGGAAATT AATACAGAAG AAATGATTCA CTTTATGCAT AAAAAATAAA
mouse   ACTGAAAATT AATACAGAAG AAATGATTCA CTTTATGCAT AAAAA.TAAA
  rat   ACTGAAGATT AATACAGA.G AAATTATTCA CTTTATGCAT AAAAA.TAAA

        601                                                         650
human   TAATAATATA GCTGAGACAT GTGGTTTGCT TCTGCTCTTG AAGATGTGAA
mouse   TAATAATATA GCTGAGACAC GTGATTTGCT TTTGCTCTTG AAGATGTGAA
  rat   TAAT...ATA GCTGAGACAT GTGATTTGCT TTTGCTCTTG AAGATGTGAA

        651                                                         700
human   CAGCTTCTAA GCATTCATTT TCTCTGACCC ATACAACAGC TTCTCAGTGA
mouse   CAGCATCTAT GCATTCATTA TCTCTGACCC CTCCAATAGC TTCTCAGTGA
  rat   CAGCATCTAT GCATTCATTA TCTCTAACCC ATCCAATAGC TTCTCGGTGA

        701                                                         750
human   TACAGGGTTT AATTTAAACA CATACAATGT CCACCCCCAA ACCTTCTGCC
mouse   TACAGGGTTT AATTTAAACA CATACAATGT CCATCCCCCA ACCTCCTGCC
  rat   TGTAGAGTTT AATTTAAACA CATACAATGT CCATCCCCAA ACCTCCTG..
```

Figure 4.5 cont'd. Homology between noncoding transcript in mouse, rat and human DNA. Identical regions have yellow background.

4.3 Expression study of the non-coding transcript in multiple tissue types.

In addition to validation of the SAGE libraries, real time PCR was used to examine the expression pattern of the non-coding transcript represented by the sage tag CATGCAATAC. A range of tissue samples from male and female mice underwent RNA isolation, DNAase treatment and reverse transcription as previously described. Real time PCR was performed following the protocols listed in chapter 3 and using the primer/probe set already described (table 3.7)
Two separate samples of each tissue were collected and again each reaction run in duplicate. Standard curves were included on the same reaction plate to determine amplification efficiency, a typical example of one curve is shown in figure 4.7.
The expression of this transcript, relative to GAPDH, is present in a number of tissues, the highest expression levels being in the spleen and adult ovary. The ovaries used in this experiment were taken from normal adult cycling females and had grossly visible follicular development; the expression of this transcript in this tissue is therefore expected. The uterus, lung and small intestine also recorded a comparatively high degree of expression, with minimal levels in the heart, epididymis, brain, liver and bladder (table 4.2 and figure 4.8).

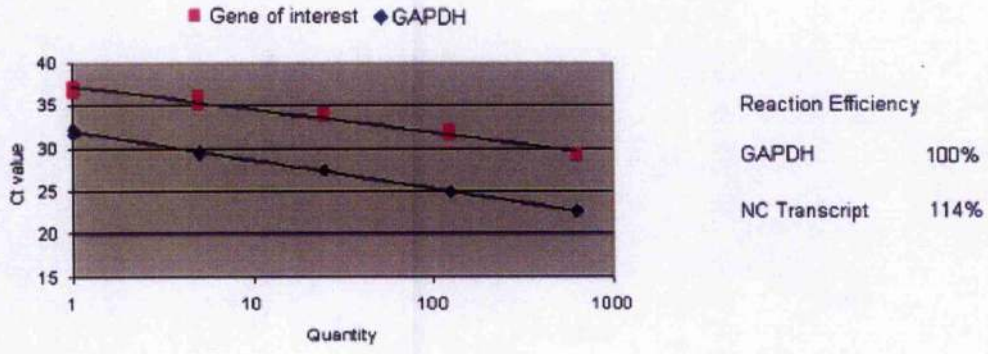


Figure 4.7 Standard curve and amplification efficiency for typical QRT PCR reaction between GAPDH and noncoding transcript.

Tissue type	Duplicate samples		Mean
Testes	0.31	0.21	0.26
Epididymis	3.47	2.99	3.23
Brain	0.62	0.41	0.52
Lung	103.7	12.8	58.2
Heart	0.81	0.68	0.74
Liver	0.69	2.18	2.87
Kidney	0.22	0.1	0.16
Adrenal	0.06	0.21	0.14
Bladder	0.98	0.77	0.88
Small Intestine	13.2	11.8	12.5
Skeletal Muscle	0.18	0.65	0.42
Spleen	76.9	280.3	178.6
Adult ovary	67.2	124.6	95.9
Uterus	13.1	21.6	17.35

Table 4.2 QRT PCR results of expression levels of noncoding transcript relative to GAPDH

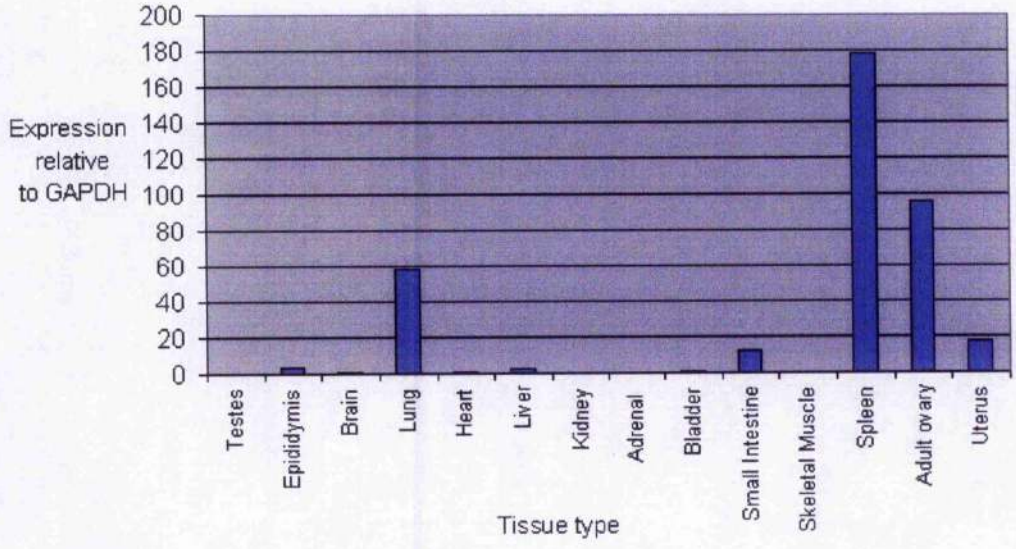


Figure 4.8 Expression levels of noncoding transcript relative to GAPDH

4.4 Temporal expression of noncoding transcript within ovarian tissue

The expression pattern of this transcript can be assessed at each stage of follicle development by using neonatal and juvenile mouse ovaries. The newborn mouse ovary contains only primordial follicles. By post-natal day 20 a number of follicles have developed to antral follicle stage. Neonatal mouse ovaries therefore contain uniform follicle populations of similar sizes as determined by the number of days after birth (Sorensen et al 1976; Epigg 1991, Mannan and O'Shaughnessy 1991). The gonadotrophin-induced mouse model using PMSG and hCG at 20 days old, before the animal has entered its own oestrus cycle, simplifies the staging and collection of uniform populations of cells. In order to study the expression of this transcript during follicular development we collected whole ovaries from day 1, 3, 5, 7, 10, 15 and 20 neonatal mice to give a series of ovaries containing follicle populations which mimic primordial to antral follicle development (Mannan and O'Shaughnessy 1991). Following on from this day 20 mice injected with PMSG intraperitoneally were taken at 6, 12, 24, 36 and 48 hours post injection and whole ovaries harvested. Mice 48 hours after PMSG were then administered hCG (following the protocol used for SAGE library generation) and ovaries recovered at 2, 4, 8 and 12 hours post injection. Ovaries were recovered from 2 animals per sample at each time point, 4 duplicate sample series were collected. This produced a collection of samples which follow primordial to preovulatory follicle development. Real time PCR was performed using the previously stated protocols and primer/probe sets, all reactions were run in duplicate.

The results show (table 4.3, figure 4.9) that expression is not detectable in the post natal mouse ovary until day 15, rapidly increasing with the development of antral follicles by day 20. Following PMSG stimulation there is a gradual increase throughout antral follicle development, peaking 48 hours post injection and then a rapid decrease during the 12 hours immediately following hCG administration as the granulosa cells undergo luteinisation.

The lower level of relative expression of the non coding transcript in the sample taken 6 hours after PMSG injection compared to the day 20 neonatal mouse can perhaps be explained by a likely increase in GAPDH expression levels associated with the hormonally induced increasingly follicle mass and cell proliferation generated by the exogenous PMSG.

	Time	4 Duplicate Tissue Sample Sets				Mean	SEM	Follicle stage
Age of mouse days after birth	day1	0	0	0	0	0	0	Primordial
	day 3	0	0	0	0	0	0	
	day 5	0	0	0	0	0	0	Primary
	day 7	0	0	0	0	0	0	
	day 10	0	0	0	0	0	0	Secondary
	day 15	36.8	18	1.3	22.6	19.7	14.6	
	day 20	88.1	100	119.1	80.8	97	16.7	Antral
5iu PMSG given i/p to d20 mice	PMSG 6h	69.1	59	25.6	81.1	58.7	23.8	
	PMSG 12h	55.2	75	88.1	133	87.8	33.0	Mature antral
	PMSG 24h	60.6	90	52	179.2	95.4	58.2	
	PMSG 36h	64.5	97	128.1	107.3	99.2	26.5	Pre-ovulatory
	PMSG 48h	178	164	131.7	185.6	164.8	23.8	
15 iu hCG given i/p 48 hours after PMSG injection	hCG 2h	115.4	118	104.4	152.8	122.6	20.9	
	hCG 4h	69.1	82	67.9	106.9	81.5	18.1	
	hCG 8h	10.3	14	1.2	30.2	13.9	12.1	
	hCG 12h	11.7	12	5.6	16.3	11.4	4.4	Luteinising

Table 4.3 Real time PCR results showing expression of the noncoding transcript relative to GAPDH in staged whole ovary samples from neonatal mice and following exogenous hormone administration.

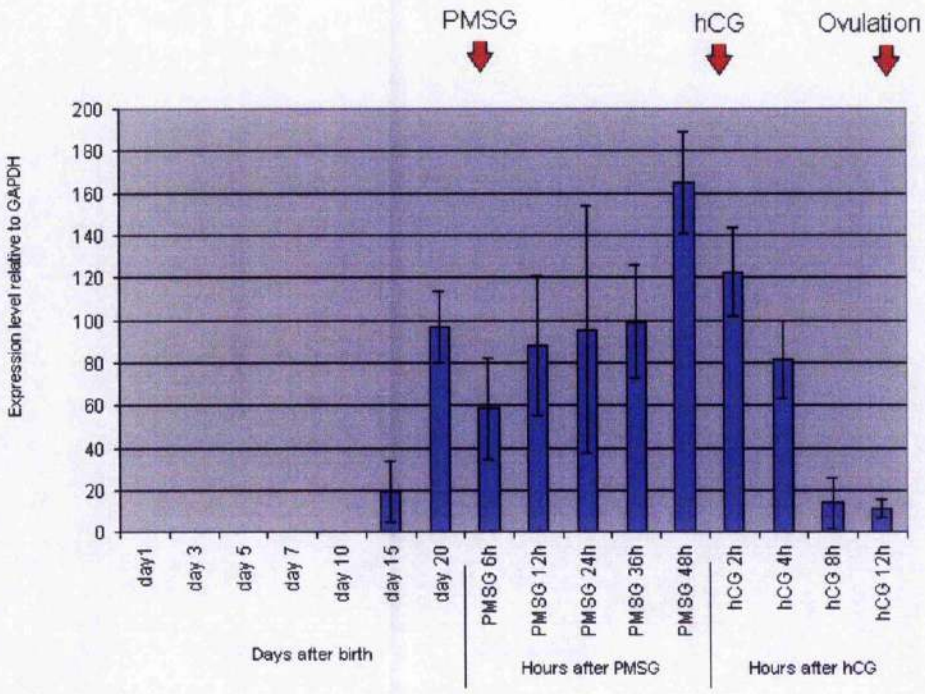


Figure 4.9 Real time PCR results showing expression of the noncoding transcript relative to GAPDH in staged whole ovary samples from neonatal mice and following exogenous hormone administration.

4.5 In situ Hybridisation

Tissue complexity is an additional factor that affects the reliability of RNA quantity measurement. In situ hybridisation is a powerful technique allowing the localisation of specific nucleic acid sequences in morphologically fixed biological samples. The ability to detect the distribution of mRNA within tissues, and indeed within cellular compartments, allows evaluation of differential and spatial gene expression. In our case it is useful in validating the SAGE and real time expression data for the non-coding transcript associated with the SAGE tag CAGTCAATAC and confirming localisation of this transcript to the granulosa cell.

Our protocol utilised synthetic oligonucleotide probes (MWG Biotech) instead of the less stable riboprobes. DEPC (diethylpyrocarbonate) treated water was used in preparation of all solutions and standard procedures (baked glassware, disposable gloves) were used to avoid RNase contamination.

Method

Tissue and slide production

Normal d20 mice were administered 5IU PMSG intraperitoneally to induce superovulation and ovaries removed following euthanasia 48hrs post injection. Normal fertile cycling adult mice and normal last third trimester pregnant adult female ovaries were also freshly collected following euthanasia. Ovaries were immersed in 4% paraformaldehyde for 90 minutes and then transferred to 70% alcohol prior to wax embedding.

Embedded tissue was sectioned in slices 7μm thick and mounted by flotation in a 37°C waterbath onto Superfrost Plus microscope slides (BDH Laboratories) and stored at 4°C until use. Sections from 4 normal, 6 PMSG treated and 2 pregnant mouse ovaries were mounted on each slide.

Probe preparation

Synthetic oligonucleotide probes corresponding to the sense and antisense sequence of the common 3' end of the cDNAs sequenced by 5' RACE were radioactively labelled with ^{35}S using terminal deoxynucleotide transferase (TdT) (Roche, UK).

Sense probe sequence (SAGE tag underlined)

TAACTGTACAAAGTATTGACTGCATGCCTCGCAAACATCA

Antisense probe sequence

ATTGACATGTTTCATAACTGACGTACGGAGCGTTTGTAGT

4µl oligonucleotide probe (25ng/µl), 5µl TdT Tail Buffer (1M Potassium cacodylate, 125 mM Tris-HCl, 1.25 mg/ml BSA) (Roche, UK), 1.5µl $CoCl_2$ (25mM), 1.5µl terminal transferase (400U/µL) (Roche, UK) and 2µl of radiolabelled^{35}S ATP (25µCi/0.925MBq) (PerkinElmer, USA) were combined in a reaction volume of 24.5µl, incubated at 37°C for 30 minutes and then placed on ice. The oligonucleotide was then separated from the reaction mix using Biospin 6 columns (Bio-Rad Laroratories) according to manufacturers instructions, recovering approximately 25µl of probe in each case.

0.5µl of radiolabelled probe was added to 5ml of Ecosinct A scintillation fluid (National Diagnostics, UK) and beta radioactivity measured using a 1600TR Liquid Scintillation Counter (Packard Bioscience, UK). Counts for the sense probe were 317628 and the antisense 259002, corresponding to a total count of 635256 counts per µl for the sense probe and 518004 counts per µl for the antisense probe. Probes were stored at -20°C until use (usually within 48 hours).

Slide Preparation

Slides were warmed to room temperature from 4°C then dewaxed by twice immersing in histoclear (National Diagnostics, UK) for 10 minutes. Hydration by immersion in decreasing concentrations of ethanol was followed by tissue digestion using hydrochloric acid and proteinase K, fixation with

paraformaldehyde and then dehydration though an alcohol series as listed in table 4.4. Slides were then air dried under a dust cover for 1 hour prior to hybridisation.

Table 4.4 Protocol for *in-situ* hybridisation slide preparation

Step	Solution	Time (minutes)
1	Histoclear	10
2	Histoclear	10
3	100% ethanol	5
4	90% ethanol	5
5	80% ethanol	5
6	70% ethanol	5
7	60% ethanol	5
8	30% ethanol	5
9	ddH_20	2
10	ddH_20	2
11	1/46 Hydrochloric acid	15
12	2xSCC	5
13	5µg/ml Proteinase K in 100mM Tris pH7.5, 50mM EDTA at 37°C	10
14	Phosphate buffered saline	1
15	Phosphate buffered saline	1
16	4% paraformaldehyde in PBS	20
17	Phosphate buffered saline	2
18	Acetylation using 0.1M Triethanolamine containing 1/400 acetic acid stirring continuously	10
19	Phosphate buffered saline	5
20	ddH_20	2
21	30% ethanol	5
22	60% ethanol	5
23	70% ethanol	5
24	80% ethanol	5
25	90% ethanol	5
26	100% ethanol	5

Hybridisation of probe

The hybridisation buffer was prepared by combining 6ml H2O, 5ml 20xSCC, 12.5ml deionised formamide, 500µl 50x Denhardts solution (Invitrogen, UK) and 1ml salmon testes DNA (Invitrogen, UK) before adding 2.5g dextran sulphate then mixing thoroughly until dissolved. Probe was added to the hybridisation buffer at a ratio of 8µl per ml and 250µl of this solution was

applied to each slide. This corresponds to just over 4 million counts per slide for both probes. Slides were covered with parafilm to prevent drying and incubated overnight at 37°C in a humidified container.

Following this the parafilm was removed and excess hybridsation buffer allowed to drain off. Slides were then rinsed in 1x SCC and mercapthethanol, H_2O and ethanol according to the protocol in table 4.5. Slides were then air dried and stored at 4°C until emulsification.

Equal number of slides were treated with the sense and antisense control probe.

Table 4.5 Washing protocol for hybridised slides

Step	Solution	Time
1	1x SCC with 0.001% mercapthethanol at room temperature	Rinse
2	1x SCC with 0.001% mercapthethanol at room temperature	Rinse
3	1x SCC with 0.001% mercapthethanol place in 55°C water bath (do not pre warm)	30 minutes
4	1x SCC with 0.001% mercapthethanol at 55°C	30 minutes
5	1x SCC with 0.001% mercapthethanol at 55°C	30 minutes
6	1x SCC with 0.001% mercapthethanol at room temperature	60 minutes
7	H_2O	Rinse
8	70% ethanol with 300mM ammonium acetate	30 seconds
9	100% ethanol	30 seconds

Emulsification

Emulsification was carried out using Ilford K5 emulsion diluted 1:1 with 2% glycerol warmed to 42°C under dark room conditions. Slides were dipped then placed on an ice cooled tray, in the presence of silica gel, to dry for 2 hours, then boxed and stored at 4°C until development. Developing and fixation was carried out using Kodak D19 developer and Kodak Unifix on days 2, 3 ,4 and 6 after emulsification. Slides were warmed to room temperature, immersed in D19 developer for 5 minutes, rinsed in water, then immersed in Unifix solution for a further 5 minutes, rinsed once more in water then allowed to dry.

Staining of Slides

Staining was performed using haematoxylin and eosin by immersing slides in haematoxylin solution for 1 minute, washing in water, dipping once in acid alcohol solution followed by a 3 minute immersion in Scott's tap water substitute, rinsed again in water, 20 seconds immersion in eosin stain, rinsed again in water then dehydrated in 70%, 80%, 90%, and 100% ethanol by dipping in each 10 times consectutively. Mounting covers were applied with DPX mounting media.

Results

In the PMSG treated ovaries there is widespread follicle development and high levels of expression of the transcript of interest throughout the granulosa cell population (figure 4.10). No hybridisation of the antisense probe is visible (figure 4.11). Ovaries taken from pregnant mice in the last trimester of gestation containing multiple mature corpus lutei do not demonstrate expression of this transcript other than in adjacent antral follicles. There is no detectable expression in the mature corpus luteum (figure 4.12). No hybridisation of the antisense probe is visible in any tissue (figure 4.13). In the normal adult ovary with no exogenous hormone treatment hybridsation can be seen on granulosa tissue within antral follicles (figure 4.14), again no hybridisation is visible with the antisense probe (figure 4.15).

Conclusion

The SAGE tag CAGTCAATAC relates to a variable length non coding transcript which shows a tissue and temporal specific expression pattern within granulosa tissue. Highest levels of expression are found within the gonadotrophin stimulated mature antral follicle prior to the LH surge. Exogenous hCG administration leads to rapid downregulation of expression.

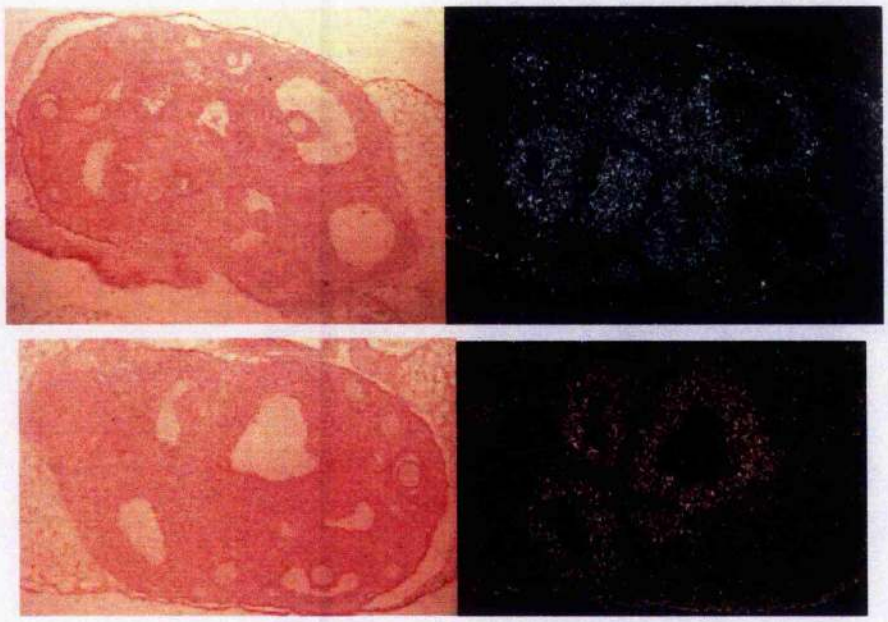


Figure 4.10 Light field and dark field views of day 20 PMSG treated mouse ovary harvested 48 hours after PMSG administration and hybridised with sense probe

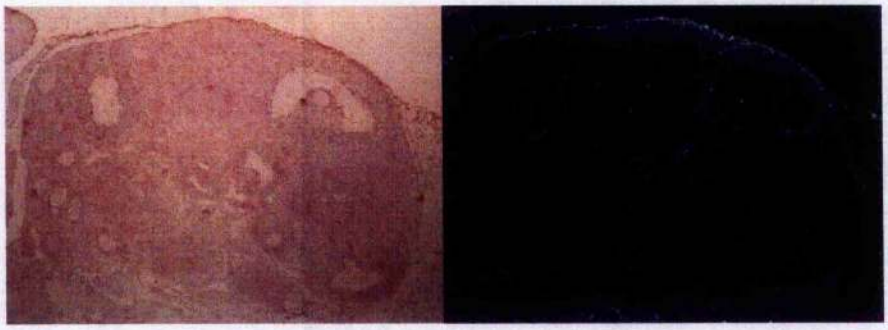


Figure 4.11 Light field and dark field views of day 20 PMSG treated mouse ovary harvested 48 hours after PMSG administration and hybridised with antisense probe.

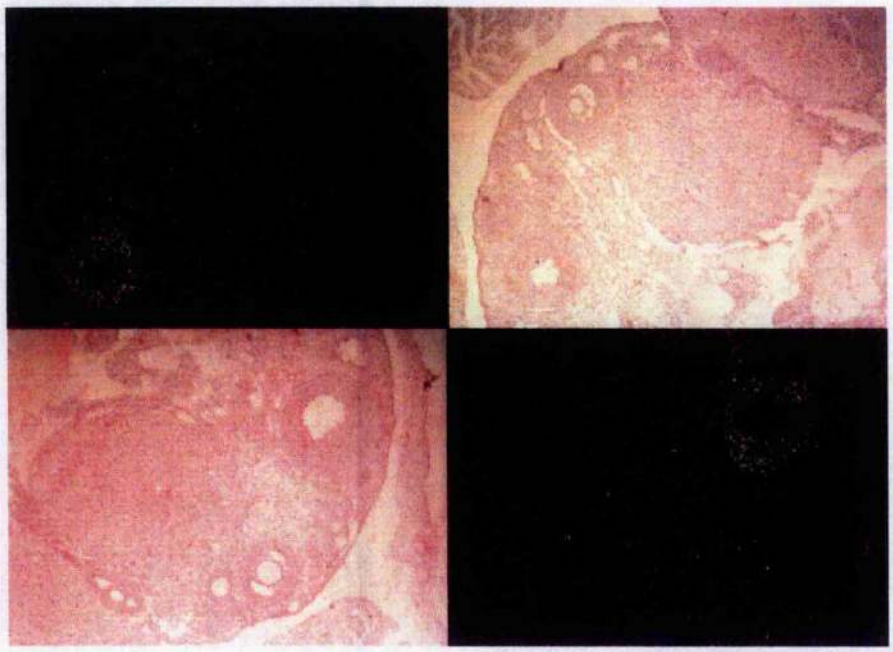


Figure 4.12 Normal adult mouse ovary during last trimester of gestation hybridised with sense probe.

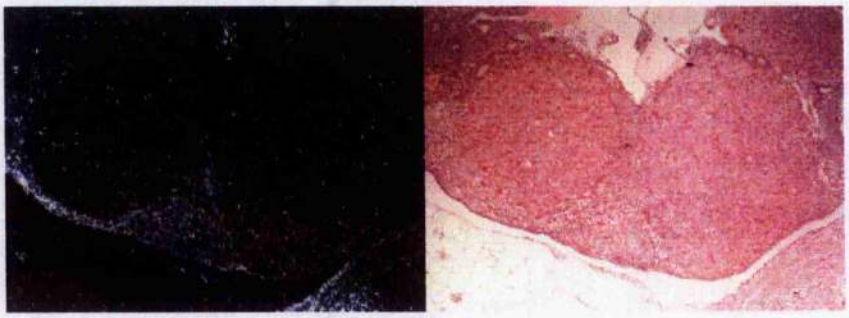


Figure 4.13 Normal adult mouse ovary during last trimester of gestation hybridised with antisense probe

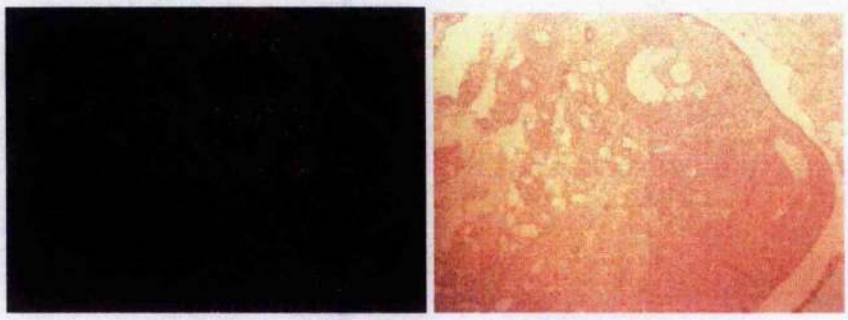


Figure 4.14 Adult mouse ovary hybridised with sense probe

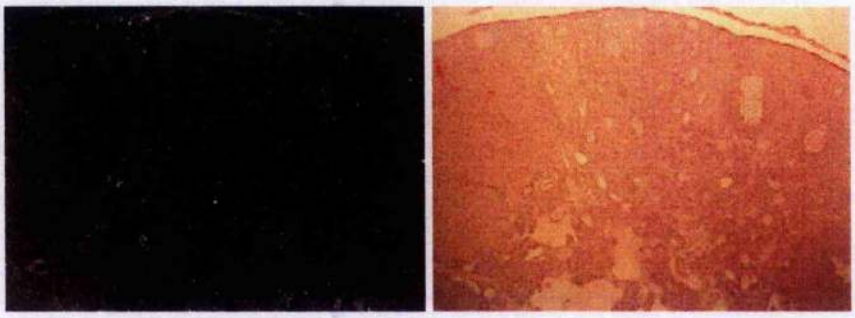


Figure 4.15 Adult mouse ovary hybridised with antisense probe

Chapter 5

Discussion

Chapter 5: General Discussion

The application of SAGE alone and in combination with other methods has been proven to provide an accurate and representative method of gene expression analysis in a number of *in vivo* and *in vitro* systems. The objective of the work presented in this thesis has been to apply the technique of serial analysis of gene expression to identify the genes associated with the molecular changes of luteinisation. This objective has, largely, been successfully achieved. The use of SAGE is effectively an efficient method of producing an EST library from a particular tissue. Each SAGE tag represents one polyadenylated RNA molecule and contains enough information to uniquely identify that molecule without the laborious cloning and sequencing required to generate true EST libraries.

The frequently quoted limitations of SAGE are based on the required labour input in comparison to microarray and the sequencing costs to generate libraries capable of analysing low expression genes. One intrinsic flaw is the possible generation of bias within a library either by preferential amplification of specific ditags during the PCR reactions, or loss of some ditag populations through melting of the double strand while handling. Ligated ditags serve as template for PCR amplification and errors generated by the DNA polymerase early in the reaction will be continuously replicated during the subsequent steps. In order to minimise this possibility our protocol adopts the use of nested primers for PCR amplification. The issue of ditag loss during handling, and in particular gel extraction, is addressed by adopting low temperature protocols. The electroelution lavage protocol need not be carried out at any higher than room temperature, and indeed can be performed at lower temperatures simply by adding chilled buffer should the operator consider it necessary.

I would also add one further criticism, that traditional data analysis of SAGE libraries, based on the generation of statistically differentially expressed tags (as performed here using the Chi squared test), takes no account of actual protein product levels, efficacy of gene product action, or gene product role within the cell. In the case of the SAGE libraries presented here many of the highly abundant or differentially expressed transcripts represent housekeeping or protein synthesis related genes (Table 3.1), while important members of the

signal transduction pathways (discussed later) are found expressed at much lower levels. This means that effective analysis of SAGE data still requires using manual curation rather than relying on the currently available bioinformatics tools if we are not to overlook frequently important transcripts expressed at lower levels.

In defence SAGE is the only technique available which has the potential to allow the simultaneous quantative analysis of the large number of genes studied here (40,248 unique transcripts) and has the ability to do so without prior knowledge of transcript sequence or expression pattern. The quantification of tag expression provides a direct measurement regarding the expression level of the corresponding transcript, this measurement is represented by absolute tag counts based on random sampling of transcripts, uniquely allowing data comparison between different experiments if adjustment is made for the total number of tags sequenced. SAGE thus has the theoretical ability to characterise the entire cellular transcriptome providing sufficient transcripts are sequenced.

5.1 Validation of SAGE method

A number of previous studies have examined the mechanisms of luteinisation (Espey and Richards 2002, Rajapaksha et al 1997, Oksjoki et al 2001, Rodgers et al 1986, Hsieh et al 2003 and Park et al 2004) but have been restricted to a limited number of genes and have generally studied the expression patterns within the whole ovary rather than within specific cellular compartments. Where comparable, the results from previous studies correlate well with results reported here. From previous work and from this study it is clear that there are many genes involved in the functional regulation of granulosa cell differentiation. It is also clear that some of the genes expressed may only contribute in a temporal fashion and expression may only be required for a short, but crucial time point. In this study the application of SAGE was restricted to time points before and after the induction of ovulation with hCG. While SAGE is unrivalled in its ability to detect unexpected and novel transcripts, even at low levels of expression, it is time-consuming and relatively expensive. This imposes a

practical limitation on its use but nonetheless the data produced has yielded a comprehensive list of genes undergoing regulation during luteinisation. This data will, serve as a baseline measurement for further studies into granulosa cell differentiation. For example, and as demonstrated in this thesis, a more complete analysis of transcripts likely to be of interest can easily be performed by studying their expression throughout the entire time course of follicle development and luteinisation using real time PCR.

This study has characterised the expression of genes novel to luteinisation, but also confirmed the expression pattern of many transcripts already known to be associated with this process. This in itself helps to validate the SAGE process. Table 5.1 lists a number of genes classically associated with either the granulosa or luteal phenotype whose changes in expression seen in the SAGE data match with previous work. Other studies (Espey and Richards 2002, Rajapaksha et al 1997, Oksjoki et al 2001, Rodgers et al 1986, Hsieh et al 2003 and Park et al 2004) examining the molecular changes during luteinisation have revealed a number of genes which are now known to be differentially expressed during this process. For example, several membrane binding and communication related components such as clusterin, annexin A2, and the gap junction membrane channel proteins (connexins) are known to show fluctuations in expression levels during granulosa cell development and luteinisation. The overwhelming level of Cx 43 expression in the PMSG SAGE library supports current thinking that this is the primary means of intercellular communication between granulosa cells and reinforces the hypothesis of a functional granulosa cell syncitium throughout folliculogenesis (Ackert et al 2001). Expression of Cx 43 decreases markedly after induction of luteinisaton, as shown previously (Itahana et al 1996), although it may continue to be expressed in the developing corpus luteum (Khan-Dawood et al 1998). In addition the steroidogenic enzymes such as aromatase, StAR, and cholesterol side chain cleavage (Espey and Richards 2002, Rajapaksha et al 1997, Rodgers et al 1986, Pescador et al 1996), genes involved in extracellular remodelling including ADAMTS-1, cathepsin L (Espey and Richards 2002, Oksjoki et al 2001), and a variety of signalling molecules including epiregulin and secreted frizzled related protein 4 (Hsieh et al 2003,

Park et al 2004) all display good correlation between SAGE and previously published data, confirming the reliability and accuracy of the SAGE method.

In approaching this I intend to break down this discussion into four main themes, focusing mainly on novel transcripts and pathways. First, approaching those genes involved in signalling and communication, secondly those responsible for granulosa cell survival, growth and regulation of proliferation, thirdly genes implicated in controlling cellular differentiation and finally I will consider some novel transcripts which may be involved in the remodelling of cellular and follicular structure.

Other areas of granulosa cell function will be mentioned only briefly, if at all, purely because the SAGE data largely supports current thinking without introducing a substantial quantity of new information. For example the steroidogenic functions of the granulosa cell are well documented and expression data presented here matches well to that previously presented elsewhere and consequently will not feature in this discussion.

Table 5.1 Comparison of SAGE transcripts with known expression profiles

Gene	Change in gene expression level during luteinisation according to SAGE data	References
Steroidogenesis		
Aromatase	Decrease	Ronen-Fuhrmann et al 1995, Richards 1994, O'Shaughnessy et al 1990, Snadhoff and Maclean 1996, Ronen-Fuhrmann et al 1998, Rodgers et al 1986.
Cholesterol side chain cleavage	Increase	
StAR	Increase	
Scavenger receptor class B member 1	Increase	Li et al 1998
11β HSD2	Decrease	Tetsuka et al 1997
Ferredoxin	Increase	Rodgers et al 1986
17β HSD1	Decrease	Rodgers et al 1986
Low Density Lipoprotein Receptor	Increase	Golos et al 1986, Golos and Strauss 1987
17β HSD4	Increase	Brown 2004
Signaling		
LH receptor	Increase	Zeleznik et al 1974
GDF 9	Decrease	Elvin et al 1999, Hayashi et al 1999, Vitt and Hseuh 2002
Epiregulin	Increase	Park et al 2004
Connexin 43	Decrease	Acket et al 2001, Itahana et al 1996
Progesterone receptor	Increase	Natraj and Richards 1993
Early growth response 1	Increase	Espey et al 2000
Serum glucocorticoid regulated kinase	Increase	Alliston 2000, Gonzalez-Robayna 1999
Remodelling		
ADAMTS 1	Increase	Robker et al 2000, Espey et al 2000
ADAMTS 4	Increase	Abbaszade et al 1999
Cathepsin L	Increase	Robker et al 2000
VEGF	Increase	Schams et al 2001
Collagen type IV	Increase	Yamada et al 1999
Integrins	Increase	Yamada et al 1999
Syndecan 1	Increase	Ishiguro et al 1999
Others		
Frizzled 1	Increase	Richards et al 2002
Secreted frizzled related protein 4	Increase	Hsieh et al 2003
CAAT enhancer binding protein beta (C/EBPβ)	Increase	Sirois and Richards 1993
Cell cycle inhibitor P21	Increase	Robker and Richards 1998

5.2 Cell signalling- novel components expressed during luteinisation

The established endocrine role of the granulosa cell has already been described in chapter 1. The essential role of steroid and peptide hormones in regulating pituitary function is supported by the expression changes of the inhibin βB and α subunits, follistatin, and the decreased oestrogen and increased progesterone production caused by change in the aromatase and cholesterol side chain cleavage enzymes. The gonadotrophin receptors are expressed in both libraries, with LH showing a two-fold upregulation in response to hCG administration.

Numerous paracrine factors are expressed at relatively lower levels than the endocrine components, for example, kit ligand, responsible for oocyte growth and maturation, antrum formation and theeal proliferation (Driancourt et al 2000), is expressed in the PMSG-treated library and downregulated by hCG. Equally, components of the IGF (IGFBP 4), TGF (TGFBP 3, TGFβR I and III), BMP (BMP 3, BMP 15, BMPR1A) and FGF (FGF 15, FGFR) systems, GDF 9, CTGF, EGFR, AmhR type II and early response factors such as epiregulin have established functions within the follicle, as outlined in chapter 1, and show expression within one or both libraries.

Novel transcripts, never previously associated with granulosa cell function, include several components of G protein coupled receptor signalling. Specifically G protein coupled receptor family C group 5, member C (GPRC5c), G protein coupled receptor 85 (GPR 85) and G protein coupled receptor 27 (GPR 27) are down regulated by hCG with G protein coupled receptor 48 (GPR 48), regulator of G protein signalling 2 and G protein coupled receptor associated sorting protein 2 showing significant upregulation by hCG. The functions of the G protein receptors listed above are unknown. GPRC5C is expressed in neurological tissue and postulated to mediate the cellular effects of retinoic acid. (Robbins et al 2000). GPR 85 is highly conserved across mammalian species, expressed in brain, spleen and placenta in man (Hellebrand et al 2001) but has only been identified in the brain of the mouse. GPR 48 is expressed in the kidney, placenta, brain and heart, and has been detected as early as 7d post coitus in mouse (Loh et al 2001). GPR 27 is highly conserved

between mammalian species and abundantly expressed in neural tissue (Matsumoto et al 2000).

Apart from a common expression pattern in neural tissue the paucity of information about these particular transcripts limits our ability to hypothesise a function in the granulosa cell. However the expression patterns would suggest a capacity for functional significance. GPR 48, for example, undergoes 6-fold upregulation in response to hCG and GPR 85 a 10 fold downregulation.

GPR5C5 undergoes an 11 fold reduction in expression level following hCG administration. In vitro transfection experiments have shown than GPRC5C is expressed at the cell surface and is inducible by retinoic acid (Robbins et al 2000). The ovary is known to express retinoic acid receptors (Zhuang et al 1994) and retinoic acid is known to be required for normal reproductive function, affecting diverse functions of ovarian tissue such as follicular development (Segweigert and Zucker 1988), steroidogenesis (Graves-Hogaland et al 1988) and oocyte maturation (Ikeda et al, 2005) as well as exerting influence on embryo development (Liu et al 1993) and the intrauterine environment (MacKenzie et al 1997). The molecular mechanisms involved in retinoic acid-mediated gene expression in the granulosa cell have not however been fully elucidated. The SAGE libraries have revealed a number of genes associated with retinoic acid metabolism not previously identified in the granulosa cell. In addition to GPR5C5, transglutaminase 2, C polypeptide and MAPK8 interacting protein 3, essential for early embryonic neurogenesis (Xu et al 2003) are both genes inducible by retinoic acid and upregulated by luteinisation while nuclear receptor coactivator 4, an enhancer of retinoic acid mediated transcription (Heinlein and Chang 2003), eukaryotic translation initiation factor 4 gamma 2, a mediator of retinoic acid induced differentiation (Yamanaka et al 2000) and chromobox homolog 2, thought to have a role in controlling access to retinoic acid response elements of several genes (Core et al 1997), are also all significantly upregulated.

GPR 27 and GPR 85 are members of the super-conserved receptor expressed in brain (SREB) family, SREB1 and SREB2 respectively (Matsumoto et al 2005). SREB2 is the most conserved receptor throughout vertebrate evolution

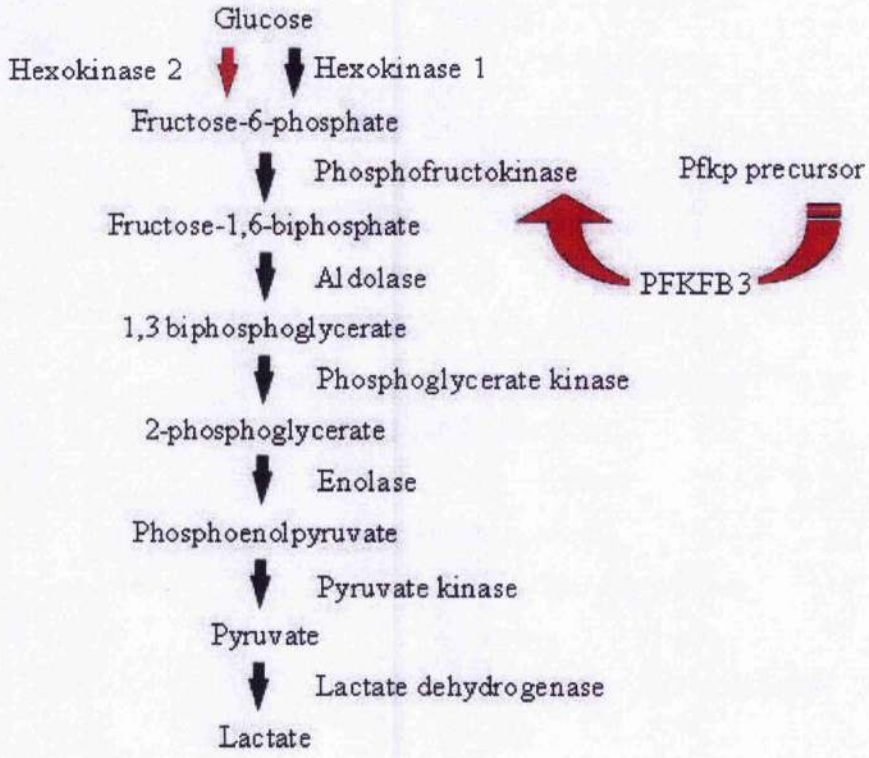
(Matsumoto et al 2000) indicating an essential function within the vertebrate group. SREB members have been identified in both the neural systems and genitalia and SREB2 is known to be temporally and spatially expressed during embryonic development and in adult brain (Hellebrand et al 2001). This suggests a specific functional role in neural development, which has been tentatively suggested to be in the maintainence of neural plasticity (Matsumoto et al 2005). Although no progress has been made towards identifying the ligands for GPR27 and GPR 85 their expression during luteinisation warrants further investigation. In addition there are cytokine related transcripts, B-cell stimulating factor 3 and stromal cell derived factor receptor 1, upregulated by hCG supporting the notion liking ovulation to an inflammatory reaction, and regulatory proteins such as suppressor of cytokine signalling 2 and 5, and protein regulator of cytokinesis 1.

5.3 Intracellular metabolic adaptations to follicular luteinisation

Follicular cell multiplication and differentiation are cellular events requiring energy. The granulosa cells of preantral follicles are known to utilise both the glycolytic pathway and Krebs cycle when under the influence of gonadotrophins (Roy and Terada 1999). When oxygen is limited cells switch from oxidative phosphorylation to anaerobic glycolysis. The hypoxic response at the cellular level is manifest by the increased expression of genes coding for glycolytic enzymes. The oocyte is known to require the expression of glycolytic enzymes in adjacent cumulus cells to supply pyruvate, obligatory for resumption of meiosis and oocyte maturation (Downs and Hudson 2000, Rose-Hellekant et al 1998). Energy production in the antral follicle, prior to the LH surge, is regulated by the oocyte in a developmentally coordinated manner (Sugiura et al 2005). Following the LH surge, during the period of oocyte maturation, cumulus cells are unresponsive to oocyte secreted factors (Sutton et al 2003, Sugiura et al 2005). FSH and LH have been reported to activate glycolysis in cumulus cells while EGF, IGF-I and TGFβ have been hypothesised to do so on the basis of activity in other tissues (Roy and Terada 1999). This work is, however, based on preantral follicles using *in vitro* culture systems and the influence of

gonadotrophins on the glycolytic pathway varies with the maturation status of the follicle. The mechanisms responsible for the maintainence of the glycolytic state, post oocyte maturation, have not yet been identified.

Figure 5.1 Glycolytic pathway in granulosa cells during luteinisation



The red arrows indicate enzymes upregulated by luteinisation. PFKFB3 is a potent activator of glycolysis, controlling expression of the rate limiting enzyme (phosphofructokinase) in this pathway. Hexokinase 2 upregulation couples glycolysis and oxidative phosphorylation

In both SAGE libraries high levels of the transcripts for hexokinase 1, GAPDH, aldolase, tirosephosphate isomerase, enolase, phosphoglycerate kinase, and lactate dehygrogenase (isoforms 1 and 2) can be found (table 5.2 figure 5.1). The rate-limiting enzyme in the glycolytic pathway is fructose-2,6-biphosphate (Fru-2,6-P2), a potent activator of glycolysis which is capable of exerting control over the rate of glucose utilisation (Kawaguchi et al 2001, Hue and Rosseau 1993, Okar and Lange 1999, Pilkis et al 1995). The enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB) controls the synthesis and

degradation of Fru-2,6-P2. Among the PFKFB mammalian isoforms PFKFB3 has the highest activity and is found highly expressed in transformed cells (Chesney et al 1999, Sakakibara et al 1997) implicating it as having some responsibility for the high glycolytic rate found in neoplastic cells. This particular isoform undergoes significant up-regulation following hCG administration, indeed while expression has not been detected by the PMSG SAGE library, 10 transcripts have been sequenced in the hCG library.

The induction of PFKFB3 has been shown to occur in response to a hypoxic cellular state, compatible with an adaptive cellular response to enhance glycolysis during periods of oxygen deficiency (Minchenko et al 2002), but also in response to certain mitogens, growth factors and cytokines (Hue and Rousseau et al 1993). PFKFB3 has been found to be highly induced by hypoxia in various cell lines and *in vivo* (Minchenko et al 2003) and regulation of the PFKFB family is controlled by hypoxia inducible factor 1 (HIF-1) (Minchenko et al 2003).

Table 5.2. SAGE expression of glycolytic enzymes

Gene	Unigene	Tag count	
		PMSG	hCG
Hexokinase 1	196605	61	33
Hexokinase 2	255848	1	12
Phosphofructokinase	1166	149	70
Fructose-2,6-biphosphatase (PFKFB)	19669	0	10
Aldolase	16763	20	25
Phosphoglycerate kinase	188	25	23
Enolase	70666	32	18
Pyruvate kinase	216135	1	5
Lactate dehydrogenase 1 A chain	29324	38	31
Lactate dehydrogenase 2 B chain	9745	18	15
Glyceraldehyde 3-phosphate dehydrogenase	5289	23	24
Triosephosphate isomerase	4222	24	12

The establishment of a rarefied oxygen gradient across the follicle from the peripheral to central granulosa cells during follicular expansion (Tsafriri et al 1976) generates the hypoxic requirement for HIF-1 induction. HIF-1 consists of α and β subunits, the β subunit is constitutively expressed, but α subunit stabilisation is hypoxia dependant (Semenza GL 1999). HIF-1α is expressed in

both SAGE libaries and upregulated by hCG administration (2.4 fold). Although hypoxia plays a major role in the activation and stabilisation of HIF-1 the amplitude of this response can be modulated by a number of growth factors including EGF, IGF-1, FSH, androgens, TNFα, and TGFβ acting through the Ras/Raf/MAPK and PI3/PKB signalling cascades (Bardos and Ashcroft 2005). These observations suggest that microenviromental hypoxia and paracrine/endocrine signals both contribute to the high rates of granulosa cell glycolysis.

Another aspect of glycolysis is the multifunctional roles served by some of the glycolytic enzymes. Hexokinase irreversibly catalyses the phosphorylation of glucose, the first step of glycolysis. Hexokinase 1 (HK1) is highly expressed in both libraries, but hexokinase 2 (HK2) is only represented by a single tag in the PMSG library, and is significantly upregulated (12 transcripts) in the hCG library. Mammalian HK2 localises to the mitochondria and uses mitochondrial ATP to phosphorylate glucose, coupling the glycolytic pathway with oxidative phosphorylation (Golshani-Hebroni and Bessman 1997). HK2 has been implicated in having involvement in the transcriptional regulation coordinating glycolysis and in the enhanced glycolysis found in malignant and immortalised cells under aerobic conditions (Arora et al 1990). The upregulation of HK2 may therefore represent a hypoxia independent method of maintaining the glycolytic pathway in the granulosa cell following the LH surge and a means of linking the glycolytic pathway to mitochondrial oxidative phosphorylation. In addition mitochondrial HK activity has been shown to be required for growth factor induced cell survival (Kim and Dang 2005), suggesting a role for HK2 in the apoptotic pathway.

The induction of HIF-1 in response to FSH occurs via the PI 3-kinase pathway and is known to be required for the up regulation of LHR and inhibin α (Alam et al 2004). The importance of HIF-1 for follicular and luteal development is further demonstrated by the subfertile phenotypes of HIF-1β knockouts (Le Provost et al 2002) and by the major role the HIF family are known to play in the regulation of genes required for angiogenesis (Wang et al 1995, Zhang et al 2003) and, in hypoxic conditions, in the suppression of proliferation and

promotion of anti-apoptotic mechanisms (Leek et al 2005). The activation of HIF-1 is a critical response in neoplastic cells undergoing hypoxic and nutritional stress allowing adaptation to a suboptimal microenviroment. In the granulosa cell these adaptations are likely to include PFKFB3 to enhance cell survival by allowing metabolic adaptation to the hypoxic conditions created by the rapid follicular expansion and the avascular nature of the granulosa compartment. (Wenger and Gassmann 1999, Semenza 2000).

Another transcription factor known to be upregulated by hypoxic conditions and significantly up regulated in the hCG SAGE library is early growth response 1 (Egr-1). This is a zinc finger transcription factor involved in a number of early responses to numerous stimuli (Cummins and Taylor 2005). Hypoxia induced Egr-1 activity is mediated through a PKC pathway and has been shown to be an important event in contributing to vascular remodelling (Semenza GL 2000, Yan et al 1999). The induction of Egr-1 by gonadotrophins is well documented (Espey et al 2000) and its essential role in female fertility has been extensively discussed elsewhere (Espey et al 2000, Russell et al 2003). The activation of such transcription factors is a critical response in granulosa cells ensuring the enhanced transcription of a number of genes that increase oxygen and nutrient supply.

Serum/glucocorticoid related kinase (sgk) is a serine/threonine kinase and another key component of the cellular stress response. It is known to exhibit a biphasic expression pattern under gonadotrophin stimulation correlating with granulosa cell proliferation and differentiation (Alliston et al 1997) with highest levels seen in differentiated non-proliferative mural granulosa cells. This confirms the expression pattern in the SAGE libraries, which show a 6.8 fold induction of sgk following luteinisation. Sgk plays a critical role in maintaining cell survival, is up regulated by multiple different stress stimuli (Leong et al 2003), and mediates survival signals via the phosphorylation and negative regulation of the pro-apoptotic forkhead transcription factor FKHRL1 (Brunet et al 2001). In addition, stimulation of sgk by glucocorticoids has been shown to cause cell cycle arrest, the opposite of the effect of serum induction which leads to cellular proliferation. These opposing effects indicate a dichotomy of function

for sgk depending on its localisation within the cell with cytoplasmic compartmentalisation in cells undergoing glucocorticoid stimulated growth arrest and nuclear localisation necessary for cell proliferation. Forced retention of sgk within the cytoplasmic compartment is sufficient to suppress cell growth (Buse et al 1999). The granulosa cell is known to modulate intracellular glucocorticoid levels, generating increased glucocorticoid levels around ovulation. The downregulation of 11βHSD2, which catalyzes the conversion of cortisol to the inactive metabolite cortisone will increase exposure to cortisone and under this influence the up-regulation of sgk may represent a mechanism contributing to the cessation of granulosa cell proliferation in the preovulatory follicle.

Glutathione transferases (GSTs) are induced by and participate in cellular defences against the oxidative damage during periods of cell stress (Hayes et al 2005). Murine GST alpha 4 is induced *in vivo* by tumor necrosis factor alpha (TNFalpha), interleukin-6 (IL-6), and epidermal growth factor (EGF) (Desmots et al 2002), and is significantly downregulated by luteinisation. These factors play crucial roles in cell survival and proliferation during cellular regeneration and GSTα4 may be considered as part of the cell survival mechanism during follicle growth. GSTα4 serves to regulate 4-hydroxynonenal (HNE) intracellular levels (reviewed in Awasthi et al 2004), a toxic end product of lipid perioxidation, which affects signal transduction pathways controlling apoptosis (Kruman et al 1997), cell differentiation (Barrera et al 1991), and can modulate cell proliferation (Zarkovic et al 1993). In vitro GSTα4 transfection results in lower levels of HNE and increases proliferation while inhibiting differentiation and apoptosis (Awasthi et al 2004). These observations suggest a previously unrecognised mechanism in the granulosa cell for maintaining the undifferentiated phenotype during follicular expansion, with the observed 9-fold down-regulation of GSTα4 by hCG contributing to the cessation of cell proliferation and encouraging development of the differentiated phenotype via increased intracellular levels of HNE.

In summary we are witnessing a dynamic balance of hormonally and environmentally regulated peptide factors and metabolic changes, co-ordinately

regulating an intricate network of intracellular processes that stringently control granulosa cell metabolism and survival during luteinisation.

5.4 Cell survival: Apoptotic/survival signalling pathways

The balance between cell growth and proliferation, cell senescence, and cell death within the follicle is delicately controlled by many different pro-survival or pro-apoptotic factors. The LH surge acts to promote cell survival but local paracrine mediators are also essential for continued follicle health (discussed in section 1.8.3.6).

Transcriptional regulators of the cell cycle include the E2F transcription factor family, whose role in transcriptional control of cell proliferation has been well documented (Zhu et al 2005, Nevins 1998). Previous work has shown that overexpression of various E2F proteins will induce cell cycle progression, while dominant negative mutants inhibit cell growth (Johnson et al 1993, Dobrowolski et al 1994). E2F factor 5 (E2F-5) shows significant upregulation in the hCG library. Initially this expression appears anomalous in a cell population exiting from mitotic activity. E2F has been considered primarily as a family that promotes cell proliferation. However high E2F levels have been recorded in post mitotic cells which have exited the cell cycle and are undergoing differentiation in Drosphilia (Brook et al 1996) suggesting E2F has functions separable from its role in cell proliferation. Further studies suggest that the E2F family might actually serve to limit proliferation by repressing the transcription of growth promoting genes (He et al 2000, Zhang et al 1999) and Gaubatz et al 2000 showed that E2F5 is specifically required for G1 arrest of cycling cells. It has been suggested on the basis of expression patterns in murine epithelium that E2F2 and E2F4 participate in maintaining an undifferentiated proliferative phenotype while E2F5 is important in maintaining a terminally differentiated state (Dagnino et al 1997). It is now understood that activator and repressor E2Fs function in an opposing manner and that E2F5 functions by recruiting the pocket protein p130 to E2F regulated promoters thereby suppressing transcriptional activity and allowing cell cycle exit (Dimova and Dyson 2005). More intriguing

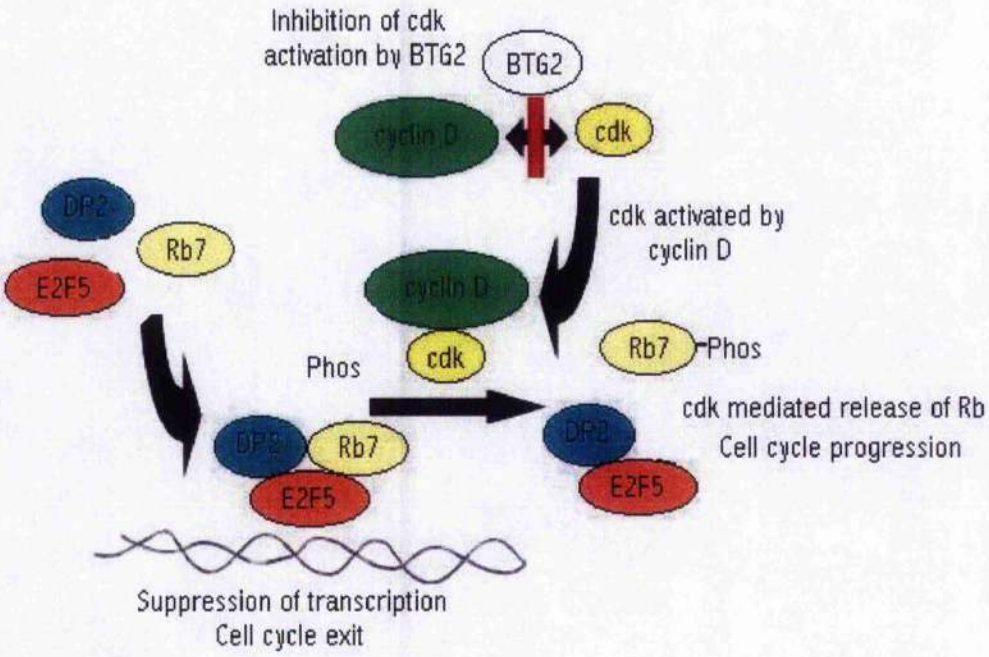
yet is the finding that mice deficient for E2F4/5 exhibit defective differentiation of numerous cell lineages in a manner which appears context specific and differs between individual cell types (Dimova and Dyson 2005).

Table 5.3 SAGE expression of components of the E2F system for cell cycle regulation

Gene	Unigene	Tag count	
		PMSG	hCG
E2F transcription factor 5	153415	0	9
Transcription factor Dp2	17977	2	13
Retinoblastoma binding protein 7	270186	7	24
Cyclin dependant kinase 4	6839	0	6
Cyclin dependant kinase 8	219645	2	10
Cyclin 1	250419	9	11
Cyclin D binding myb-like transcription factor 1	22480	0	13
Cyclin dependant kinase inhibitor 1A (P21)	195663	0	9
B Cell translocation gene 2	239605	0	12

Also significantly upregulated is transcription factor DP2, a dimerisation partner of E2F (Zhang and Chellappan 1995). Numerous alternatively spliced forms of DP2 exist with either cytoplasmic or nuclear location, suggesting that DP binding may control E2F localisation. (Dyson 1998). Binding of DP2 to E2F can directly enhance heterodimer DNA affinity and transcriptional activity (Hitchens and Robbins 2003), however binding of DP/E2F complexes to members of the retinoblastoma (Rb) family of proteins can yield a complex which actively suppresses transcriptional activity (Dyson 1998). The concomitant 3.4 fold upregulation of retinoblastoma binding protein 7 in the SAGE libraries is likely to be related to this E2F/DP2 system and requires further investigation. Known to be a core component of two co-repressor complexes it has been shown to inhibit cell growth in vitro and in vivo (Yang et al 2002). It thus provides a link between gonadotrophin stimulation and cell cycle control having previously been shown to increase expression in the ovaries of FSHβ null mice (Burns et al 2003). The precise mechanisms utilised to carry out this role in vivo have not yet been identified.

Figure 5.2 Proposed action of E2F5 and associated genes in regulating cell cycle exit in the granulosa cell



E2F5 dimerises with DP2 and retinoblastoma protein 7 to suppress transcription and induce cell cycle exit. Phosphorylation of Rb7 by cdk would allow release of E2F and result in cell proliferation. Inhibition of cdk activation by BTG2 may be an early response inhibiting cdk action and inducing cell cycle exit. All components shown here, except cyclin D, demonstrate significant upregulation in the SAGE libraries in response to hCG administration.

The expression levels of cyclin dependant kinase 4 (cdk4), cyclin dependant kinase 8 (cdk8), and cyclin G associated kinase (Cgak) are all up-regulated by hCG. Cdk4 is responsible for phosphorylation of retinoblastoma protein (pRb). This is a process thought to result in the release of E2F and the expression of genes allowing S phase entry and cell proliferation (Cobrinik 2005), increased expression in a quiescent non proliferative cell cannot easily be explained using the current models. We also have a significant increase in expression of cyclin-dependant kinase inhibitor 1A (P21), perhaps serving to counteract the activity of the cdk's. B cell translocation gene 2 (BTG2) negatively regulates cell growth

and promotes differentiation (Guehenneux et al 1997) and can do so through its effects on pRb activity. Cyclin D1 is a target of, and negatively influenced by, BTG2 (Guardavaccaro et al 2000). D type cyclins bind and activate the cyclin dependant kinases that phosphorylate pRb (Matsushime et al 1992). Inhibition of this phosphorylation will prevent the release of E2F and discourage progression of the cell cycle. Inhibition of cyclin D function by BTG2 may therefore be an early response inhibiting Rb phosphorylation and cell cycle progression. This gene is also significantly up regulated by hCG.

Although this is the first report implicating the E2F system in granulosa cell luteinisation E2F5 has previously been shown to influence FSH-R expression with ectopic expression increasing FSH-R promoter activity (Putowski et al 2001). This may function as a feedback mechanism with increasing FSH sensitivity via increased FSHR expression negating the effects of E2F5 to maintain a proliferating phenotype during rapid follicle growth. Further work is required to elucidate fully the roles played by different components of the E2F mechanism during follicular expansion and luteinisation, and in particular the reasons behind the apparently anomalous increase in cdk expression.

A number of other transcripts implicated in the control of cell proliferation and/or senescence are also included among those tags significantly upregulated. Growth arrest specific 1 (Gas1) (no expression in PMSG library, 14 transcripts in hCG library) is a membrane protein up-regulated during cell quiescence and whose ectopic expression exerts a growth suppressing effect (Del Sal et al 1992). It has also shown induction in response to endothelial cadherin and VEGF and to inhibit endothelial cell apoptosis (Spagnuolo et al 2004). Two members of the mortality factor 4 (MORF 4) family (MORF 4 like 1 and MORF 4 like 2) are significantly upregulated in response to luteinisation (no expression in PMSG library, 9 and 11 transcripts respectively following luteinisation). MORF 4 has been shown to induce replicative senescence in immortalised cells. Due to its nuclear location, and leucine zipper motif allowing DNA binding it is postulated to acts as, or interact with, a transcription factor influencing genes regulating cell cycle progression (Bertram et al 1999). Salvador is a gene influencing both cell cycle control and apoptosis in Drosophilia and in human cell lines. Loss of

Salvador leads to increased cyclin E levels and delayed cell cycle exit. (Tapon et al 2002). The SAGE tag corresponding to Salvador homolog 1 (Drosophila) undergoes 40 fold upregulation in the granulosa cell in response to hCG administration.

This multilevel regulation permits several layers of control that can simultaneously or individually converge on the same cellular component in response to appropriate extracellular signals to regulate cell survival.

5.5 Cell Differentiation and Transcription factors

A relatively small group of essential molecular signals are repeatedly used to regulate cellular development. Wingless, Hedgehog, TGFβ, Receptor tyrosine kinase/phosphatase (RTK/P) and Notch pathways are the central molecular pathways acting individually and cooperatively to coordinate the transcriptive cellular response (Gerhart 1999). Within the granulosa cell the Wnt, TGFβ, and RTK/P signaling pathways have been recorded but the influence of the Notch and Hedgehog pathways on luteal development is as yet unknown. Both Indian and Desert Hedgehog are expressed in the PMSG treated library (7 and 3 transcripts respectively) and both are down regulated by hCG (1 and 0 transcripts respectively). Both are highly conserved and known to influence gonadal development and function, regulating thecal cell gene expression during folliculogenesis (Wijgerde et al 2005). The downregulation following hCG suggests they do not have an active role in luteinisation.

Mastermind like 1 (Drosophila) (Maml1) undergoes considerable up-regulation under hCG influence, no tags are recorded in the PMSG library yet 33 transcripts are present following luteinisation. Maml1 acts as a regulator of Notch signalling (Wu et al 2004). Notch receptors modulate the development of a broad spectrum of tissues and it has been firmly established that developing embryos use Notch signalling to amplify and consolidate molecular differences between adjacent cells. Notch is involved in the regulation of cellular differentiation, proliferation and specification, (Iso et al 2003, Hoyne 2003, Kojika and Griffin 2001). Binding of membrane Notch receptors by ligand initiates proteolytic cleavage

and release of the Intracellular Domain of Notch (ICN or Ncid) which translocates to the nucleus and activates the DNA binding transcription factor CSL by displacement of co-repressors and recruitment of co-activators (Figure 5.3). These in turn regulate expression of tissue specific transcription factors that influence lineage commitment, apoptosis and proliferation (Wu et al 2002, Artavanis-Tsakonas et al 1999, Mumm and Kopan 2000). Johnson et al (2001) has previously demonstrated the presence of components of the Notch signalling pathway during folliculogenesis but recent transcript profiling has also revealed Notch expression in primordial follicles (Serafica et al 2005) while the essential role of Notch signalling in female fertility has been proven by the infertility, abberant folliculogenesis and failure of oocyte meiosis in lunatic fringe-null female mice (Hahn et al 2005). This is however the first report of differential expression of components of the Notch pathway during luteinisation.

Maml1 is a highly conserved critical component in the transcriptional activation induced by Notch signalling and is dynamically expressed at different developmental stages suggesting tight regulation and a role in the modulation of cell fate determination (Wu et al 2000 and 2004). The effects of Notch signalling have been shown to extend beyond differentiation, influencing both growth potential (Weng et al 2003) and apoptosis (Artavanis-Tsakonas et al 1999). Maml1 inhibition has been shown to result in growth inhibition and death of Notch1-induced human and murine cell lines, indicating that signals transduced by nuclear Notch 1 via Maml1 are required for growth and survival. In addition to the induction of Maml1, A Disintegrin and Metalloproteinase domain 10 and 17 (Adam10, Adam17) are also differentially expressed, both are involved in the proteolytic degradation of the Notch receptor. Adam 10 undergoes 2.2 fold upregulation and Adam 17 significant downregulation in response to hCG. Intracellular cleavage is mediated by presenilin 1 or 2, expressed at a much lower levels. A large number of other components of Notch signalling are also expressed in one or both libraries (table 5.4).

These findings expose the luteinisation process of mammalian granulosa cells as a novel site of active Notch signalling. Given that both Notch and its ligands are transmembrane proteins and that signalling only occurs between closely apposed

cells it is logical to conclude that Notch must be acting in either an autocrine manner, influencing adjacent granulosa cells, or paracrine manner, influencing thecal and/or oocyte development. The Notch signalling pathway has been repeatedly demonstrated to play a crucial developmental role in tissues where it is expressed and consequently the proposition that it is involved in modulating final follicular maturation is reasonable. One problem with an intrafollicular role is presented by the rapid follicular expansion induced by hCG, loss of cell-cell and cell-oocyte contact is an inevitable prequel to ovulation, as demonstrated by the changes in Cx43 and cell adhesion proteins. Equally the separation of the thecal and granulosa compartments by the follicular basement membrane would surely hinder thecal/granulosa interaction by this method. The role of a signalling system dependant on contact with adjacent cells to operate in rapidly disintegrating tissue structure seems incongruous. Is induction of Maml1 in granulosa cells perhaps a final effort on the part of the oocyte to maintain cell survival during ovulation? Or is Notch responsible for the differentiation within the granulosa compartment of mural and cumulus cells?

In order to understand the potential role of Notch signalling in the granulosa cell we have to glance further afield and consider the synergistic manner in which signalling pathways interact to exert transcription control. Notch interacts with both TGFβ and Wnt signalling to regulate the implementation of particular developmental programs (Jacobsen 2005, Klüppel and Wrana 2005). To understand the potential interactions we have to draw comparisons with functions in other tissue types. Notch1 is essential for early embryonic development of haematopoietic stem cells (HSC) but dispensable for later differentiation (Radtke et al 2004). Notch signalling is active in phenotypically defined HSCs but absent following differentiation (Duncan et al 2005). Prevention of Notch cleavage, CSL transcription factor binding and Maml1 activation all resulted in enhanced lineage differentiation. In the same system it was found that Wnt stimulation upregulated expression of Notch target genes and the ability of Wnt signalling via Wnt3a to inhibit differentiation is dependant on Notch signalling (Duncan et al 2005) demonstrating that HSCs undergo continuous self renewal *in vivo* with involvement of both Notch and Wnt

signalling pathways. These interactions between signalling pathways also incorporate the TGFβ superfamily. Recent work has revealed the interactions between TGFβ and Notch (Dahlqvist et al 2003, Blokzijl et al 2003, Itoh et al 2004) in the transcriptional regulation of Notch target genes and the inhibition of cellular differentiation and migration. TGFβ signalling induces the expression of Hes-1, a known Notch target gene via a CSL/Nicd pathway mediated by Smad 2 and/or Smad3 (Bolkzijl et al 2003). It is now known that Samd and Ncid proteins can interact directly and that this interaction is enhanced by TGFβ (Bolkzijl et al 2003, Klüppel and Wrana 2005). This raises the prospect of TGFβ and Notch signals combining through recruitment of Smad 3 to activate Notch target sequences. During myogenesis this integration of signals leads to inhibition of myogenic regulatory factors and thus inhibition of myogenesis (Bolkzijl et al 2003). This work is supported by the interaction of the BMP family with Notch regulating myogenic differentiation and endothelial migration (Dahlqvist et al 2003, Itoh et al 2004).

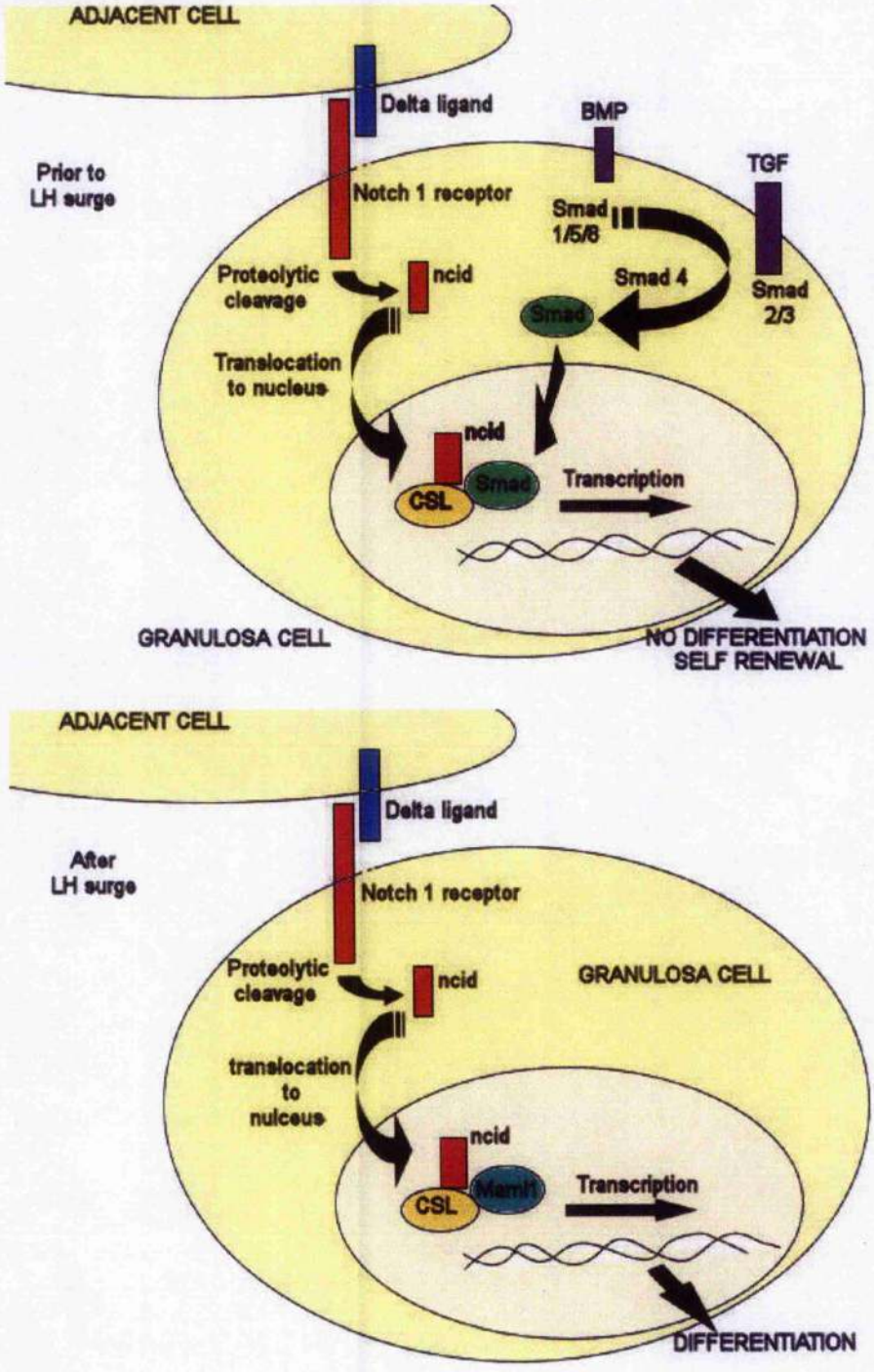
Consequently, it becomes apparent that a major biological function of Notch is the suppression of cellular differentiation and the maintainence of a self renewing cell population. It also appears to be the case that, in some tissues at least, this function is mediated by exposure to members of the TGFβ superfamily. As discussed earlier (Introduction) the oocyte produces members of this signalling family (GDF9, BMP15) during follicular development and through them regulates the differentiation of granulosa cells in a paracrine manner leading to a differentiative phenotypic gradient within the follicle. It would appear possible that oocyte-produced paracrine members of the TGFβ family could interact with Notch signalling pathways to maintain the undifferentiated proliferative granulosa phenotype most apparent in cumulus cells, and that terminal luteinisation is allowed to proceed via a change in the transcriptional activity of the Notch pathway following reduced exposure to TGFβ ligands created by a combination of follicular matrix expansion, downregulated connexin expression by the granulosa cell and reduced ligand expression by the oocyte.

This suggestion leaves us with unresolved questions as to the mechanisms by which Notch exerts its influence during the regulation of granulosa cell differentiation. Intrafollicular localisation of ligand/receptor pairs, particularly looking for oocyte expression of Notch ligands, and functional disruption of this pathway will be required to provide further insights into its role.

Table 5.4 Genes present in SAGE libraries known to be involved in or regulated by Notch signalling.

Gene	Unigene	Tag count	
		PMSG	hCG
Adam 10	3037	5	11
Adam 17	27681	23	1
Presenilin 1	998	4	3
Presenilin-like protein 4	246376	14	7
Mastermind like-1 (Drosophila)	51116	0	33
Strawberry notch homolog 1 (Drosophilia)	104898	5	0
Notch gene homolog 1 (Drosophila)	31255	4	3
Delta/notch-like EGF-related receptor	39067	1	0
Notch gene homolog 3 (Drosophila)	4945	0	1
Notch regulated ankyrin repeat protein	46539	0	1
Adaptor related protein complex 3, delta subunit	28463	10	7
Jagged 2	186146	2	0
Jagged 1	22398	1	0
Hairy and enhancer of split 5 (Drosophila)	137268	0	1
Hairy/enhancer-of-split related with YRPW motif 2	103573	6	3
Numb gene homolog (Drosophila)	4390	3	2
Recombining binding protein suppressor of hairless (Drosophila)	180561	7	3
SKI interacting protein	27094	6	7
A pending anterior pharynx defective 1A homolog (C. elegans)	268053	14	1
Cir-pending CBF1 interacting corepressor	268053	0	1
Deltex 2 homolog (Drosophila)	29343	0	2
Dishevelled, dsh homolog 1 (Drosophila)	3400	0	2
Dishevelled associated activator of morphogenesis 1	87417	0	2
Protein O-fucosyltransferase 1	216045	0	1

Figure 5.3 Suggested Notch signalling pathway in the granulosa cell before and during luteinisation



Activation of the Notch 1 receptor on the granulosa cell generates release of ncid which translocates to the nucleus. BMP/TGFβ signalling acts via Smad proteins to form a complex with CSL and ncid in the nucleus to control transcription and maintain the cellular phenotype. Following luteinisation the withdrawal of the TGFβ influence and complexing of ncid/CSL with Maml1 changes the transcriptive response and allows cellular differentiation.

5.6 Cellular and follicular remodelling: Angiogenic and cytoskeletal remodelling

Angiogenesis

The high vascularity required by the corpus luteum necessitates the rapid development of a vascular system generated by the invasion of thecal microvessels and extensive angiogenesis. The angiogenic response is associated with changes in cellular adhesive interactions exemplified by the well documented changes in integrin β expression, the secretion of proteolytic enzymes and the remodelling of the adjacent ECM as previously discussed (section 1.8.3.7). Endothelial cell migration through the follicular basement membrane requires the local production of angiogenic factors, endothelial cell proliferation, orientation, differentiation, establishment of the basement membrane and lumen formation all of which eventually leads to the formation of intact microvessels.

Recent studies suggest that angiogenesis is mediated by 3 important ligand/receptor systems, namely VEGF/VEGFR, angiopoietins/Tie receptors and ephrins/Eph receptors (Yancopoulos et al 2000). The VEGF system has been well documented in luteinising granulosa cells, the angiopoietin and ephrin mediated systems less so. Components of all 3 mechanisms are found within the SAGE libraries with angiopoietin like 4 (Angptl4) and ephrin B class 2 both significantly upregulated by hCG administration. Ephrin B2 is an endothelial marker suggested to be involved in the formation of the arterial muscular wall in adults (Yancopoulos et al 2000). It serves to distinguish arteries from veins before any structural, physiologic, or functional distinctions can be made (Urness et al 2000). Upregulation prior to ovulation suggests a role marking the early development of the nascent corpra luteal arteriolar system.

Angiopoietins are major players in the formation and stabilisation of new blood vessels (Yancopoulos et al 2000). Angptl4 has recently been implicated as an angiogenic mediator in pathologic processes, to exert anti-apoptotic effects specifically on endothelial cells, and to function as a secreted protein likely to have paracrine effects (Hermann et al 2005). The expression pattern of Angptl4

is limited to specific tissue types (inflamed synovium, kidney, liver and adipose tissue) and induction of its expression has been recorded primarily in neoplastic and inflammatory tissue (Hermann et al 2005), expression in the granulosa cell not previously having been recognised. Expression and secretion of Angptl4 from luteinising granulosa cells could therefore provide a paracrine angiogenic signal to promote and stabilise blood vessel formation within the developing CL.

In addition to the ephrin/angiopoietin/VEGF mechanisms we have identified other genes significantly differentially expressed within the SAGE libraries with known functions related to angiogenesis and endothelial remodelling.

Blood vessel epicardial substance (bves) is significantly upregulated following luteinisation, not showing expression in the PMSG treated library. It is a transmembrane protein thought to have involvement in cellular adhesion. Expressed during embryogenesis and in specific regions of developing epithelium (Osler and Bader 2004) it has never previously been detected in ovarian tissue. It is one of the first adhesion proteins to traffic to points of cell-cell contact in forming epithelium (Wada et al 2001) and consequently is postulated to have a role in cell development and orientation through its function in morphogenesis. Its expression was first recorded during coronary blood vessel development in mesenchymal cells recruited to the blood vessel wall (Reese et al 1999), a unique occurrence in the developing embryo since the endothelial cells are usually derived from the endothelial sheet connected to the endocardium rather than local mesenchyme (Coffin and Poole 1988). This raises the intriguing possibility that it may be involved in the morphogenetic organisation of developing capillary structures within the new CL through the recruitment of mesenchymal cells to the vessel walls.

Fibroblast inducible growth factor 14 (Fn 14), undergoes a 4.3 fold upregulation after hCG and functions as a TNF-related weak inducer of apoptosis (TWEAK) receptor. TWEAK promotes angiogenesis and endothelial migration (Jakubowski et al 2002, Wiley et al 2001) and has been shown to stimulate vascular formation in vivo (Lynch et al 1999). Interestingly TWEAK retains a functional ambiguity, promoting endothelial cell survival and regulating endothelial cell proliferation, migration, and morphogenesis by modulating the

response to bFGF and VEGF. In conjunction with bFGF TWEAK demonstrates pro-angiogenic behaviour but inhibits the endothelial cell morphogenesis induced by VEGF suggesting that its role regulating angiogenesis is dependant on the local microenviroment (Jakubowski et al 2002).

Cytoskeletal elements

Several cytoskeletal associated transcripts are differentially regulated during luteinisation including actin, vinculin, cofilin, tubilin and tropomyosin. It is known that gonadotrophins regulate structural gene expression in granulosa cells (Sasson et al 2004, Grieshaber et al 2003) and cytoskeletal remodelling during luteinisation is likely to be an essential part of the movement and morphological development of the cells. One transcript does show an unexpected upregulation. Vimentin is a cytoplasmic intermediate filament expressed in a variety of mesenchymal cell types during development (Evans 1998). Vimentin undergoes a dramatic 48-fold upregulation following hCG administration, yet appears to have little effect on the organisation of cytoplasmic structures other than filament associated proteins (Evans 1998). The explanation for such marked upregulation is found instead in the role of vimentin in lipid metabolism, vimentin filaments form structures around nascent lipid droplets (Franke et al 1987) and have been suggested to have involvement in the transport of cholesterol required for steroidogenesis (Almahbobi et al 1992). In cell culture there is a striking correlation between the presence of a vimentin If network and the ability of the cell to utilise lysosomal cholesterol. (Sarria et al 1992). However vimentin null mice appear phenotypically normal and can reproduce without obvious deficiencies suggesting that even this function has considerable redundancy (Colucci-Guyon et al 1994).

5.7 Genes with a poorly defined role in luteinisation

In addition to confirming changes in gene expression previously reported or predicted, the SAGE data set reported here also identifies a number of genes of interest that have not previously been linked to the process of early luteinisation.

This list is made up of genes with unknown function and genes with known function but with no previous association to granulosa luteinisation. Genes with known function and differentially regulated during luteinisation include syndecan-1, secreted phosphoprotein 1 (Spp1), secreted acidic cysteine-rich glycoprotein (SPARC), prosaposin, and vanin 1.

Syndecan-1 is a heparin sulphate-rich integral membrane proteoglycan, and it is expressed in a developmental and cell type-specific pattern (Bellin et al 2002), but it has not previously been indentified as having a role in folliculogenesis or luteinisation. Syndecans have major roles as matrix and cell surface receptors, coreceptors for growth factor signalling, internalisation receptors and soluble paracrine effectors (Bellin et al 2002). In addition, syndecan-1 appears to be capable of independent signalling and may play a role in regulation of Wnt signalling (Bellin et al 2002). Syndecan-1 is not expressed in the PMSG treated library but shows a high level of transcript expression after hCG administration. Syndecans have well-established involvement in the regulation of cytoskeletal organisation (Bellin et al 2002) and a likely function of syndecan-1 in the luteinising follicle is in the regulation of cytoskeleton assembly.

Spp1 (also known as osteopontin or Eta-1) is among the most highly up-regulated tags after hCG administration. It is a multifunctional protein expressed in various cell types and involved in a number of physiological and pathological processes including biomineralisation, inflammation, leukocyte recruitment, cell survival, tissue repair, cell proliferation and proliferation of vascular smooth muscle cells (Mazzali et al 2002). It is possible therefore that Spp1 has multiple functions during luteinisation. For example, it may act as a survival factor preventing onset of apoptosis during the critical phase of ovulation and luteinisation. Equally the effects on vascular smooth muscle suggest a possible role in the angiogenic process that accompanies formation of the corpus luteum.

Prosaposin is expressed at a medium level in the antral follicle but shows a 6-fold upregulation after hCG administration. The protein is either secreted or acts as a precursor of smaller saposins, and it has been shown to have diverse functions including involvement in the MAPK and Akt signalling pathways and maintainence of cell growth, differentiation and survival (Morales et al 2000).

The likely role of prosaposin in development of the corpus luteum is uncertain but may, again, relate to overall function as a survival factor.

Vanin 1 is a glycosylphosphatidylinositol-anchored cell surface molecule involved in thymic and gonadal development (Bowles et al 2000, Aurrand-Lions et al 1996). As with Spp1, it is also highly up regulated in granulosa cells after hCG administration. Vanin 1 is expressed specifically in the Sertoli cells of the developing foetal gonad, and it has been suggested that it may be involved in the migration of mesenchymal cells from the mesonephros into the developing gonad (Bowles et al 2000). The likely function of vanin 1 in the developing corpus luteum is unclear but, by analogy with developing thymic and gonadal tissue, it is possible that it may be involved in the cell migration that occurs early in corpus luteum formation to integrate both thecal and endothelial cells into the developing tissue.

Secreted acidic cysteine-rich glycoprotein (SPARC, osteonectin, basement membrane protein 40) is a highly expressed transcript which undergoes a 7-fold up regulation during luteinisation. SPARC is expressed *in vivo* where cells are undergoing proliferative or reorganisational activity (Lane and Sage 1994) and it has previously been identified in follicular granulosa cells after the LH surge (Bagavandoss et al 1998). It is possible that SPARC may play an essential role in the development of the corpus luteum because specific peptide fragments of the protein are strongly angiogenic (Reed et al 1993). In the follicle, SPARC is found in both granulosa cells and oocytes, although expression in the oocyte may derive from adjacent granulosa cells (Bagavandoss et al 1998). Calmodulin, a protein with strong functional and structural similarities to SPARC, has been implicated in the resumption of meiosis in the starfish oocyte (Santella and Kyozuka 1997), and it is possible that up-regulation of SPARC after hCG may play a role in allowing resumption of meiosis in the oocyte.

For those genes already discussed in this thesis it is possible to hypothesise a putative function in ovulation and corpus luteum development based on their known properties and functions in other tissues. The SAGE libraries described here also contain many other highly expressed or differentially expressed transcripts for which this is not currently possible. Included among this latter

group are proline rich protein MP-5, tumour protein translationally controlled 1, testes enhanced gene transcript and polycystic kidney disease 2. In addition the list of tags differentially regulated after hCG contains a considerable number that are unmatched in the SAGEmap database or are matched only to EST clusters or uncharacterised transcripts. The challenge now will be to identify those genes from this list that are fundamentally involved in the process of luteinisation and those genes that have a more downstream role in the development of the corpus luteum.

5.8 Non coding transcript

A final consideration has to be given to the role of non-coding transcripts in the regulation of luteinisation. Chapter 4 details the further investigations we have taken into the characterisation and expression of the RNA transcript represented by the SAGE tag CAGTCAATAC. We have demonstrated the expression, within the granulosa cell and other tissue types, of a variable length RNA without translation initiation sites that we propose acts as an RNA. Increasingly, evidence is mounting that the role of cellular RNA extends far beyond that of an intermediary in protein synthesis. The high conservation of the genomic sequence for this transcript across the mammalian genomes and the specific temporal and spatial expression pattern suggest a significant cellular function. The abundant and differential expression exhibited in response to gonadotrophin stimulation suggests that this function may be associated with folliculogenesis.
A growing number of RNAs lacking open reading frames have been identified as transcriptional or protein function regulators (Szmanski et al 2003) and accumulating evidence suggests critical roles for noncoding RNAs in a variety of cellular processes, including developmental decisions relating to gene dosage, silencing or genome imprinting (Erdmann et al 2001). Among the groups are naturally occurring antisense RNAs (Brandl 2002), small interfering RNAs (siRNA) and small temporary RNAs (stRNA) which mediate down-regulation of gene expression either through interference and degradation of perfectly complementary mRNA (siRNA) or translational inhibition while retaining

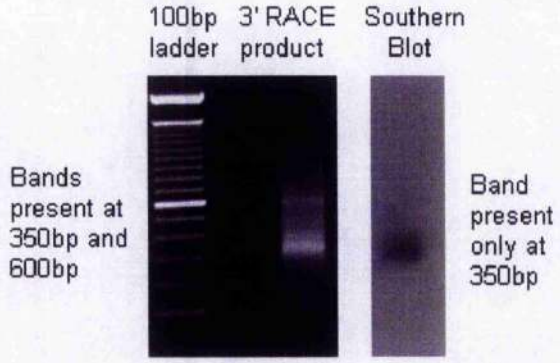
stability of imperfectly complementary mRNA (stRNA) (Hutvagner and Zamore 2002). A further model suggests that ncRNAs function to define the domains of open chromatin necessary to facilitate transcriptional activation (Morey and Avner 2004). The essential nature of RNA metabolism to development and survival is demonstrated by the early developmental arrest of Dicer enzyme null mice (Bernstein et al 2003). The discovery that ncRNAs are implicated in a disparate variety of regulatory systems, and likely to be integral to the overall molecular architecture of organisms, suggests that they are part of a cellular efficiency drive utilising signalling molecules which can be produced and destroyed at a lower metabolic cost than protein molecules. Comparison of mouse, human and other mammalian genomes shows a considerable degree of homology out-with protein coding regions (Mayor et al 2000, Mattick 2001).

We have demonstrated that the expression of this non-coding RNA is strongly induced by PMSG in granulosa cells, but not by hCG. The expression was first detected in early antral follicles and peaked 48hrs after PMSG administration before rapidly declining to basal levels 8 hrs following hCG administration. Sequencing analysis of the cDNA revealed a polyadenylated transcript with no extensive open reading frames. In addition several unexpected features are associated with this transcript, these include a 23 nucleotide hairpin fold centred on base 233 and several small 5-6 base complementary sequences located either side of the hairpin fold (Figure 4.2b) which may be involved in secondary folding of the RNA. While a high degree of homology is maintained by this transcript throughout mammalian, and even avain genomes, indicating an evolutionary conservation, no significant antisense match can be found to any known gene suggesting that an RNAi related function is unlikely.

The genomic location raises the possibility that this transcript may be part of the 3' UTR of the inhibin βA subunit. 3' RACE carried out using primers for inhibin βA failed to produce a band greater than 600bp long (personal communication, Prof. PJ O'Shaughnessy) (Figure 5.4, provided courtesy of Prof PJ O'Shaughnessy) Southern blot showed that only the 350bp band was derived from the inhibin βA subunit. This band yielded matching sequence (data provided courtesy of Prof PJ O'Shaughnessy) to the 3' genomic region of

inhibin βA. It is highly unlikely therefore, that the non-coding RNA is part of the inhibin βA 3' UTR.

Figure 5.4 Agarose gel showing product of 3' RACE reaction and southern blot on inhibin βA subunit



Bands of 350bp and 600bp produced from 3' RACE are of insufficient size to demonstrate continuity between the inhibin subunit and the noncoding transcript

Although the biological functions of this transcript remain unknown the observation that induction occurs during PMSG stimulated folliculogenesis and downregulation preceeds hCG stimulated luteal differentiation suggests a possible role in granulosa cell proliferation or differentiation. The induction of a non-coding RNA transcript in cells undergoing TGFβ driven differentiation has previously been recognised. The bone morphogenic proteins (BMP) and osteogenic proteins (OP), members of the TGFβ superfamily and responsible for the induction of bone formation in vivo, have been shown to induce expression of a non-coding transcript. Two proteins BMP-2 and OP-1, specifically induce transcription of the 3Kb non coding BORG RNA (BMP/OP-responsive gene), which has been suggested to play a key role in osteoblast differentiation (Takeda et al 1998) although its precise function is unknown.

The potential for hairpin folding in the secondary structure of our transcript draws comparison with the E.coli thi-box structure. These are conserved RNA structures that co-regulate the expression of genes involved in thiamine

metabolism (Miranda-Rios et al 2001, Lesnik et al 2005) and are utilised for small molecule-mRNA interactions involved in translation regulation in prokaryotes (Stormo and Ji 2001). Riboswitches consist of RNA that forms a selective binding site for a target metabolite, binding creates an allosteric structural changes that leads to transcriptional change. Such riboswitches are common in prokaryotes and archaea and there is some evidence for them in eukaryotes. A motif similar to the thiamine riboswitch has been found in eukaryotic genes involved in thiamine biosynthesis (Sudarsan et al 2003). In the eukaryotic examples the proposed riboswitches are contained within 3' UTR or intronic sequences of the genes in question.

Regardless of the mechanism of action the obvious question arises regarding the genes and/or cellular functions that may be affected by the expression of this transcript. The obvious candidate is the adjacent inhibin βA subunit which serves as a component of inhibin A (βA:βB) and activin A (βA:βA), TGFβ family members regulating follicle development. The expression profiles of these during follicle development and ovulation have been studied elsewhere (Newton et al 2002). In summary as follicles develop under FSH stimulation inhibin A production begins to predominate over inhibin B. Following the LH surge there is a dramatic fall in inhibin A and B with a concomitant increase in activin A expression. The action of activin A on the oocyte is critical for maturation (Alak et al 1998) and has been suggested to mediate the LH surge in this regard (Newton et al 2002). In the rat the transcription of the βA subunit is reported to increase progressively within the follicle following recruitment from the primordial follicle pool (Meunier et al 1998, Arai et al 2002), following a pattern identical to that of the non-coding transcipt. This raises the question as to whether the non-coding transcript may act to control transcription and/or translation of the inhibin βA subunit, or to affect the post-translational availability of the inhibin βA subunit.

An additional clue to the possible function of this transcript is its global expression profile throughout the mammalian tissue range. Highest levels of expression are found in the spleen and ovary with lower levels in intestine, lung and uterus (figure 4.6). These tissues have in common the presence of a

proliferating and differentiating cell population, in ovary the granulosa cell, in spleen the haematopoietic cell lines, and in intestine, lung and uterus the constant turnover of epithelial tissue necessitates the continual regeneration and proliferation of cellular stock. The inhibin βA subunit has been heavily implicated in cellular differentiation and especially in haematopoietic and epithelial differentiation (Shav-Tal and Zipori 2002, Zhang et al 2004, Ball and Risbridger 2001), in particular negative regulation of activins during pancreatic epithelial development has been shown to induce cellular proliferation and inhibit terminal differentiation. Whether the non coding transcript identified here has a role in this modulation of activin signaling requires investigation.

Much of this is highly speculative and further work is required to disclose the precise functional role of this transcript and its target and mechanisms of action in granulosa cell development. An analysis of expression patterns of both the noncoding transcript and the inhibin βA subunit in a variety of tissues and development situations where one or both is known to be expressed in conjunction with up/down regulation of the non coding transcript in cell or follicle culture is required. At the time of writing siRNA knockdown of this transcript in granulosa cell culture is being carried out and this study should provide some insight into the functional significance of the expressed RNA.

5.9 Conclusion

A large number of differentially expressed transcripts have been successfully identified using SAGE as well as many more genes abundantly, if not differentially, expressed. A body of literature has already characterised the individual cellular events activated by either steroid hormones, peptide hormones and growth factors which trigger the principle signal transduction pathways employed by the granulosa cell to respond to external stimuli. We have isolated a large number of candidate genes related to the cellular differentiation processes occurring within the granulosa cell during luteinisation. In particular the finding of a number of novel transcription factors and signalling receptors with altered expression profiles in response to hCG requires further investigation. Equally we

have demonstrated that there are a variety of potential mechanisms of cross talk and interaction between different signalling and/or metabolic pathways controlling the development of the terminal luteal phenotype. The data generated and presented here constitutes a new base for the testing of hypotheses in the field of follicle development and luteinisation.

Appendices

Appendix 1: Significantly differentially expressed genes

Appendix 2: Abbreviations

Appendix 3: Reference list

Appendix 1: Identified significantly differentially expressed transcripts with annontations briefly describing gene function.

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
TTGTTGCTAC	387	8	395	$1.29x10^{-77}$	268000	Vimentin
A member of a well characterised class of cytoskeletal elements. Class III intemediate filament specific for mesenchymal tissue, undergoes significant reorganisation during cell division.						
TTGCTACTTT	367	6	373	$1.23x10^{-74}$	27961	Leprecan 1
Extracellular matrix protein located in plasma membrane whose human homolog Gros1 has been shown to have growth suppressor activity						
CCTTTAATCC	61	397	458	$1.5x10^{-58}$	347445	Hyaluronidase 1
A lysosomal hyaluronidase intracellularly degrades hyaluronan, one of the major components of the extracellular matrix.						
CAAACACCGT	278	2	280	$2.02x10^{-58}$	288474	Secreted phosphoprotein 1
Forms part of the cell-ECM interaction and can also act as a cytokine, enhancing interferon and interleukin production						
AACTGAGGGG	306	49	355	$6.38x10^{-40}$	277498	Prosaposin
Glycoprotein which is a precursor for 4 saposins involved in the lysosomal degradation of sphingolipids						
TAATGTAGAC	116	405	521	$2.43x10^{-39}$	370184	Gap junction membrane channel protein alpha 1
A member of the connexin gene family and a component of gap junctions.						
AGCAAGAATT	226	28	254	$1.95x10^{-33}$	1061	Ferredoxin 1
An iron-sulfur protein found in steroidogenic tissues, transfers electrons from NADPH through ferredoxin reductase to a terminal cytochrome P450.						
AGGCAATAAA	143	3	146	$1.95x10^{-29}$	27154	Vanin 1
This is a member of a family that includes secreted and membrane-associated proteins. This protein is a GPI-anchored cell surface molecule.						
TAACTGACAA	140	11	151	$2.62x10^{-24}$	147226	Metallothionein 2
Metallothioneins are small cysteine-rich proteins with highly specific roles in fundamental zinc-regulated cellular processes.						
GGTTAAATGT	206	56	262	$6.41x10^{-19}$	930	Cathepsin L
A component of the lysosomal proteolytic system believed to participate in intracellular degradation and turnover of proteins.						
ATACTAACGT	99	6	105	$1.73x10^{-18}$	34102	Ornithine decarboxylase, structural 1
The rate-limiting enzyme of the polyamine biosynthesis pathway. An increase in the activity level of this enzyme is often seen in reponse to growth promoting stimuli.						
TACAGTATAA	9	98	107	$2.62x10^{-18}$	3092	Inhibin beta-B
In combination with the inhibin alpha subunit forms a pituitary FSH secretion inhibitor also shown to regulate gonadal stromal cell proliferation negatively. Also forms a homodimer, activin B, which in combination with the beta A subunit forms activin which stimulates FSH secretion. Both compounds are involved in regulating a number of functions including hormone secretion (hypothalamic, pituitary, gonadal) and germ cell maturation.						
GGGCATTTGA	108	11	119	$9.31x10^{-18}$	302865	Cytochrome P450 11a1
Cytochrome P450 cholesterol side chain cleavage, catalyzes the conversion of cholesterol to pregnenolone, the rate-limiting step in the synthesis of the steroid hormones						
CAAACTCTCA	116	16	132	$4.98x10^{-17}$	291442	Secreted acidic cysteine rich glycoprotein
Also called osteonectin. This is a matrix-associated protein inhibits cell-cycle progression and influences the synthesis of extracellular matrix (Bradshaw et al., 2003)						
GATACTTGGA	73	3	76	$9.97x10^{-15}$	297	Actin, beta, cytoplasmic
Major cytoskeleton component and mediator of internal cell motility						
AAAACAGTGG	16	91	107	$1.68x10^{-13}$	21529	Ribosomal protein L37a
Structural component of ribosomes						
GCTCTGGGAG	56	152	208	$5.48x10^{-12}$	140811	Hydroxysteroid dehydrogenase-1 delta<5>-3-β
The 3beta-HSD enzyme plays a crucial role in the biosynthesis of all steroid hormones						
ACTGAAGCAA	146	46	192	$6.56x10^{-12}$	282242	Scavenger receptor class B, member 1

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
Facilitates the cellular uptake of cholesterol from high density lipoprotein						
GACTCAGGGC	47	0	47	$4.98x10^{-11}$	2580	Syndecan 1
Mediates cell-matrix and cell-cell adhesion and inhibits cell invasion.						
GAAAAGTGGA	69	9	78	$7.91x10^{-11}$	15295	Epoxide hydrolase 2, cytoplasmic
This protein binds to specific epoxides and converts them to the corresponding dihydrodiols. Mutations in this gene have been associated with familial hypercholesterolemia.						
GGATGGGGAG	54	3	57	$9.79x10^{-11}$	283926	Seizure related 6 homolog (mouse)-like 2
An extracellular protein previously shown to have a role in CNS development, most abundant during/after neuronal differentiation and during cell specification or axogenesis.						
TACCTTGACA	45	0	45	$1.33x10^{-10}$	4791	Epiregulin
Growth factor acting as a paracrine mediator of the LH signal throughout the follicle.						
TTGAAATTAC	45	0	45	$1.33x10^{-10}$	362063	Proline-rich protein MP5
Unknown function						
AATCACTGTG	44	0	44	$2.17x10^{-10}$	33240	Epithelial V-like antigen 1
A cell membrane protein expressed during early thymic embryogenesis, mediates homophilic cell-cell adhesion						
TGGCTCGGTC	53	4	57	$5.46x10^{-10}$	300639	Actin, gamma, cytoplasmic 1
Major cytoskeleton component and mediator of internal cell motility						
TACATTCCAA	45	1	46	$5.54x10^{-10}$	3401	Proprotein convertase subtilisin/kexin type 5
This encoded protein mediates post-translational endoproteolytic processing for several integrin alpha subunits.						
TACTTTATAA	39	0	39	$2.54x10^{-09}$	1421	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1
Active metalloprotease, cleaves proteoglycans, aggrecan, versican and brevican. Has angiogenic inhibitor activity. They have been associated with various roles in connective tissue organization, coagulation, inflammation, arthritis, angiogenesis and cell migration as well as follicular rupture.						
GGTCAAGATA	4	46	50	$2.74x10^{-09}$	188939	Hydroxysteroid (17-beta) dehydrogenase 1
In ovarian granulosa cells the activity of 17HSD1 is essential for gonadal estradiol biosynthesis and is also involved in the modulation of steroid hormone action.						
TTAGAAGTGA	40	1	41	$6.44x10^{-09}$	373563	Salvador homolog 1 (Drosophila)
Involved in regulating both cell proliferation and cell death. In Drosphila salvador is a gene that promotes both cell cycle exit and cell death and mutatons in the mammalian homolog have been implicated in three cancer cell lines.						
GATTGTCAGA	42	2	44	$9.18x10^{-09}$	25613	Immediate early response 3
This gene functions in the protection of cells from Fas- or tumor necrosis factor type alpha-induced apoptosis. Alternative splicing of this gene results in two transcript variants.						
ACTTCCTTTC	36	0	36	$1.12x10^{-08}$	282242	Scavenger receptor class B, member 1
Facilitates the cellular uptake of cholesterol from high density lipoprotein						
GCTGCCCTCC	409	566	975	$1.45x10^{-08}$	104368	Ribosomal protein L32
Structural component of ribosome						
GTGGCGCACG	10	54	64	$2.98x10^{-08}$	214645	Hyaluronidase 3
A lysosomal hyaluronidase intracellularly degrades hyaluronan, one of the major components of the extracellular matrix						
TCCACCAGAT	41	3	44	$5.22x10^{-08}$	279361	Vinculin
Vinculin is a highly conserved F-actin anchoring cytoskeletal protein associated with cell-cell and cell-matrix junctions.						
AGACACTTCC	48	6	54	$5.62x10^{-08}$	238343	Annexin A2
Members of this family of membrane proteins have a role in the regulation of cellular growth and signal transduction. This protein functions as an calcium binding autocrine factor known to enhance osteoclast formation and bone resorption.						
TCCCGGATCA	2	35	37	$7.12x10^{-08}$	18962	Catenin alpha 1
Cadherin associated actin binding cell adhesion protein found at cell-cell and cell-matrix boundaries						
AATTTCAAAA	5	41	46	$1.14x10^{-07}$	371577	Ribosomal protein S17
Structural component of ribosome						

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
TATGAATGCT	48	7	55	$1.58x10^{-07}$	158700	Chondroitin sulfate proteoglycan 2
Secreted hyaluronic acid binding extracellular matrix protein which may play a role in intercellular signaling						
GGGGGAGCAT	6	42	48	$2.03x10^{-07}$	275973	Sialyltransferase 4C
Catalyses conversion of glycoproteins and glycolipids. Highly expressed in adult placenta, ovary and testes						
TTTGTAATAA	30	0	30	$2.18x10^{-07}$	284855	Endothelin 2
Peptide hormone involved in signal transduction and cell communication						
ATCAGTGTGC	32	1	33	$3.29x10^{-07}$	275555	Calponin 3, acidic
Cytoskeletal binding protein associated with cell growth and maintainence. Capable of binding to actin, calmodulin, troponin and tropomyosin						
TAAATGTGCA	8	45	53	$3.44x10^{-07}$	4913	Follistatin
Follistatin is a gonadal protein that specifically inhibits FSH release. Binds directly to activin and functions as an activin antagonist.						
AAGATCAAGA	44	6	50	$3.6x10^{-07}$	360115	Actin, alpha, cardiac
Major cytoskeleton component and mediator of internal cell motility						
TGCTGTGCAT	60	14	74	$4.26x10^{-07}$	289662	Fibroblast growth factor inducible 14
Fibroblast growth factor-inducible 14 mediates multiple pathways of induced cell death, proliferation and angiogenesis through its function as a TWEAK (a TNF family member) receptor.						
CTCTGAATAC	43	6	49	$5.71x10^{-07}$	2442	Calcium binding protein, intestinal
Belongs to a family of calcium-binding proteins that includes calmodulin, parvalbumin, troponin C, and S100 protein. Its exact function is unknown. In the intestine its expression correlates with calcium transport activity.						
TCCCCCCCCC	28	0	28	$5.88x10^{-07}$	35088	Cholinergic receptor, nicotinic, beta polypeptide 2 (neuronal)
After binding acetylcholine responds by opening an ion-conducting channel across the plasma membrane. Involved in synaptic transmission, signal transduction and ion transport. Picciotto et al. (1995) disrupted the CHRNB2 mouse homolog in embryonic stem (ES) cells to generate 'knockout' mice deficient in this subunit. Homozygous mice were viable and had no physical deficits.						
ACCGGGTCAT	30	1	31	$8.83x10^{-07}$	206919	Male sterility domain containing 2
Unknown function						
ATTTGACTGG	30	1	31	$8.83x10^{-07}$	29677	Myosin heavy chain IX
Structural component of cytoskeleton.						
TTGTCAGGTA	68	20	88	$1.44x10^{-06}$	148155	Malic enzyme, supernatant
Malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+) activity						
TCTCGTAATG	29	1	30	$1.45x10^{-06}$	42095	Secreted frizzled-related sequence protein 4
Acts as a soluble modulator of Wnt signaling. In ventricular myocardim expression of SFRP4 correlates with apoptosis related gene expression. In the mouse ovary expression has previously been shown to be up-regulated in granulosa cells of large antral follicles after hCG administration.						
CACATTATCA	25	0	25	$2.62x10^{-06}$	28099	Sterol O-acyltransferase 1
An endoplasmic reticulum integral membrane protein. Catalyzes the formation of cholesterol esters from long-chain fatty acyl CoA and cholesterol						
CCCTTCTTCT	10	44	54	$3.41x10^{-06}$	196110	Hemoglobin alpha, adult chain 1
Involved in oxygen transport to the various peripheral tissues.						
GGATTTGGCT	51	107	158	$3.5x10^{-06}$	341719	Ribosomal protein, large P2
Structural component of ribosome						
AAGAGGCAAG	37	87	124	$3.6x10^{-06}$	288212	Ribosomal protein S15a
Structural component of ribosome						
TTAAATGCAG	27	1	28	$3.89x10^{-06}$	273188	Coagulation factor III
Cell surface glycoprotein, enables cells to initiate the coagulation cascades and functions as a high-affinity receptor for the coagulation factor VII.						
TATAGTGTAA	43	8	51	$3.91x10^{-06}$	158700	Chondroitin sulfate proteoglycan 2
Secreted hyaluronic acid binding extracellular matrix protein which may play a role in intercellular signaling						
GGCTTTGGTC	60	119	179	$3.92x10^{-06}$	3158	Ribosomal protein, large, P1
Structural component of ribosome						

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
TTTTTGATAA	32	3	35	$3.97x10^{-06}$	42095	Secreted frizzled-related sequence protein 4
Acts as a soluble modulator of Wnt signaling. In ventricular myocardium expression of SFRP4 correlates with apoptosis related gene expression. In the mouse ovary expression has previously been shown to be up-regulated in granulosa cells of large antral follicles after hCG administration.						
GGGAGCGAAA	28	2	30	$8.46x10^{-06}$	34871	Inhibitor of DNA binding 2
This gene encodes a helix-loop-helix (HLH) protein that can form heterodimers with members of the basic HLH family of transcription factors and therefore can inhibit the DNA binding and transcriptional activation ability of proteins with which it interacts. This protein may play a role in cell growth, senescence, and differentiation.						
GGTTATAATA	34	5	39	$1.32x10^{-05}$	28405	Serum/glucocorticoid regulated kinase
This kinase has been shown to be important in activating certain potassium, sodium, and chloride channels. Expression of is stimulated by TGF-beta. Plays an important role in cellular stress response, mediates cell survival signals, phosphorylates and negatively regulates pro-apoptotic foxo3a						
TGGGTTGTCT	253	156	409	$1.43x10^{-05}$	296922	Tumor protein, translationally-controlled 1
Molecular function unknown. Found in several healthy and tumoral cells including erythrocytes, hepatocytes, macrophages, platelets, keratinocytes, erythroleukemia cells, gliomas, melanomas, hepatoblastomas, and lymphomas.						
GAAAGCCTCT	24	1	25	$1.72x10^{-05}$	8245	Tissue inhibitor of metalloproteinase 1
The proteins encoded by this gene family complex with metalloproteinases (such as collagenases, a group of peptidases involved in degradation of the extracellular matrix) and irreversibly inactivate them. TIMP1 is highly inducible at the transcriptional level in response to many cytokines and hormones and is also known to suppress angiogenesis						
ATCACACACT	21	0	21	$1.94x10^{-05}$	62886	UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 7
This gene catalyzes the initial reaction in o-linked oligosaccharide biosynthesis controlling the initiation step of mucin-type O-linked protein glycosylation and transfer of N-acetylgalactosamine to serine and threonine amino acid residues.						
GGGAAGTCTG	33	5	38	$2.09x10^{-05}$	347009	Peroxiredoxin 2
This gene is involved in redox regulation of the cell. May play an antioxidant protective role in cells by reducing peroxides. Speculated to participate in the signaling cascades of growth factors and tumor necrosis factor-alpha by regulating the intracellular concentrations of hydrogen peroxide.						
CTTGCTCTGT	35	6	41	$2.21x10^{-05}$	263396	Integrin beta 1 (fibronectin receptor beta)
Integrin family members are membrane receptors involved in cell adhesion and recognition in a variety of processes including embryogenesis, hemostasis, tissue repair, immune response and metatastatic diffusion of tumor cells. Integrin-beta(1) has also been shown to be necessary for inducing angiogenesis by regulating cell survival and differentiation after implantation into ischemic tissue						
GATATGGTCT	23	1	24	$2.83x10^{-05}$	2863	Integral membrane protein 1
Involved in protein glycosylation						
TTATCAAGTG	4	28	32	$2.86x10^{-05}$	358946	Similar to development- and differentiation-enhancing factor 2; PYK2 C terminus-associated protein
PYK2 C is a GTPase activator activity, signal transduction and cell communication						
AACAGGTTCA	19	54	73	$3.21x10^{-05}$	292027	Ribosomal protein S25
Structural component of ribosome						
CACGGCTTTC	2	23	25	$4.05x10^{-05}$	306548	Ribosomal protein L26
Structural component of ribosome						
TCTTTAATCC	2	23	25	$4.05x10^{-05}$	196638	CDC23 (cell division cycle 23, yeast, homolog)
Part of the APC complex that functions as a protein ubiquitin ligase and governs exit from mitosis.						
CAGGCAAAAC	3	25	28	$4.52x10^{-05}$	171378	Uncoupling protein 2, mitochondrial
Facilitates the transfer of anions from the inner to the outer mitochondrial membrane and the return transfer of protons from the outer to the inner mitochondrial membrane.						
GATTCCGTGA	36	78	114	$4.84x10^{-05}$	10474	Ribosomal protein L37
Structural component of ribosome						
CTTAGATGTT	19	0	19	$5.32x10^{-05}$	309193	Ropporin 1-like

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
Interacts with both A-kinase anchoring protein and the Rho signalling pathway, present on sperm and thought to be sperm specific.						
TTAATTACAG	19	0	19	$5.32x10^{-05}$	28405	Serum/glucocorticoid regulated kinase
This kinase has been shown to be important in activating certain potassium, sodium, and chloride channels. Expression of is stimulated by TGF-beta. Plays an important role in cellular stress response, mediates cell survival signals, phosphorylates and negatively regulates pro-apoptotic foxo3a						
AATTAGTTGT	8	34	42	$6.53x10^{-05}$	353	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F
Catalyzes ATP synthesis during oxidative phosphorylation.						
CAACCATCAT	26	3	29	$7.02x10^{-05}$	30071	Lysosomal-associated protein transmembrane 4A
Suggested to have a role in the transport of small molecules across endosomal and lysosomal membranes.						
GATAATGCAC	6	30	36	$7.54x10^{-05}$	253142	NADH-ubiquinone oxidoreductase 18 kDa subunit, mitochondrial precursor
Nuclear gene coding for the 18 kD (IP, AQDQ) subunit of respiratory complex I. Known to have a critical role in the differentiation and functional activity of brain cells						
TGCACCACCT	30	5	35	$8.27x10^{-05}$	182470	Ribonuclease H2, large subunit
Of the multiple RNases H in mammals, shows increased activity during DNA replication.						
CTCCCACCCA	18	0	18	$8.83x10^{-05}$	249318	Frequenin homolog (Drosophila)
This gene regulates G protein-coupled receptor phosphorylation in a calcium-dependent manner and can substitute for calmodulin.						
GATGACACCA	39	80	119	$9.84x10^{-05}$	371603	Ribosomal protein S28
Structural component of ribosome						
CCTACCAAGA	17	48	65	0.0001	328529	Ribosomal protein S20
Structural component of ribosome						
AAAAGAAAAT	20	1	21	0.0001	276831	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 8
High-affinity transport of large neutral amino acids.						
TTTTCTATTT	3	23	26	0.0001	25594	Protein kinase, cAMP dependent regulatory, type II beta
cAMP-dependent protein kinase (AMPK) transduces signals through phosphorylation of different target proteins. Four different regulatory subunits and three catalytic subunits of AMPK have been identified in humans. The protein encoded by this gene is one of the regulatory subunits. This subunit has been shown to interact with and suppress the transcriptional activity of the cAMP responsive element binding protein 1 (CREB1) in activated T cells.						
TACAATATAC	35	8	43	0.0001	31403	Tumor necrosis factor, alpha-induced protein 9
Located on plasma membrane and involved in electron transport						
CCTGGCCAAG	29	5	34	0.0001	28099	Sterol O-acyltransferase 1
An endoplasmic reticulum integral membrane protein. Catalyzes the formation of cholesterol esters from long-chain fatty acyl CoA and cholesterol						
AACAATTTGG	42	83	125	0.0001	300271	Ribosomal protein L9
Structural component of ribosome						
ACTCGGAGCC	9	34	43	0.0001	285993	Calmodulin 1
Calcium binding protein involved in cell signal transduction and communication						
GTTGTTAACA	22	2	24	0.00015	143768	F-box only protein 3
Constitutes one of the four subunits of the ubiquitin protein ligase complex whose function is phosphorylation-dependent ubiquitination. Involved in ubiquitin protein ligase activity						
AGCAAGATGG	40	11	51	0.00016	290578	Aminolevulinic acid synthase 1
5-Aminolevulinate synthase (ALAS) catalyzes the first step of the heme biosynthetic pathway in mammalian cells						
TGTATCCAGT	8	32	40	0.00016	373561	Nucleosome assembly protein 1-like 5
Nucleosome assembly						
AACCTCGCTG	26	4	30	0.00019	30221	Insulin induced gene 1

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
Regulated by peroxisome proliferator- activated receptor gamma (PPAR gamma) which regulates lipid metabolism and glucose homestasis, providing a link between insulin sensitization/glucose homeostasis and lipid homeostasis. Also a key regulator in the processing of the sterol regulatory element-binding proteins (SREBPs)						
GTGCTGTTGT	26	4	30	0.00019	196189	Angiopoietin-like 4
This gene ia a transcriptional target of PPAR alpha and gamma and is a proangiogenic factor produced during ischemia having been demonstrated to induce a strong angiogenic response even in the absence of VEGF						
ATGACATAGA	28	5	33	0.0002	235182	Calcium/calmodulin -dependent protein kinase II gamma
The influx of Ca^{2+} has been shown to activate multiple intracellular protein kinases pathways, such as PKA, PKC, PKG, nitric oxide synthase, as well as CaM kinase II. CaM kinase II plays a regulatory role in the maintenance of phosphorylation of CREB protein.						
CAAACAATGT	19	1	20	0.0002	277351	G protein-coupled receptor 48
G-protein-coupled receptor involved in signal transduction. Also expressed in human pancreas, placenta, kidney, brain, and heart. Additionally expressed as early as 7 days post coitus in the mouse, suggesting some potential involvement in development.						
GAACATTGCA	19	1	20	0.0002	291442	Secreted acidic cysteine rich glycoprotein
Also called osteonectin. This is a matrix-associated protein inhibits cell-cycle progression and influences the synthesis of extracellular matrix						
GTGGCTCTCA	0	15	15	0.00022	355306	A disintegrin and metalloproteinase domain 17
A member of the ADAM protein family implicated in a variety of biologic processes involving cell-cell and cell-matrix interactions also functions as a tumor necrosis factor-alpha converting enzyme cleaving the membrane-bound precursor of tnf-alpha to its mature soluble form and is responsible for the proteolytic release of several other cell-surface proteins.						
TCCAACTCCT	0	15	15	0.00022	371616	Mitochondrial ribosomal protein S21
Mitochondrial ribosomal structural protein						
TGGAACAATG	18	1	19	0.00034	338790	Proteoglycan 1, secretory granule
This gene encodes a protein best known as a hematopoietic cell granule proteoglycan.						
TGTGCCGGCC	18	1	19	0.00034	293314	Steroidogenic acute regulatory protein
Key function in the regulation of steroid hormone synthesis by enhancing the conversion of cholesterol into pregnenolone through mediation of cholesterol transport across the mitochondrial membrane.						
AGAAGACAGA	15	0	15	0.00041	16769	Testis enhanced gene transcript
A novel, conserved gene of the rat that is developmentally regulated in the testis. Analysis of rat RNA from different stages of spermatogenesis indicated that accumulation of the short transcript occurred mainly postmeiotically. Function unknown.						
AGAGGACTAG	15	0	15	0.00041	358930	G protein-coupled receptor associated sorting protein 2
G protein associated cell signalling molecule						
TAAAGAGGCC	51	91	142	0.00043	261679	Ribosomal protein S26
Structural component of ribosome						
TTCTTGTATT	22	3	25	0.00047	276405	FK506 binding protein 51
Expression of FKBP5 is strongly enhanced by glucocorticoids, progestins, and androgens. It has diverse biochemical functions. Best studied is its role as a component of steroid hormone receptors. It has been suggested that increased expression of FKBP5 through progestin stimulation may attenuate progestin responsiveness in hormone-conditioned cells						
GATGTGGCTG	15	41	56	0.00047	2718	Eukaryotic translation elongation factor 1 β 2
This gene encodes a translation elongation factor, acts as a guanine nucleotide exchange factor involved in the transfer of aminoacylated tRNAs to the ribosome.						
GTTTGTACAA	24	4	28	0.0005	182396	Latent transforming growth factor beta binding protein 3
An extracellular matrix form of TGF-β binding protein, which requires complexing with Cys33 of the TGF-beta propeptide for secretion after coexpression with TGF-β. Null mice show growth retardation, splenic and thymic involution, multiple skeletal abnormalities and high falatity levels 3-4 weeks after birth.						
AGGATCAATG	28	6	34	0.0005	205601	Cortactin
Cytoskeleton binding cytoplasmic protein regulating interactions between adherens junctions and cytoskeletal organisation.						

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
TAAATGATAA	28	6	34	0.0005	1421	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1
Known to be hormonally regulated in the ovary by LH and the progesterone receptor (PR). Protease involved in regulation of the ECM. Expression pattern related to that of versican and hyaluronan.						
ATAATACATA	231	154	385	0.00051	200362	Cytochrome b-245, beta polypeptide
Cytochrome b is comprised of a light chain (alpha) and a heavy chain (beta).						
ATGATGGTAG	24	54	78	0.00052	353171	Eph receptor A6
Eph receptors and their ligands, the ephrins, represent a class of cell-cell communication molecules with organ-site-specific expression patterns with proven roles in regulation of tissue development						
GCTCAGCACC	3	20	23	0.00059	218957	Gene regulated by estrogen in breast cancer protein
The expression pattern of this gene has been shown to correlate with that of oestrogen receptor, no function has been determined.						
AGGAATCCAC	14	0	14	0.00067	22701	Growth arrest specific 1
Growth arrest protein which blocks entry to s phase preventing mitotic cycling of normal and transformed cells.						
TGAGGCCTCG	14	0	14	0.00067	21671	Eukaryotic translation initiation factor 3, subunit 9 (eta)
EIF3 binds to 40S ribosomal subunits and stimulates recruitment of Met-tRNAiMet and mRNA to the pre-initiation complex.						
TGATGTGTGA	14	0	14	0.00067	260988	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 11
Membrane bound solute transport						
AGCCAAATAC	19	2	21	0.00067	261831	Basic leucine zipper and W2 domains 1
Regulation of translational initiation						
TTTCAAGGCA	19	2	21	0.00067	46067	Solute carrier family 25, member 30
Membrane bound solute transport						
CTATCCTCTC	29	7	36	0.00073	200916	Glutathione peroxidase 3
Glutathione peroxidase catalyzes the reduction of hydrogen peroxide and lipid peroxides and protects cells against oxidative damage.						
TTTCATTGCC	25	5	30	0.00078	308452	Transforming, acidic coiled-coil containing protein 1
The function of this gene has not yet been determined, it is expressed at high level during early embryogenesis and is known to interact with microtubules and tubulin and to be involved oncogenic transformation.						
TTCATCTGTC	16	1	17	0.00092	272675	Solute carrier family 20, member 1
Membrane bound solute transport						
GTGATGTTTC	26	55	81	0.00097	31018	Cytochrome b-5
Membrane bound hemoprotein which functions as an electron carrier for several membrane bound oxygenases.						
GAGGAGAAGA	15	39	54	0.001	290899	Ribosomal protein L3
Structural component of ribosome						
TATAGTATGT	30	8	38	0.001	210745	Glutamine synthetase
Cytoplamsic enzyme, central role in cellular nitrogen metabolism, coverts glutamate to glutamine						
GGGAAGGCGG	22	49	71	0.0011	331113	Ribosomal protein S3a
Structural component of ribosome						
AGAAGGACCT	13	0	13	0.0011	21002	Solute carrier family 2 (facilitated glucose transporter), member 1
Membrane bound solute transport						
ATGAGAACAG	13	0	13	0.0011	236123	Splicing factor 3b, subunit 3

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
Forms part of a small nuclear ribonucleoproteins complex (U2 snRNP). These complexes may function in chromatin modification, transcription, splicing, and DNA repair.						
TAATAAAATT	13	0	13	0.0011	276831	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 8
Membrane bound solute transport						
TAGCCAACTT	13	0	13	0.0011	4509	Runt related transcription factor 2
Transcription factor involved in osteoblastic and skeletal differentiation and morphogenesis. Runx2 overexpression upregulates several genes in osteoblasts, including collagen type I and osteocalcin.						
TAGCTTTAGG	13	0	13	0.0011	22480	Cyclin D binding myb-like transcription factor 1
DNA dependant regulation of transcription						
GAAACTGAAC	0	12	12	0.0011	42196	Ubiquitin-like, containing PHD and RING finger domains, 1
Interacts with topoisomerase II-alpha which introduces transient double-stranded breaks in DNA, required during the cell cycle. (Hopfner et al., 2000).						
CAAGGTGACA	20	3	23	0.0012	328846	Phosphodiesterase 6A, cGMP-specific, rod, alpha
Hydrolase activity. Metabolism and energy pathways.						
TCGCTGCTGC	20	3	23	0.0012	276647	Cyclin G associated kinase
Governs the cell cycle, expression oscillates slightly during the cell cycle, peaking at g1. Cyclin G is a transcriptional target of, and functions downstream of, p53. Also regulates epidermal growth factor receptor signaling.						
CCCTCACCCA	22	4	26	0.0012	371591	Tubulin, alpha 1
Major constituent of microtubules.						
GAGCGTTTTG	28	57	85	0.0012	5246	Peptidylprolyl isomerase A
Encodes a member of the cyclophilin family. They have been proposed to act either as catalysts or as molecular chaperones in protein-folding events through catalyzation of cis-trans isomerization of peptidylprolyl imide bonds in oligopeptides..						
AGGAAGATCA	1	14	15	0.0014	4078	Antigen identified by monoclonal antibody Ki 67
Cell surface protein used as cellular marker of apoptosis						
TTCTCTCCCT	1	14	15	0.0014	306954	Carbonic anhydrase 14
Carbonic anhydrases (CAs) are a large family of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide. They participate in a variety of biological processes.						
CAGAACAATG	15	1	16	0.0015	282084	Adhesion regulating molecule 1
Plasma membrane protein promoting cell adhesion.						
GCCTAATGTA	36	67	103	0.0015	371575	Ribosomal protein L21
Structural component of ribosome						
CCAAGAGACC	4	20	24	0.0015	46561	Leukocyte cell derived chemotaxin 1
Bifunctional glycoprotein that stimulates the growth of chondrocytes and inhibits the tube formation of endothelial cells. Serves as a growth regulator that stimulates the growth of cultured chondrocytes in the presence of basic fibroblast growth factor (fgf) but inhibits the growth of cultured vascular endothelial cells.						
TGACCCCGGG	72	112	184	0.0015	297372	Ribosomal protein L40
Structural component of ribosome						
CTGCTCTGAC	17	2	19	0.0017	153566	BC019776 Meteorin, glial cell differentiation regulator-like
Unknown function						
TAGAATCCTA	12	33	45	0.0018	4132	Suppressor of cytokine signaling 2
Cytokine-inducible negative regulator of cytokine signaling.						
CGACCTTTAC	12	0	12	0.0019	263396	Integrin beta 1 (fibronectin receptor beta)
Critical cell adhesion molecule for inducing therapeutic angiogenesis. Has been shown to regulate cell survival and differentiation in ischemic tissue						
CTTAAATCTT	12	0	12	0.0019	239605	B-cell translocation gene 2, anti-proliferative

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
A member of the BTG/Tob family. This family has structurally related proteins that appear to have antiproliferative properties. This encoded protein is involved in the regulation of the G1/S transition of the cell cycle. Modulates transcription regulation mediated by esr1.						
GAGTGATTAT	12	0	12	0.0019	256422	Zinc finger protein 162
Also known as splicing factor 1, belongs to a new and growing family of genes dubbed STAR (signal transduction and activator of RNA) proteins. Thought to play a downstream role in cell signaling and RNA binding.						
GGAATTTAGA	12	0	12	0.0019	332793	Blood vessel epicardial substance
This gene is expressed in cardiac and skeletal muscle and is involved in the regeneration of adult skeletal muscle and may act as a cell adhesion molecule in coronary vasculogenesis. Bves is an early marker of developing vascular smooth muscle cells						
TGCAGGAGCT	12	0	12	0.0019	333388	Chromodomain helicase DNA binding protein 4
Part of the nucleosome remodelling and histone deacetylation (nurd) complex. Expression increases in vascular endothelium in response to TNF alpha secretion						
GTGAAACTAA	9	28	37	0.002	66	Ribosomal protein S4, X-linked
Structural component of ribosome.						
AAAATGTACT	0	11	11	0.002	3752	RAN binding protein 1
Interacts with GTP metabolism and consequently may act in an intracellular signaling pathway with possible involvement in progression through the cell cycle by regulating the transport of protein and nucleic acids across the nuclear membrane.						
AATCCAGCCC	0	11	11	0.002	20206	Aquaporin 2
This gene encodes a water channel protein located in the kidney collecting tubule. Forms a water-specific channel that increases plasma membranes permeability to water, thereby permitting water to move in the direction of an osmotic gradient.						
GCTATACAGA	0	11	11	0.002	286830	Leucine aminopeptidase 3
Catalyzes the removal of unsubstituted n-terminal amino acids from various peptides						
ATTGCTTAGA	4	19	23	0.0025	371574	RNA binding motif protein 3
RNA binding and processing						
AACAAAATCT	1	13	14	0.0025	3903	RAS, dexamethasone-induced 1
This gene encodes a novel GTP-binding protein (G protein) that is stimulated by glucocorticoids. Thought to function as an inhibitor of the ERK mitogen activated protein kinase cascade. It may play a role in dexamethasone-induced alterations in cell morphology, growth and cell-extracellular matrix interactions.						
CGCTGGTTCC	21	45	66	0.0026	371622	Ribosomal protein L11
Structural component of ribosome						
GCCCGGGAAT	33	61	94	0.0028	322491	Ribosomal protein L17
Structural component of ribosome						
TACTTGTGTT	18	3	21	0.003	15125	Stromal cell derived factor receptor 1
Small cytokine that belongs to the intercrine family, members of which activate leukocytes and are often induced by proinflammatory stimuli such as lipopolysaccharide, TNF, or IL1. Chemoattractant active on lymphocytes and monocytes, but not neutrophils.						
ACTCCTTAGT	11	0	11	0.0032	336400	Syntrophin, gamma 1
Syntrophins are cytoplasmic peripheral membrane proteins. Involved in actin and protein binding						
CCTGTGTATG	11	0	11	0.0032	293605	Tumor protein p53 inducible nuclear protein 2
Function unknown						
CTGGAGACGC	11	0	11	0.0032	26743	Apolipoprotein A-I
Apolipoprotein A-I (apoA-I) is the major protein in high density lipoprotein (HDL).						
GCTTGCCTCC	11	0	11	0.0032	232293	Ubiquitin specific protease 36
Ubiquitin-mediated proteasomal degradation comprises a major proteolytic pathway in eukaryotes. Usp36 has growth-suppressing activity and induces arrest in g1 phase upon controlled expression.						
TAAATTCAGG	11	0	11	0.0032	275909	Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4B
Semaphorins have been implicated in neuronal growth and differentiation. Sema4b is expressed differentially in the olfactory pathway both during development and regeneration						
TTAGAACGTG	11	0	11	0.0032	27218	Mortality factor 4 like 2
Involved in growth regulation and replicative senescence. The human homolog MRGX can repress or activate the B-myb promoter depending on the cell type studied, suggesting that there may be tissue-specific functions of this protein.						

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
CCCCTATATT	37	14	51	0.0033	288639	RAB guanine nucleotide exchange factor (GEF) 1
Regulatory protein RabGEF1 binds to Ras and negatively regulates Ras activation. RabGEF1 null mice develop skin inflammation in vivo.						
GGGTTTGGAG	27	8	35	0.0034	274463	Endothelin converting enzyme 1
Converts big endothelin-1 to endothelin-1 type ii membrane protein. Mice null for Ece 1 have numerous craniofacial and cardiac abnormalities.						
ATTAATCAGT	32	11	43	0.0035	46754	Solute carrier family 38, member 2
Membrane bound solute transport						
ACCCTGCTTA	0	10	10	0.0035	206417	Cystathionine beta-synthase
This enzyme has an important role of this enzyme in glutathione-dependent redox homestasis						
GAAGCTGTAT	0	10	10	0.0035	5079	Hydroxysteroid 11-beta dehydrogenase 2
Catalyzes the conversion of cortisol to the inactive metabolite cortisone.						
GTCTGCTTGT	0	10	10	0.0035	4375	Fatso
Thought to be involved in the processes of programmed cell death, craniofacial development, and establishment of left-right asymmetry during embryonic development.						
TGCCACCACT	0	10	10	0.0035	327037	Similar to Phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing beta polypeptide (Phosphoinositide 3-Kinase-C2-beta) (PtdIns-3-kinase C2 beta) (PI3K-C2beta) (C2-PI3K)
GTTCACTTTC	5	20	25	0.0036	20841	ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit
Energy metabolism						
GGCAATAATG	53	25	78	0.004	9925	Isocitrate dehydrogenase 1 (NADP+), soluble
The protein encoded by this gene is the NADP(+)-dependent isocitrate dehydrogenase which serves a significant role in cytoplasmic NADPH production.						
GGAGTAAGAA	4	18	22	0.0041	371563	H3 histone, family 3B
Histones are basic nuclear proteins that are responsible for the nucleosome structure of the chromosomal fiber. Histone h3, along with histone h4, plays a central role in nucleosome formation.						
GTGTTAACCA	13	1	14	0.0041	2050	Ribosomal protein L15
Structural component of ribosome						
TCATTCTCCA	13	1	14	0.0041	257837	ATPase, class VI, type 11A
Integral membrane ATPase. The encoded protein is probably phosphorylated in its intermediate state and likely drives the transport of ions such as calcium across membranes						
GTGGTGCACA	2	14	16	0.0046	28779	Ubiquitin specific protease 54
Member of the ubiquitin-specific protease (USP) family functions in the extremely complex and diverse USP proteolytic system.						
TGAAGTACTG	15	2	17	0.0047	17519	Zfp259 Zinc finger protein 259
Interacts with the survival motor neurons (smn) gene in mice, essential for embryonic viability. Required for the localization of SMN in nuclear bodies.						
GGGTTTTTAT	36	14	50	0.0047	258204	Nuclease sensitive element binding protein 1
Also called MSY 1. Regulates expression of the murine growth hormone receptor gene.						
GGTATCAGTC	19	4	23	0.0047	21281	Rng finger protein 4
Transcription regulator, interacts with, and inhibits the activity of, TRPS1, a transcription suppressor of GATA-mediated transcription. Studies in the mouse suggest a role for this protein in spermatogenesis, also highly expressed in human testes. Enhances steroid receptor-mediated transcriptional activation as well as activating basal transcription.						
ACAGTTAAGC	10	0	10	0.0054	209813	Ephrin-B class 2
The ephrins and EPH-related receptors comprise the largest subfamily of receptor protein-tyrosine kinases and have been implicated in mediating developmental events, especially in the nervous system and in erythropoiesis. The ephrin-B (EFNB) class are transmembrane proteins implicated in a control system integrating blood vessel and tissue morphogenesis.						
ACGAAAACCA	10	0	10	0.0054	4509	Runt related transcription factor 2

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
Transcription factor involved in osteoblastic and skeletal differentiation and morphogenesis. Runx2 overexpression upregulates several genes in osteoblasts, including collagen type I and osteocalcin.						
CCACTGTACA	10	0	10	0.0054	267473	PHD finger protein 20-like 1
Nucleic acid binding						
CTTCCCCGGG	10	0	10	0.0054	34903	FSH primary response 1
Function as yet unknown. May function as a transcriptional regulator.						
GAGGAGGAGG	10	0	10	0.0054	136604	Nuclear factor, interleukin 3, regulated
The NFIL3 (E4BP4) transcription factor has been identified as a key regulation protein affecting murine interleukin-3 (IL-3)-dependent cell survival. Expression of NFIL3 is regulated by oncogenic Ras mutants through both the Raf-mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways. NFIL3 inhibits apoptosis without affecting Bcl-xL expression. E4BP4 may play a role in the glucocorticoid repression of genes.						
GCGAAGGCTG	10	0	10	0.0054	2718	Eukaryotic translation elongation factor 1 beta 2
Translation elongation is the process of adding amino acylated tRNAs to the growing polypeptide chain. Translation elongation factor 1A, eEF1A, transfers aminoacylated tRNAs to the A site of the ribosome. This is a GTP-dependent process catalyzed by eEF1B, a guanine nucleotide exchange factor.						
GTGTACTTTC	10	0	10	0.0054	20615	Peroxisomal biogenesis factor 11a
A PPAR alpha target gene PEX11 proteins play a direct role in peroxisome division and that their loss inhibits peroxisome metabolism indirectly, perhaps due to altered membrane structure or dynamics.						
TACTGCTAAG	10	0	10	0.0054	273915	Gene rich cluster, C3f gene
Unknown function						
TGGACATTTG	10	0	10	0.0054	276656	Low density lipoprotein receptor-related protein 8, apolipoprotein e receptor
Apolipoprotein E is a component of lipoproteins such as chylomicron remnants, very low density lipoprotein (VLDL), and high density lipoprotein (HDL). Binds lipoprotein and transports it into cells by endocytosis.						
TTCTGTGTCA	10	0	10	0.0054	19669	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
Hypoxia inducible factor-1 inducible gene. A key regulator of glycolytic flux. Up regulated in response to hypoxia by HIF-1 to provide glycolytic metabolism.						
AATGCTTGAT	24	7	31	0.0057	270186	Retinoblastoma binding protein 7
Together with metastasis-associated protein-2, it deacetylates p53 and modulates its effect on cell growth and apoptosis and functions in the regulation of cell proliferation and differentiation. High levels of RbAp46 expression inhibit the transformation of tumor cells through interfering with normal cell cycle and/or enhancing apoptotic cell death.						
TGTATAAAAA	12	30	42	0.0058	87773	Tumor rejection antigen gp96
Molecular chaperone that functions in the processing and transport of secreted proteins.						
CCCCAGCCAG	35	61	96	0.0058	236868	Ribosomal protein S3
Structural component of ribosome						
AGAGCGAAGT	44	72	116	0.0063	290786	Ribosomal protein L41
Structural component of ribosome						
AATATGGATG	0	9	9	0.0063	30012	High density lipoprotein (HDL) binding protein
High density lipoprotein (HDL) plays a key role in the transportation of cholesterol to extrahepatic tissues including steroidogenic tissues and in the reverse transportation of cholesterol from extrahepatic tissues to the liver. Previously shown to be present in the rodent ovary and regulated by gonadotrophins						
AGCAGTGCTT	0	9	9	0.0063	274715	Coiled-coil domain containing 3
Unknown function						
CCTTGACACC	0	9	9	0.0063	1262	Cytochrome P450, family 17, subfamily a, polypeptide 1
Cholesterol side chain cleavage enzyme, progesterone synthesis						
GCAGAAAGCA	0	9	9	0.0063	268397	Cdc42 GTPase-activating protein
GTPase activating protein involved in reorganisation of the actin cytoskeleton.						
TGAAACACTG	0	9	9	0.0063	1605	Programmed cell death 4
Thought to play a role in apoptosis but the specific role has not yet been determined.						
AAGTAATGTG	12	1	13	0.0069	10516	Prolactin receptor

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
Prolactin has been implicated in physiological functions such as immunoregulation and ovarian steroid production. Prlr has been shown to be present in luteinized granulosa cells						
AGTTCATAAG	12	1	13	0.0069	359654	Similar to airway trypsin-like 3
GTGTCCGTAC	12	1	13	0.0069	294882	F11 receptor
Regulator of tight junction assembly in epithelia. Can also act as a receptor for reovirus, a ligand for the integrin LFA1 and a platelet receptor.						
TGATGTTAAC	12	1	13	0.0069	255848	Hexokinase 2
Hexokinases phosphorylate glucose to produce glucose-6-phosphate, thus committing glucose to the glycolytic pathway. Expression of this gene is insulin-responsive, and studies in rats suggest that it is involved in the increased rate of glycolysis seen in rapidly growing cancer cells.						
TGTTGGTTGA	12	1	13	0.0069	246990	Reticulon 3
Retinal expressed protein implicated in axon development, precise function unknown						
TTACCACATA	20	5	25	0.0069	28262	Regulator of G-protein signaling 2
Known to regulate membrane signaling pathways, expressed in ovarian follicles in response to an ovulatory dose of gonadotropin.						
TCTCCAGGCG	31	55	86	0.0075	200608	Clusterin
An anti-apoptotic factor regulated by IGF-1R/Src/MAPK/Egr-1 signaling						
ACAGCCAGGG	1	11	12	0.0075	242413	G protein-coupled receptor, family C, group 5, member C
Member of the type 3 G protein-coupled receptor family. The specific function of this protein is unknown						
ATAGTAAGCT	1	11	12	0.0075	289707	Fascin homolog 1, actin bundling protein (Strongylocentrotus) purpuratus)
Crosslinks F-actin into highly ordered bundles within dynamic cell extensions, acts as an actin bundling protein.						
GAAATGTTGT	1	11	12	0.0075	22117	Polymerase (RNA) II (DNA directed) polypeptide G
Subunit of the polymerase responsible for synthesizing messenger RNA in eukaryotes.						
GCCTTTATGA	16	35	51	0.0076	16775	Ribosomal protein S24
Structural component of ribosome						
ATAAGGGATT	14	2	16	0.0076	4593	UDP-galactose translocator 2
GTCAACGTAC	25	8	33	0.0076	179189	Ribosomal protein L36a-like
Structural component of ribosome						
ATACACCAGA	16	3	19	0.0077	27286	Protein tyrosine phosphatase-like (proline instead of catalytic arginine), member b
ATGTTCGTGG	16	3	19	0.0077	1791	Dual specificity phosphatase 6
Also known as Mitogen-activated protein kinase phosphatase 3. Inactivates target kinases by dephosphorylating both the phosphoserine/threonine and phosphotyrosine residues. This gene product inactivates ERK2 and mediates the response to fibroblast growth factor signalling.						
CTAATAAAGC	28	51	79	0.0078	329631	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed (fox derived)
Function unknown						
GGATATGTGG	42	19	61	0.0078	181959	Early growth response 1
Functions as a transcriptional regulator. Activates target genes required for differentitation and mitogenesis.						
GTCTTGGGCG	36	15	51	0.0079	30156	Protease, serine, 11 (Igf binding)
Proposed to regulate the availability of insulin-like growth factors (IGFs) by cleaving IGF-binding proteins. It has also been suggested to be a regulator of cell growth. Binds to various TGF-beta proteins and inhibits the signaling of BMP-4, -2 and TGF-beta 1						
TGACTGGGAG	36	15	51	0.0079	333849	Nur77 downstream gene 2
(Nur77=NGFIB) Novel gene of unknown function whose expression is regualted by Nur77, a nuclear orphan steroid receptor that has been implicated in negative selection.						
TGGGCAAAGC	8	23	31	0.0085	371625	Eukaryotic translation elongation factor 1 gamma

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
Translation elongation factor-1 (EF-1), a ubiquitously expressed protein that regulates the efficiency and fidelity of mRNA translation in eukaryotic cells, EF-1 binds aminoacyl-tRNAs and then transfers them to 80 S ribosomes, while binding and hydrolyzing GTP.						
TGTACCCAGG	23	7	30	0.0086	3196	Alpha glucosidase 2 alpha neutral subunit
Structural gene for lysosomal enzymes						
AAGGAAATGG	26	48	74	0.0087	285021	Ribosomal protein L31
Structural component of ribosome						
ACACTCTTTG	9	0	9	0.0091	309193	Ropporin 1-like
Interacts with both A-kinase anchoring protein and the Rho signalling pathway, present on sperm and thought to be sperm specific.						
CAAACACCGG	9	0	9	0.0091	30208	Mitochondrial ribosomal protein S27
Structural component of mitochondrial ribosome.						
CACTGTCTTC	9	0	9	0.0091	4114	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2
Membrane bound solute transport						
CTGTAGAGTG	9	0	9	0.0091	196508	Mortality factor 4 like 1
Component of the nua4 histone acetyltransferase (hat) complex which is involved in transcriptional activation of select genes. This may both alter nucleosome - dna interactions and promote interaction of the modified histones with other proteins which positively regulate transcription. May be required for the activation of transcriptional programs associated with oncogene and proto-oncogene mediated growth induction, tumor suppressor mediated growth arrest and replicative senescence, apoptosis, and dna repair. Interestingly, MRG15 is most highly expressed in adult testis and Sp1 is a testis-enriched protein, suggesting a potential role for MRG15 in meiosis and/or germ cell differentiation.						
CTTTAGAAAA	9	0	9	0.0091	268798	Solute carrier organic anion transporter family, member 3a1
Membrane bound solute transport						
GCTGGATGTG	9	0	9	0.0091	2580	Syndecan 1
Mediates cell-matrix and cell-cell adhesion and inhibits cell invasion.						
GGAAATGACT	9	0	9	0.0091	46497	Ras homolog gene family, member E
Members of the Rho family of Ras-related GTPases, such as ARHE, regulate the organization of the actin cytoskeleton in response to extracellular growth factors. Rho family members appear to cycle between an inactive GDP-bound form and an active GTP-bound form.						
GGGGCAATCC	9	0	9	0.0091	296202	Inositol polyphosphate-5-phosphatase B
Involved in sertoli cell vacuolization and germ cell adhesion in mouse testes						
GGGGGAGGGA	9	0	9	0.0091	273915	Gene rich cluster, C3f gene
A PPARalpha regulated gene involved in lipid metabolism and known to be upregulated in cells with peroxisome proliferation.						
GTGCTGCCTT	9	0	9	0.0091	295565	Echinoderm microtubule associated protein like 4
Cytoskeletal component						
GTGTCTGATA	9	0	9	0.0091	738	Procollagen, type IV, alpha 1
Major type IV alpha collagen chain of basement membranes.						
TAACATTGTA	9	0	9	0.0091	22225	Zinc finger protein 313
Unknown function						
TAACCGAGAC	9	0	9	0.0091	277812	Villin 2
Involved in connections of major cytoskeletal structures to the plasma membrane. It plays a key role in cell surface structure adhesion, migration, and organization.						
TAGACAAAGG	9	0	9	0.0091	276815	Adenosine deaminase, RNA-specific, B1
Responsible for pre-mRNA editing of the glutamate receptor subunit B						
TATTGTGGCT	9	0	9	0.0091	195663	Cyclin-dependent kinase inhibitor 1A (P21)
Transcription factor/regulator responsive to activin A signalling and previously shown to regulate sertoli cell proliferation						

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
TCAGTTTAAT	9	0	9	0.0091	275266	Rho guanine nucleotide exchange factor (GEF) 12
Rho GTPases mediate cellular processes that are initiated by extracellular stimuli through G protein coupled receptors. This particular transcript is highly expressed in haematopoietic stem cell fractions and has been shown to demonstrate colocalization with the insulin-like growth factor-1 (IGF-1) receptor, suggesting a potential physiologic as an activator of RhoA in response to IGF-1						
TGCAATATGG	9	0	9	0.0091	260084	Eukaryotic translation initiation factor 4A2
Subunit of a protein complex involved in cap recognition and is required as a single polypeptide chain for mRNA binding to ribosome.						
TGCTACTTTA	9	0	9	0.0091	153415	E2F transcription factor 5
The E2F family plays a crucial role in the control of cell cycle. This is an transcriptional activator of many genes whose products are involved in cell proliferation. E2F5 is dispensable for cell cycle progression but necessary for G1 arrest of cycling cells and while other members of the E2F family participate in maintaining a proliferative undifferentatied phenotype, E2F5 serves to maintain the differentiated state.						
TGGATTTGCT	9	0	9	0.0091	248337	Slit-like 2 (Drosophila)
Unknown function						
TTGAGGTAGA	9	0	9	0.0091	1421	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1
Active metalloprotease, cleaves proteoglycans, aggrecan, versican and brevican. Has angiogenic inhibitor activity. They have been associated with various roles in connective tissue organization, coagulation, inflammation, arthritis, angiogenesis and cell migration as well as follicular rupture.						
GTTGCTGAGA	30	53	83	0.0092	100113	Ribosomal protein 10
Structural component of ribosome						
AATGGCTAGC	5	18	23	0.0092	35389	Cytochrome c, somatic
Component of the electron transport chain in mitochondria. Cytochrome c is also involved in initiation of apoptosis.						
TGTGAAGTAG	37	16	53	0.0093	371546	ADP-ribosylation factor 1
The ARF1 protein is localized to the Golgi apparatus and has a central role in intra-Golgi transport.						
TCCTTATAIT	73	42	115	0.0099	290285	RAB39, member RAS oncogene family
Rab39 is a novel Golgi-associated Rab GTPase involved in cellular endocytosis.						
GAATTAACAT	7	21	28	0.01	234700	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide
One of a family of ubiquitous phosphoserine/threonine-binding proteins. Mice deficient in Ywhae have defects in brain development and neuronal migration. There is a crucial role for 14-3-3epsilon in neuronal development through sunstance of the effects of CDK5 phosphorylation						
TCCTGTGGGA	7	21	28	0.01	4533	Apolipoprotein A-IV precursor
Apolipoprotein A-IV precursor.						
GAAATATATG	11	27	38	0.01	2966	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 3
Energy metabolism.						
CCTCGGAAAA	9	24	33	0.01	371629	Ribosomal protein L38
Structural component of ribosome.						
AGAGAGAGAG	19	5	24	0.01	9277	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)
The secretory PLA2 (sPLA2) family, in which 10 isozymes have been identified, consists of low-molecular weight, Ca2+-requiring secretory enzymes that have been implicated in a number of biological processes, such as modification of eicosanoid generation, inflammation, and host defense.						
GTGCTTTCAA	4	16	20	0.01	289645	GTL2, imprinted maternally expressed untranslated mRNA
The mouse Gtl2 gene is differentially expressed during embryonic development, encodes multiple alternatively spliced transcripts, and may act as an RNA.						

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
GTTGAGGTTT	32	13	45	0.01	331964	DNA segment, Chr 4, Wayne State University 53, expressed
Unknown function						
GGCAGCACAA	24	8	32	0.011	9043	Heterogeneous nuclear ribonucleoprotein L
Plays a major role in the formation, packaging, processing, and function of mRNA.						
AAGGTCTTTA	0	8	8	0.011	298893	Casein kinase II, alpha 1 polypeptide
Casein kinase II is a serine/threonine protein kinase that phosphorylates acidic proteins such as casein.						
CGTGGTGGCC	0	8	8	0.011	45071	Cartilage oligomeric matrix protein
Cartilage oligomeric matrix protein is a noncollagenous extracellular matrix protein previously thought to have a relatively cartilage-specific expression pattern.						
TAAAGGATAC	0	8	8	0.011	1213	Mannan-binding lectin serine protease 1
Also known as the Ra-reactive factor (RARF), this is a complement-dependent bactericidal factor that binds to the Ra and R2 polysaccharides expressed by certain enterobacteria. This gene is involved in the mannan-binding lectin (MBL) pathway of complement activation.						
TTCCTATATT	59	32	91	0.011	179011	Vav2 oncogene
VAV2 is the second member of the VAV oncogene family universally expressed in most tissues. Acts as a guanine nucleotide exchange factor for the rho family of ras-related gtpases. Vav-2 has a role in B cell antigen receptor calcium signaling and is critical to B cell development and function						
ATCACTCCAA	11	1	12	0.011	273142	Membrane interacting protein of RGS16
A mammalian glycerophosphoinositol phosphodiesterase regulated by stimulation of G protein-coupled receptors. MIR16 provides a link between phosphoinositide metabolism and G protein signal transduction.						
GTGCTACTCC	11	1	12	0.011	738	Procollagen, type IV, alpha 1
Cytoskeletal component						
TGCAGTGTTA	11	1	12	0.011	249342	7-dehydrocholesterol reductase
Catalyzes the reduction of 7-dehydrocholesterol (DHC), the terminal reaction in cholesterol biosynthesis.						
TTGTTACTGC	11	1	12	0.011	280231	Annexin A7
Calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis. Suggested to have a function in electromechanical coupling, probably through Ca(2+) homoeostasis.						
CCTTTAATTC	3	14	17	0.012	154306	Ubiquitin specific protease 45
Has a role in the negative regulation of gluconeogenesis. Required for proteosome-dependent catabolite degradation of fructose-1,6-bisphosphatase (fbpase). Accelerates proteosomal breakdown of ubiquitinated proteins as it disassembles free ubiquitin chains that would compete with ubiquitinated proteins to bind to the proteosome.						
AGGGGCCGGT	15	3	18	0.012	232930	Neuritin 1
Neuritin is an immediate-early gene induced by Ca(2+) influx through NMDA receptors and L-type voltage-sensitive calcium channels. Expression is mediated through the CaM kinase and MAP kinase pathways and is induced by camp. In neuronal tissue this is an effector gene targeted by signal transduction pathways mediating synaptic plasticity. It has also been shown to undergo androgenic regulation						
TTGATGTACA	15	3	18	0.012	223946	Splicing factor, arginine/serine-rich 11
The function of this protein is not yet known but structure and immunolocalization data suggest that it may play a role in pre-mRNA processing.						
ATTGTAATAT	13	2	15	0.012	4509	Runt related transcription factor 2
A member of the Core Binding Factor gene family (Runx 1, Runx 2 and Runx 3) of transcription factors with distinct tissue specific gene expression patterns which operate to regulate development in different cellular lineages. Runx genes have been identified as potential oncogenes.						
GGGGCAGGGA	13	2	15	0.012	204670	Thioredoxin domain containing 1
Modifies molecules with its oxidoreductase activity and be involved in the redox regulation in the endoplasmic reticulum.						
GTATGTATGG	13	2	15	0.012	17977	Transcription factor Dp 2
Can stimulate e2f-dependent transcription by binding DNA cooperatively with e2f transcription factor family members. The dp2/e2f complex functions in the control of cell-cycle progression from G1 to S phase and appears to mediate both cell proliferation and apoptosis.						
TCCCGATATC	13	2	15	0.012	263414	Poliovirus receptor-related 4
Expressed on differentiating neuronal cells during neurogenesis, may play a role in early neuronal differentiation and axon outgrowth.						

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
TGTTCATCTT	13	2	15	0.012	249555	Procollagen, type III, alpha 1
Component of cytoskeleton						
CAAATGCTGT	1	10	11	0.013	20943	FK506 binding protein 9
Also known as Peptidyl-prolyl cis-trans isomerase, accelerates the folding of proteins during protein synthesis						
GGAGATCTTT	1	10	11	0.013	167625	G protein-coupled receptor 85
G-protein coupled receptor with identical protein sequence in man and mouse, previously believed to be confined to the brain in the mouse						
GGAGCCATTG	1	10	11	0.013	275780	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5
Energy metabolism						
AATAAACACG	20	6	26	0.014	257629	Protease, serine, 35
Chymotrypsin and trypsin activity, proteolysis and peptidolysis						
AACGTGAGGT	8	0	8	0.015	258773	Zinc finger, MYND domain containing 11
Functions as a transcriptional repressor, inhibits the transcriptional activity of c-Myb.						
AAGCTCCGAC	8	0	8	0.015	12917	Multiple endocrine neoplasia 1
Men1 encodes menin, a nuclear protein. Interacts with several transcription factors and inhibits their activities and is essential for repression of the endogenous IGFBP-2, a gene that can regulate cell proliferation.						
AGAATATTTT	8	0	8	0.015	253564	Actinin, alpha 1
Alpha actinin is an f-actin cross-linking protein with multiple roles in different cell types. In nonmuscle cells, the cytoskeletal isoform is found along microfilament bundles and adherens-type junctions, where it is involved in binding actin to the membrane.						
AGATCATCTA	8	0	8	0.015	246003	Frizzled homolog 1 (Drosophila)
Members of the 'frizzled' gene family are receptors for Wnt signaling proteins. The FZD1 protein contains a signal peptide and belongs to the g-protein coupled receptor fz/smo family.						
ATCCGGCGCC	8	0	8	0.015	153758	Transcription elongation factor B (SIII), polypeptide 2
Encodes the protein elongin B, which is a regulatory subunit of the transcription factor B (SIII) complex. This complex activates elongation by RNA polymerase II.						
ATGTTTGGGG	8	0	8	0.015	17461	Serine/threonine kinase 16
CCCTCTGGAT	8	0	8	0.015	100144	S100 calcium binding protein A6 (calcyclin)
Implicated in the regulation of cell growth and proliferation.						
CTCAGATAAC	8	0	8	0.015	34399	Zinc metalloproteinase, STE24 homolog (S. cerevisiae)
A multispanning membrane protein widely distributed in mammalian tissues. Disruption caused severe growth retardation and premature death in homozygous-null mice. Zmpste24-null mice are defective in the proteolytic processing of prelamin A.						
GGGGGGAAGA	8	0	8	0.015	27308	ADP-ribosylation factor 6
A small guanine nucleotide-binding protein that plays a role in vesicular trafficking and as an activator of phospholipase D.						
GTAGCGCTCA	8	0	8	0.015	5121	Peptidylglycine alpha-amidating monooxygenase
The protein encoded by this gene localizes to perinuclear endosomes and associates with peptidylglycine alpha-amidating monooxygenase, and may be involved with the trafficking of this enzyme through secretory or endosomal pathways.						
TCCCCCCCTT	8	0	8	0.015	4946	Ins2 Insulin II
Insulin upregulated gene						
TCTCTCAGTC	8	0	8	0.015	1620	Annexin A5
The protein encoded by this gene belongs to the annexin family of calcium-dependent phospholipid binding proteins some of which have been implicated in membrane-related events along exocytotic and endocytotic pathways. Annexin 5 is a phospholipase A2 and protein kinase C inhibitory protein with calcium channel activity and a potential role in cellular signal transduction, inflammation, growth and differentiation.						
TGACATCCAT	8	0	8	0.015	233889	Solute carrier family 39 (zinc transporter), member 10
Membrane bound solute transport						
TGTCCACACA	8	0	8	0.015	276656	Low density lipoprotein receptor-related protein 8, apolipoprotein e receptor

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
This gene encodes an apolipoprotein E receptor, a member of the low density lipoprotein receptor (LDLR) family. Apolipoprotein E is a small lipophilic plasma protein and a component of lipoproteins such as chylomicron remnants, very low density lipoprotein (VLDL), and high density lipoprotein (HDL). The apolipoprotein E receptor is involved in cellular recognition and internalization of these lipoproteins. Alternative splicing generates three transcript variants for this gene; additional variants have been described, but their full length nature has not been determined.						
TGTGGATGGC	8	0	8	0.015	30602	Ubiquitin specific protease 22
Belongs to the peptidase c19 family. Invovled in the synthesis and degradation of proteins.						
TTGCTGCCCT	8	0	8	0.015	247764	Peroxisome biogenesis factor 19
PEX genes encode the machinery required to assemble the peroxisome. Membrane assembly and maintenance requires three of these (PEX 3, 16, and 19)						
TTCATTCTAG	18	5	23	0.016	122366	Heterogeneous nuclear ribonucleoprotein F
RNA binding protein complexes with heterogeneous nuclear RNA (hnRNA). These proteins are associated with pre-mRNAs in the nucleus and appear to influence pre-mRNA processing and other aspects of mRNA metabolism and transport.						
TATCCCACGC	23	8	31	0.016	280038	S100 calcium binding protein A11 (calizzarin)
Proinflammatory cytokine and a chemoattractant for monocytes and granulocytes. Strongly expressed at sites of apoptosis in the mouse embryo and in tumor necrosis factor alpha (TNF)-treated murine cells, High level expression correlates predominantly with hypoxic and apoptotic cells. It is also an anti-angiogenic protein which inhibits fetal lung neovascularization and leads to the arrest of lung airway epithelial morphogenesis and apoptosis						
TAACAGTTGT	38	18	56	0.017	248827	Calnexin
Calcium-binding protein that interacts with newly synthesized glycoproteins in the endoplasmic reticulum. It may act in assisting protein assembly and/or in the retention within the ER of unassembled protein subunits. Seems to play a major role in the quality control apparatus of the ER by the retention of incorrectly folded proteins						
GCACAACTTG	12	27	39	0.017	329243	Calmodulin 2
Calmodulin is the principal mediator of the intracellular calcium signa and is therefore involved in regulation of numerous processes.						
AAGCAGAAGG	16	4	20	0.017	1	S100 calcium binding protein A10 (calpactin)
The protein encoded by this gene is a member of the S100 family of proteins and is involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. May function in exocytosis and endocytosis.						
CTGAACATCT	16	4	20	0.017	5286	Acidic ribosomal phosphoprotein P0
Structural component of ribosome						
TCCATATATT	21	7	28	0.018	259667	RNA binding motif, single stranded interacting protein 1
This gene encodes a member of a small family of proteins which bind single stranded DNA/RNA. This protein interacts with the region upstream of the c-myc gene and may have a role in DNA replication. Has been shown to suppres hypoxia inducible factor 1 and VEGF expression blocking angiogenesis						
AAGGGTGCTG	10	1	11	0.019	265347	Annexin A6
Annexin VI belongs to a family of calcium-dependent membrane and phospholipid binding proteins. Although their functions are still not clearly defined, several members of the annexin family have been implicated in membrane-related events along exocytotic and endocytotic pathways. Annexin VI has been implicated in mediating the endosome aggregation and vesicle fusion in secreting epithelia during exocytosis						
ACAACAGAGG	10	1	11	0.019	24807	Prostaglandin F2 receptor negative regulator
Induces a decrease in receptor number, suggesting a non-competitive means of inhibition of prostaglandin receptors						
ATAGAGAAGG	10	1	11	0.019	250419	Cyclin I
Controls cell-cycle progression by regulating the activity of cyclin-dependent kinases.						
CCTCTAGCTG	10	1	11	0.019	29346	Guanosine monophosphate reductase 2
A guanosine monophosphate reductase, can promote the monocytic differentiation of HL-60 leukemia cells.						
CCTGATCTTT	10	1	11	0.019	4071	Laminin receptor 1 (ribosomal protein SA)
Structural constitutent of ribosome						
GCCACTTCCT	10	1	11	0.019	371598	Nuclear receptor coactivator 4
ARA70 enhances androgen receptor transactivation through the increase of receptor expression, protein stability, and nuclear translocation. Also regulates induction and repression of PPAR alpha transcription.						
GTTGTTTGTT	10	1	11	0.019	20827	Homeodomain interacting protein kinase 1

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
The protein encoded by this gene belongs to the Ser/Thr family of protein kinases. Also modulates localization, phosphorylation, and transcriptional activity of Daxx, a transcriptional regulator important for transducing growth regulatory signals.						
TCACATAAAT	10	1	11	0.019	31752	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)
This gene encodes a membrane protein involved in the increase in intracellular calcium concentration that occurs upon cell adhesion to extracellular matrix.						
TCATTTGGTG	10	1	11	0.019	172	Lysyl oxidase
Initiates the crosslinking of collagens and elastin.						
TCTAGCCAGA	10	1	11	0.019	35253	Solute carrier family 12 (potassium/chloride transporters), member 8
Mediates sodium and chloride reabsorption, plays a role in the regulation of ionic balance and cell volume						
TGCCGTATGC	10	1	11	0.019	6442	Polycystic kidney disease 2
PKD2 encodes a protein of unknown function, mutated in 15% of autosomal dominant polycystic kidney disease families.						
TGTACAAATG	10	1	11	0.019	15622	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1
Binds to specific glycoside residues on multiple extracellular ligands, mediates cell-cell and cell-matrix interactions in a variety of cells. Also acts as a sperm surface receptor binding to the zona pellucida glycoprotein during fertilisation.						
TGTTATGTAA	10	1	11	0.019	277376	ATP-binding cassette, sub-family A (ABC1), member 1
ABC proteins transport various molecules across extra- and intracellular membranes. Functions as a cholesteral efflux pump in the cellular lipid removal pathway. Essential for high density lipoprotein (HDL) formation and considered rate-controlling for reverse cholesterol transport.						
CTGAGGAAGT	14	3	17	0.019	29027	SPARC-like 1 (mast9, hevin)
An extracellular calcium-modulated protein that binds collagen I. Similar to SPARC (secreted protein acidic and rich in cysteine), a matricellular protein that regulates cell adhesion, cell cycle, and matrix assembly and remodeling.						
CAAAATACAT	8	21	29	0.019	27578	Melanoma antigen, family D, 1
Mediates p75 neurotrophin receptor-dependent apoptosis binding in neuronal cells. Inhibits cell cycle progression, and facilitates p75ntr-mediated apoptosis. Closely related to the magphinin proteins known to be present in the male and female germ cells during gametogenesis.						
GTGTTTTGTG	25	44	69	0.019	201455	Secretory carrier membrane protein 1
Functions as a carrier to the cell surface in post-golgi recycling pathways.						
AAGGTCGAGC	3	13	16	0.019	282814	Ribosomal protein L24
Structural component of ribosome						
CCCTGATTTT	3	13	16	0.019	185453	Eukaryotic translation initiation factor 4, gamma 2
A novel translational repressor mRNA. This translation initiation factor homolog for eIF4G controls specific gene expression pathways required for cellular differentiation. Null cells exhibit an impaired ability to differentiate.						
GTGCATTTCA	3	13	16	0.019	5199	Cytochrome P450, family 19, subfamily a, polypeptide 1
Cytochrome P450 aromatase catalyzes the last steps of estrogen biosynthesis.						
GTGCTATTCA	12	2	14	0.019	1639	Myeloid cell leukemia sequence 1
Belongs to the Bcl-2 family. Alternative splicing occurs and two distinct isoforms have been identified. The longer gene product (isoform 1) enhances cell survival by inhibiting apoptosis while the alternatively spliced shorter gene product (isoform 2) promotes apoptosis and is death-inducing. Acts via the MAPK pathway and can prolong cell viability under a variety of cytotoxic conditions.						
TACATTAATA	12	2	14	0.019	225505	Choline kinase alpha
Implicated in hematopoietic development but null mice develop normally suggesting action is compensated by activity of a closely related protein tyrosine kinase Csk.						
TCAACTTGGG	12	2	14	0.019	156727	Hyperparathyroidism 2 homolog (human)
Unknown function						
TCCTTATTGC	12	2	14	0.019	14547	Chromobox homolog 2 (Drosophila Pc class)

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
Transcriptional repressor. Homozygous null mice show greatly retarded growth, homeotic transformations of the axial skeleton, sternal and limb malformations and a failure to expand in vitro of several cell types including lymphocytes and fibroblasts.						
AACTTTTAAA	0	7	7	0.02	354643	Heterochromatin protein 1, binding protein 3
Necessary for the condensation of nucleosome chains into higher order structures. This product appears during meiosis in spermatogenesis, expressed in pachytene spermatocytes during meiotic prophase 1.						
AATTGTATTT	0	7	7	0.02	10651	GTP cyclohydrolase 1
Amino acid synthesis						
CCAATGAACT	0	7	7	0.02	235123	Inner membrane protein, mitochondrial
Structural component of mitochondrial membrane						
CCTTTAATGC	0	7	7	0.02	219648	THO complex 1
Unknown function						
GCATCCAGCT	0	7	7	0.02	277091	Poly(A)-binding protein, cytoplasmic pseudogene
Binds to the poly(A) tail present at the 3-prime ends of most eukaryotic mRNAs.						
GCTCACAACC	0	7	7	0.02	272930	Component of oligomeric golgi complex 4
Component of Golgi apparatus						
GGTTTTCAAG	0	7	7	0.02	259278	SMT3 suppressor of mif two 3 homolog 1 (yeast)
Has a key role in the modification of many transcription factors including PPAR gamma.						
GTCTATGTTG	0	7	7	0.02	4952	Insulin receptor substrate 1
The principal substrate for the insulin and insulin-like growth factor-1 (IGF-1) receptors. Disruption of IRS1 results in insulin resistance, but not DM, because of compensatory hyperinsulinemia.						
AAAATGTCAA	1	9	10	0.022	208554	Oogenesin 1
Mouse protein expressed in oocytes during follicle development and in early cleavage-stage embryos specific function unknown.						
GGCTTTTTCG	1	9	10	0.022	27832	V-ral simian leukemia viral oncogene homolog B (ras related)
This gene encodes a GTP-binding protein that belongs to the small GTPase superfamily and Ras family of proteins. GTP-binding proteins mediate the transmembrane signaling initiated by the occupancy of certain cell surface receptors.						
TATGCAGATG	1	9	10	0.022	2662	Glutathione S-transferase, alpha 4
In mice GSTA4 has been shown to be induced by TNFalpha, IL-6 and EGF, factors that play crucial roles in cell survival and proliferation suggesting an antiapoptotic role.						
CTAGTCTTTG	68	94	162	0.025	154915	Ribosomal protein S29
Structural component of ribosome						
AAATGCACTA	7	0	7	0.026	57734	LIM and senescent cell antigen-like domains 1
Involved in the regulation of integrin-mediated cell adhesion						
ACTCTAAGTT	7	0	7	0.026	347919	B-cell stimulating factor 3
Recently indentified cytokine acts as a novel myeloma growth and survival factor						
AGAAAGGATA	7	0	7	0.026	296814	Phosphodiesterase 7A
Plays a role in signal transduction by regulating the intracellular concentration of cyclic nucleotides, particularly cAMP						
AGACAAGCTG	7	0	7	0.026	43331	Splicing factor, arginine/serine-rich 5 (SRp40, HRS)
Plays a role in constitutive splicing and can modulate the selection of alternative splice sites.						
AGATGTACTG	7	0	7	0.026	21767	Cadherin 5
Interacts with filamentous actin forming a functional complex controlling endothelial tube formation.						
AGCCAAACAA	7	0	7	0.026	172897	Mitogen activated protein kinase kinase kinase 12
Protein serine/threonine kinase which plays an active part in cellular processes related to terminal differentiation, With cell-type-specific expression in the seminiferous tubules of mature testes, and other tissues.						
AGTGTGACGT	7	0	7	0.026	43081	Mitogen-activated protein kinase 8 interacting protein 3
Encodes a scaffold protein for c-Jun N-terminal kinase cascades.						

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
ATCAGTACTA	7	0	7	0.026	46401	Son cell proliferation protein
Unknown function						
CGAAAGAACA	7	0	7	0.026	23335	YME1-like 1 (S. cerevisiae)
The protein encoded by this gene is the mouse ortholog of yeast mitochondrial AAA metalloprotease, Yme1p. It is proposed that this gene plays a role in mitochondrial protein metabolism.						
CTGTAGACTG	7	0	7	0.026	3158	Ribosomal protein, large, P1
Structural component of ribosome						
GCTGACTCCG	7	0	7	0.026	34374	Chymotrypsinogen B1
Inactive precursor of alpha-chymotrypsin, one of a family of serine proteases						
GTACTTGTCT	7	0	7	0.026	29658	Chemokine-like factor super family 4
Function unknown						
TAAATAAGAT	7	0	7	0.026	158700	Chondroitin sulfate proteoglycan 2
May play a role in intercellular signaling and in connecting cells with the extracellular matrix. Binds hyaluronic acid.						
TATCTATACA	7	0	7	0.026	258475	Zinc finger and BTB domain containing 24
Regulator of Bax-mediated signaling pathways for apoptosis. Bax is a proapoptotic member of the Bcl-2 protein family that commits the cell to undergo programmed cell death in response to apoptotic stimuli.						
TGCCAATAAT	7	0	7	0.026	23156	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 4
Member of the ADAMTS protein family. Responsible for the degradation of aggrecan, a major proteoglycan of cartilage, and brevican, a brain-specific extracellular matrix protein.						
TGCCTGTCTG	7	0	7	0.026	3064	properdin
A positive regulator of the alternate pathway of complement. Binds to and stabilizes the c3- and c5-convertase enzyme complexes. The structural locus for murine tissue inhibitor of metallothionine proteases (Timp) and properdin are located together.						
TGGTCGCTGA	7	0	7	0.026	295031	DEAD (Asp-Glu-Ala-Asp) box polypeptide 27
Probable ATP-dependent RNA helicase DDX27						
TGTGCTGTTG	7	0	7	0.026	32700	Sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog (S. cerevisae)
Fatty acid biosynthesis and sterol metabolism						
TTGCTAAGAA	7	0	7	0.026	272675	Solute carrier family 20, member 1
Sodium-phosphate symporter. Active in early growth phase.						
TTGGCTGGAT	7	0	7	0.026	3815	Syndecan 4
Member of the syndecan gene family of heparan sulfate proteoglycans with the ability to bind and activate protein kinase C-alpha. Production stimulated by TNF alpha in ischaemic environments and it is an important cell-surface receptor in wound healing and angiogenesis						
TGTAACTGGT	15	4	19	0.027	233009	RAS related protein 1b
Belongs to a superfamily of RAS-like small GTP-binding proteins involved in cell signalling. Promotes integrin-dependent cell adhesion and can modulate integrin alpha(IIb)beta(3) interactions with the actin cytoskeleton.						
TGTGAACGAA	15	4	19	0.027	18522	Carnitine palmitoyltransferase 1a, liver
Responsible for the initiation of the mitochondrial oxidation of long-chain fatty acids.						
GAGTGGATTC	20	7	27	0.027	277857	Hydroxysteroid (17-beta) dehydrogenase 4
Bifunctional enzyme acting on the peroxisomal beta- oxidation pathway for fatty acids. Catalyzes the formation of 3- ketoacyl-coa intermediates from both straight-chain and 2-methyl- branched-chain fatty acids.						
TTCATTATAA	6	17	23	0.028	19187	Prothymosin alpha
Nuclear protein thought to play a critical role in cellular proliferation. Transcription activated by the proto-oncogene c-myc. During mouse foetal development the temporal, spatial and tissue-specific expression patterns of both myc proto-oncogenes coincide with the pattern of prothymosin alpha.						
CTGTAGGTGA	36	56	92	0.029	295618	Ribosomal protein S23

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
Structural component of ribosome.						
AACTGCTTCA	13	3	16	0.03	30010	Actin related protein 2/3 complex, subunit 1B
Part of a complex implicated in the control of actin polymerization in cells						
ATACGGAGCA	13	3	16	0.03	333183	Mitochondrial ribosomal protein L53
Structural component of ribosome						
ATCTCAAACC	13	3	16	0.03	248163	Mitochondrial ribosomal protein L52
Structural component of ribosome						
ATTATACAGT	13	3	16	0.03	29192	Asparaginyl-tRNA synthetase
Protein synthesis						
CTCTGGGGTT	13	3	16	0.03	271711	Transgelin 2
The protein encoded by this gene is a homolog of the protein transgelin, which is one of the earliest markers of differentiated smooth muscle. It is an actin-associated protein with a unique cell lineage-restricted pattern of expression.						
TGAATTCCCT	13	3	16	0.03	373569	Chemokine-like factor super family 3
Unknown function						
CTGCTATCCG	20	36	56	0.03	4419	Ribosomal protein L5
Structural component of ribosome.						
ACCACTGATA	9	1	10	0.031	50424	Tax1 (human T-cell leukemia virus type I) binding protein 1
Cellular myosin, appears to play a role in cytokinesis, cell shape, and specialized functions such as secretion and capping. Also implicated in Rho signalling pathways.						
ACTCCTCCCT	9	1	10	0.031	333868	RAS-related protein-1a
RAP1A belongs to a superfamily of RAS -like small GTP-binding proteins involved in cell signaling. Involved in interactions with beta 1 and beta 2 integrins						
ATACTGAAGC	9	1	10	0.031	319719	Ribosomal protein L13
Structural component of ribosome						
ATCAAAGTTC	9	1	10	0.031	10299	Col5a2 Procollagen, type V, alpha 2
Alpha chain for one of the low abundance fibrillar collagens.						
ATGCACAGAT	9	1	10	0.031	2551	SEC22 vesicle trafficking protein-like 1 (S. cerevisiae)
Cytoplasmically oriented integral membrane protein involved in vesicle docking/fusion fidelity between the endoplasmic reticulum and Golgi.						
ATTTCCCGAG	9	1	10	0.031	288974	Actin related protein 2/3 complex, subunit 5
This gene encodes a subunit of the human Arp2/3 protein complex which has been implicated in the control of actin polymerization in cells and is highly conserved across species.						
CCCGTAGCCC	9	1	10	0.031	121878	Tropomyosin 1, alpha
Ubiquitous protein associated with the actin filaments of myofibrils and stress fibers. In nonmuscle cells is implicated in stabilizing cytoskeleton actin filaments.						
GGTAACCTAA	9	1	10	0.031	3213	Low density lipoprotein receptor
Cell surface protein involved in receptor-mediated endocytosis. Low density lipoprotein (LDL) is normally bound at the cell membrane and taken into the cell. Providing the substrate for cholesterol synthesis.						
TGAACTGTAA	9	1	10	0.031	270647	Solute carrier family 39 (zinc transporter), member 14
No literature information available						
TGATGCTAAA	9	1	10	0.031	304976	Down-regulated by Ctnnb1, a
DRCTNNB1A has been shown to be involved in the beta-catenin-Tcf/Lef signaling pathway.						
TTCAGGCACT	9	1	10	0.031	38436	Tetratricopeptide repeat domain 13
No literature information available						

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
GCAGCTCACA	3	12	15	0.031	7236	Dolichyl-di-phosphooligosaccharide-protein glycotransferase
Essential subunit of n-oligosaccharyl transferase enzyme which catalyzes the transfer of a high mannose oligosaccharide.						
TCTGTGCACC	3	12	15	0.031	196538	Ribosomal protein S11
Structural component of ribosome.						
ATGAGTGAGC	11	2	13	0.031	27183	Phenylalkylamine Ca2+ antagonist (emopamil) binding protein
Emopamil-binding protein (EBP) is an integral membrane protein of the endoplasmic reticulum. It is similar to sigma receptors and may be a member of a superfamily of high affinity drug-binding proteins in the endoplasmic reticulum of different tissues. EBP shares structural features with bacterial and eukaryontic drug transporting proteins.						
CAGACCTCAA	11	2	13	0.031	250605	Sel1 (suppressor of lin-12) 1 homolog (C. elegans)
Impacts on the expression of genes involved in regulation of cellular growth, possibly through the TGF-beta signaling pathway. Expression has been associated with a reduction in both proliferative activity in vitro and in vivo neoplastic aggressiveness						
CAGATTGTGA	11	2	13	0.031	317701	Thioredoxin domain containing 4 (endoplasmic reticulum)
May be involved in the control of oxidative protein folding.						
GGGAACAACT	11	2	13	0.031	8687	CAP, adenylate cyclase-associated protein 1 (yeast)
Highly conserved actin monomer binding protein present in all eukaryotes, promotes rapid actin filament depolymerization and is important for cell morphology, migration, and endocytosis. CAP promotes rapid actin dynamics in conjunction with ADF/cofilin and is required for several central cellular processes in mammals.						
TGAGTTCCCT	11	2	13	0.031	38390	Copine III
Ubiquitously expressed, intrinsic kinase activity. Represents a novel unconventional kinase family.						
GAACTTGCAA	24	10	34	0.034	39472	Farnesyl diphosphate synthetase
Catalyzes the sequential condensation of isopentenyl pyrophosphate with the allylic pyrophosphates, dimethylallyl pyrophosphate, and then with the resultant geranylpyrophosphate to the ultimate product farnesyl pyrophosphate Involved in cholesterol biosynthesis.						
ATGTCTCAAA	30	48	78	0.035	324696	Tubulin, alpha 2
Cytoskeletal component						
AGGCAGACAG	131	94	225	0.036	335315	Eukaryotic translation elongation factor 1 alpha 1
Tissue-specific isoform of peptide elongation factor-1A (eEF1A-1); its mRNA is expressed only in, tissues dominated by terminally differentiated, long-lived cells such as brain, heart, and skeletal muscle. Has been shown to be a specific translational target of TrkA signalling with eEF1A-1 mRNA translation stimulated by the NGF-signaling pathway						
AATGTTTCTG	2	10	12	0.036	7141	Proliferating cell nuclear antigen
This protein is an auxiliary protein of DNA polymerase involved in the control of eukaryotic dna replication.						
CACTGACGAG	2	10	12	0.036	2871	DnaJ (Hsp40) homolog, subfamily C, member 9
Novel member of the DnaJ family, is expressed in the germinal zone of the rat brain, liver lung and kidney.						
AAGAAAACAT	0	6	6	0.036	218851	Eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked
eIF-2 is composed of three subunits, [alpha], [beta] and [gamma], which, in the presence of GTP, form a ternary complex with Met-tRNA, recruiting it to the 40S ribosomal complex						
AAGAGAAAAG	0	6	6	0.036	240066	Proteasome (prosome, macropain) activator subunit 4
Nuclear proteasome activator involved in DNA repair.						
CAAACTGTAT	0	6	6	0.036	196405	Hydroxysteroid dehydrogenase-2, delta<5>-3-beta
Bifunctional enzyme, catalyzes the oxidative conversion of delta(5)-ene-3-beta-hydroxy steroid, and the oxidative conversion of ketosteroids. Plays a crucial role in the biosynthesis of all classes of hormonal steroids.						
CGCTCTAACG	0	6	6	0.036	25594	Protein kinase, cAMP dependent regulatory, type II beta

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene

cAMP-dependent protein kinase (AMPK) transduces signals through phosphorylation of different target proteins. Four different regulatory subunits and three catalytic subunits of AMPK have been identified in humans. The protein encoded by this gene is one of the regulatory subunits. This subunit has been shown to interact with and suppress the transcriptional activity of the cAMP responsive element binding protein 1 (CREB1) in activated T cells.

GTCTTTGTGA	0	6	6	0.036	200775	Transforming growth factor, beta receptor III

Transforming growth factor beta (TGF-beta) signaling is mediated by the cell surface TGF-beta type I (ALK5), type II, and the accessory type III receptors endoglin and betaglycan. The type III receptor may regulate the ligand-binding ability or surface expression of the type II receptor

TCTGGCAGTC	0	6	6	0.036	221452	RAN binding protein 5

Involved in nucleocytoplasmic transport. The protein encoded by this gene is a member of the importin beta family.

TGAAATGGCC	0	6	6	0.036	28020	Cofactor required for Sp1 transcriptional activation, subunit 3

Sp 1 transcriptional factor cofactor implicated in metastasis suppression in neoplastic cells.

AACAAATTCT	1	8	9	0.039	35628	Fucosyltransferase 8

The product of this gene catalyzes the transfer of fucose from GDP-fucose to N-linked type complex glycopeptides. The expression of this gene may contribute to the malignancy of cancer cells and to their invasive and metastatic capabilities.

CAGGGCTCCG	1	8	9	0.039	193096	Stearoyl-Coenzyme A desaturase 2

Integral membrane protein, belongs to the fatty acid desaturase family. Terminal component of the microsomal stearyl-coa desaturase system, that utilizes o(2) and electrons from reduced cytochrome b5 to catalyze the insertion of a double bond into a spectrum of fatty acyl-coa substrates.

TACAAAATTA	1	8	9	0.039	355306	A disintegrin and metalloproteinase domain 17

This gene encodes a disintegrin and metalloprotease (ADAM) domain 17, which is a member of the ADAM protein family. Members of this family are membrane-anchored proteins implicated in a variety of biologic processes involving cell-cell and cell-matrix interactions, including fertilization, muscle development, and neurogenesis. The member encoded by this gene functions as a tumor necrosis factor-alpha converting enzyme; binds mitotic arrest deficient 2 protein; and also plays a prominent role in the activation of the Notch signaling pathway. Also identified as the major convertase of epiregulin, TGF alpha, amphiregulin and EGF-like growth factor.

TTTACTGTGT	1	8	9	0.039	28897	Pyrophosphatase

Regulates constituency of extracellular matrix

CGTCTGTGGA	19	7	26	0.04	30155	ATPase, H+ transporting, V0 subunit C

Encodes a component of vacuolar ATPase (V-ATPase), an enzyme that mediates acidification of organelles, necessary for processes such as protein sorting, zymogen activation, endocytosis, and synaptic vesicle proton gradient generation.

TGCACTGCTG	19	7	26	0.04	44151	Cofactor required for Sp1 transcriptional activation, subunit 6

Nuclear receptor cofactor for Sp 1

CATTGCGTGG	22	9	31	0.041	27955	Williams-Beuren syndrome chromosome region 1 homolog (human)

Translation initiation factor, functions to stimulate the initiation of protein synthesis at the level of mRNA utilization.

GTTCTGACAG	14	4	18	0.041	261025	Carnitine deficiency-associated gene expressed in ventricle 3

Localized mainly in cytoplasm, phosphorylated preferentially by Abl. Phosphorylation of tyrosine 120 in this protein leads to an apparent mobility shift in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, suggesting conformational change in the phosphorylated protein. Thus, appears to be a novel substrate of Abl tyrosine kinase. Function unknown.

GTTTAGTGGA	6	16	22	0.043	252255	Proteasome (prosome, macropain) subunit, alpha type 2

Part of a multicatalytic proteinase complex that plays a role in the generation of peptides for presentation by major histocompatibility complex class I molecules.

AAAGACACTA	6	0	6	0.046	360445	Interferon regulatory factor 2 binding protein 2

Interacts specifically with Interferon Regulatory Factor-2 (IRF-2). Nuclear protein with the properties of IRF-2-dependent transcriptional co-repressors that can inhibit both enhancer-activated and basal transcription

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
AACTGCACAC	6	0	6	0.046	246965	Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)
Complex II of the respiratory chain, which is specifically involved in the oxidation of succinate, carries electrons from FADH to CoQ.						
AGGCGTGGCT	6	0	6	0.046	30849	Prenylcysteine oxidase 1
Involved in the degradation of prenylated proteins to yield free cysteine and the aldehyde of the isoprenoid lipid, expressed at high levels in mouse liver, kidney, heart, and brain. Homozygous null mice are both healthy and fertile.						
AGGTGGCATT	6	0	6	0.046	2442	Calcium binding protein, intestinal
Belongs to a family of calcium-binding proteins that includes calmodulin, parvalbumin, troponin C, and S100 protein. Its exact function is unknown. In the intestine its expression correlates with calcium transport activity						
AGGTGTACAG	6	0	6	0.046	29397	Splicing factor, arginine/serine-rich 15
Acts to physically and functionally link transcription and pre-mrna processing (by similarity).						
ATAAACTGCA	6	0	6	0.046	230654	Testis derived transcript
Unknown function						
ATCAGTGTGA	6	0	6	0.046	275555	Calponin 3, acidic
ATGCTTCTCA	6	0	6	0.046	248313	RAB12, member RAS oncogene family
Localized to the Golgi complex with a role in the specification of the apicolateral junctional complex sites and the formation and segregation of apical tight junctions						
ATTAGGATGT	6	0	6	0.046	22347	Pinin
Involved in translation initiation by promoting the binding of the formylmethionine-tRNA to ribosomes.						
CAAGAATTAA	6	0	6	0.046	294083	Annexin A11
Calcium-dependent phospholipid-binding protein. Distinctive properties include calcyclin binding, autoantigenicity, organelle transport, nuclear localization, extracellular targeting, and tyrosine phosphorylation.						
CAGAACTTTG	6	0	6	0.046	4352	Procollagen, type XVIII, alpha 1
Alpha chain of type XVIII collagen, one of the multiplexins, an extracellular matrix proteins that contains multiple triple-helix domains interrupted by non-collagenous domains. Potently inhibits endothelial cell proliferation and angiogenesis. May inhibit angiogenesis by binding to the heparan sulfate proteoglycans involved in growth factor signalling such as FGF-2.						
CAGCAATAAA	6	0	6	0.046	291326	Mahogunin, ring finger 1
C3HC4 RING-containing protein with E3 ubiquitin ligase activity.						
CAGTCTTGAG	6	0	6	0.046	22560	Diaphorase 1 (NADH)
Oxidoreductase participating in NO production from vasorelaxants. Can convert selected NO donors, glycerol trinitrate (GTN) and formaldoxime (FAL) to nitrites and nitrates with NO as an intermediate.						
CTCTCCAGAA	6	0	6	0.046	274810	HRAS like suppressor 3
Growth inhibitory RAS target gene, target of interferon-regulatory factor-1 and is involved in IFNgamma-induced cell death in human ovarian carcinoma cells.						
CTGAATATCT	6	0	6	0.046	371545	Acidic ribosomal phosphoprotein P0
60S acidic ribosomal protein P0, structural component of ribosomes.						
CTGAGAATGC	6	0	6	0.046		Unmatched tag
GAGTCTCTTC	6	0	6	0.046	184021	Protein tyrosine phosphatase, receptor type, D
Regulates the tyrosine phosphorylation that controls cell activities and proliferation.						
GTAATCACGT	6	0	6	0.046	2654	WD repeat domain 1
The encoded protein may help induce the disassembly of actin filaments.						
TACAATAAAC	6	0	6	0.046	40321	Progesterone receptor membrane component 2
Steroid binding integral membrane protein						
TACCCCACAA	6	0	6	0.046	41849	Brain expressed, associated with Nedd4
No literature available						
TACTATAGTC	6	0	6	0.046	123211	Polymerase (DNA directed), beta
DNA polymerase beta is involved in DNA base excision repair. mRNA most abundant in spermatocytes at early pachytene may be involved in the repair-type DNA synthesis associated with the recombination process.						

Tag sequence	hCG	PMSG	Total tags	p-value	Unigene	Gene
TGAACACTGA	6	0	6	0.046	330731	Transglutaminase 2, C polypeptide
Catalyzes the cross-linking of proteins and the conjugation of polyamines to proteins						
TGAATGAATG	6	0	6	0.046		Unmatched tag
TGAATGAGCG	6	0	6	0.046	1401	Chemokine (C-X-C motif) receptor 4
Chemokine receptor specific for stromal cell-derived factor-1. Involved in neoplastic metastasis and in endothelial cell survival						
TGGAGCGTTG	6	0	6	0.046	6839	Cyclin-dependent kinase 4
Member of the Ser/Thr protein kinase family, catalytic subunit of the protein kinase complex that is important for cell cycle G1 phase progression. The activity of this kinase is restricted to the G1-S phase. Regulates cell proliferation and hypertrophy and the activity of the c-Myb transcription factor						
TGGCTCCATC	6	0	6	0.046	316652	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase catalyses the rate-limiting step for cholesterol synthesis. HMG-CoA reductase inhibitors have been shown to upregulate the cyclin-dependent kinase inhibitor p27Kip1 and to block cell proliferation through cholesterol-independent pathways. Growth hormone significantly increases both the LDL-r and HMG-CoA reductase transcript levels						
TGTGTCCCGC	6	0	6	0.046	287810	Importin 13
Importin beta-related receptors mediate translocation through nuclear pore complexes. Co-operation with the RanGTPase system allows them to bind and subsequently release their substrates on opposite sides of the nuclear envelope, which in turn ensures a directed nucleocytoplasmic transport. Imp13 also shows export activity towards the translation initiation factor eIF1A and is thus a case where a single importin beta-like receptor transports different substrates in opposite directions.						
TGTTAGCTCC	6	0	6	0.046	21667	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 12
Receptor protein, mediates TNF alpha membrane transport						
TTTGAGGATT	6	0	6	0.046	333893	Non imprinted in Prader-Willi/Angelman syndrome 2 homolog (human)
Novel gene, ubiquitously expressed, highly conserved with predicted function as receptor or transporter protein						
AGGAAGGCGG	48	68	116	0.046	11376	Ribosomal protein L36
Structural component of ribosome						
CTCCTGCAGA	12	3	15	0.046	275489	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 1
High-affinity, low capacity permease involved in the transport of the cationic amino acids (arginine, lysine and ornithine) in non-hepatic tissues.						
TCTTCATCAA	12	3	15	0.046	229151	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1
Members of the Imitation Switch (ISWI) family of chromatin remodeling proteins. Predominantly expressed in terminally differentiated neurons after birth and in adult animals and in terminally differentiated cells within ovaries and testes of adult mice. Suggested to have distinct functions associated with cell maturation or differentiation.						
TGCCTACAGT	12	3	15	0.046	1815	Cytidine 5'-triphosphate synthase
The catalytic conversion of UTP to CTP, important in the biosynthesis of phospholipids and nucleic acids, key role in cell growth, development, and tumorigenesis.						
CAAAAATAAA	18	32	50	0.047	70666	Enolase 1, alpha non-neuron
Glycolytic enzyme under the inducible control of hypoxia inducible factor 1						
TAAGTGGAAT	26	12	38	0.047	3360	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide
This highly conserved gene product belongs to a family of proteins which mediate signal transduction by binding to phosphoserine-containing proteins. Found in both plants and mammals, this protein is 99% identical in the mouse, rat and sheep. Involved in initiating the embryonic cellular communication system						

Appendix 2: List of abbreviations

ADAM	A disintegrin and metalloproteinase
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AhR	Aryl hydrocarbon receptor
Amh	Antimullerian hormone
Apaf-1	Apoptotic protease activating factor 1
Ar	Amphiregulin
AR	Androgen receptor
ARC	Apoptotic repressor
ATP	Adenosine triphosphate
Bad	Bcl-2 antagonist of cell death
BAG1	BAG family molecular chaperone regulator-1
Bax	Bcl-2 associated X protein
Bcl-2	B cell leukaemia/lymphoma 2
BDNF	Brain derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenic protein
Bok	Bcl-2 related ovarian killer protein
Boo	Apoptosis regulator Bcl-B
BORG	BMP/OP-responsive gene
BSA	Bovine serum albumin
B+W	Binding and washing buffer
BTC	Betacellulin
BTG2	B cell transclocation gene 2
C/EBPβ	CAAT enhancer binding protein β
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
cdk	Cyclin dependant kinase
C-fos	Proto-oncogene protein c-fos
Cgak	Cyclin G associated kinase
c-jun	Transcription factor AP-1
c-kit	Kit ligand receptor
CL	Corpus luteum
C-myc	Myc proto-oncogene protein
COC	Cumulus oocyte complex
COX-2	Prostaglandin endoperoxidase synthetase 2
CTGF	Connective tissue growth factor
Cx	Connexin
CYP 51	Lanosterol 14α-demethylase
Cyp11a1	Cytochrome P450 side chain cleavage enzyme
Cyp19	Cytochrome P450 aromatase enzyme
Dax-1	Nuclear receptor 0B1
DES	Diethylstilbestrol
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
dNTPs	DNA nucleotides
DTT	Dithiothreitol

E2	Oestrogen
eCG	Equine chorionic gonadotrophin
ECM	Extracellular matrix
EDTA	Ethylenediaminetetracetic acid
EGF	Epidermal growth factor
Egr-1	Early growth regulatory factor
EMBL	European Molecular Biology Laboratory
EPI	Epiregulin
ER	Oestrogen receptor
ERK	Extracellular regulated kinase
EST	Expressed sequence tag
FABP	Fatty acid binding protein
FIGα	Factor in the germline alpha
Fru-2,6-P2	Fructose-2,6-biphosphate
FS	Follistatin
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone
Fz	Frizzled
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
RACE	Rapid amplification of complementary ends
G-CSF	Granulocyte colony-stimulating factor
G-CSFR	Granulocyte colony-stimulating factor receptor
GDF-9	Growth and differentiation factor 9
GnRH	Gonadotrophin releasing hormone
GnSAF	Gonadotrophin surge attenuating factor
GPR	G protein coupled receptor.
SREB	Super-conserved receptor expressed in brain
GSTs	Glutathione S transferases
GVBD	Germinal vesicle breakdown
hCG	Human chorionic gonadotrophin
HDL	High density lipoprotein
HIF-1	Hypoxia inducible factor 1
HK	Hexokinase
HNE	4-hydroxynonenal
HSC	Haematopoietic stem cell
HSD	Hydroxysteroid dehydrogenase
ICN	Intracellular domain of notch
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IGF-R	Insulin-like growth factor receptor
IHOP	Information hyperlinked over proteins
GLGI	Generation of longer gene fragments for gene identification
Il	Interleukin
IL-1-ra	Interleukin 1 receptor antagonist
INSL3	Insulin like 3
JNK	c-Jun N-terminal kinases
Jun D	Jun proto-oncogene related gene d1
KGF	Keratinocyte growth factor

KL	Kit ligand
LB	Luria bertani
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LGR8	Leucine-rich repeat-containing G protein-coupled receptor 8
LH	Luteinising hormone
LHR	Luteinising hormone
Maml1	Mammalian homolog 1 (Drosphilia)
MAPK	Mitogen activated protein kinase
MAS	Meiosis activating sterol
MCAM	Melanoma cell adhesion molecule
MGF	Mast cell growth factor
MMP	Matrix metalloproteinase
MORF 4	Mortality factor 4
MPF	Maturation promoting factor
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NGF	Nerve growth factor
NGFI-B	Nerve growth factor induced
p21 CIP/KIP	Cyclin dependant kinase inhibitors
P4	Progesterone
P450scc	Cytochrome P450 side chain cleavage enzyme
PACAP	Pituitary cyclase-activating polypeptide
PAGE	Polyacrylamide gel electrophoresis
PAPP-A	Pregnancy associated plasma protein A
PCR	Polymerase chain reaction
PDE4D	cAMP specific phosphodiesterase 4D
PFKFB	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase
PGS-2	Prostaglandin endoperoxide synthase-2
PI	Phosphatidyllinositol
PI3	Phosphatidyllinositol-3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PMSG	Pregnant mare serum gonadotrophin
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone receptor
pRb	Retinoblastoma protein
PRL	Prolactin
QRT-PCR	Quantative reverse transcription polymerase chain reaction
RIP 140	Nuclear receptor interacting protein 1
RNA	ribonucleic acid
RTK/P	Receptor tyrosine kinase/phosphatase
RT-PCR	Reverse transcription polymerase chain reaction
SAGE	Serial analysis of gene expression
SF-1	Steroidogenic factor 1
SFRP-4	Secreted frizzled related protein 4
Sgk	Serum/glucocorticoid regulated kinase

Smad	Mothers against decapentaplegic homolog 2
Snk	Serum induced kinase
SPARC	Secreted acidic cysteine-rich glycoprotein
Spp1	Secreted phosphoprotein 1
SR-B1	Scavenger receptor B1
StAR	Steroidogenic acute regulatory protein
STC	Stanniocalcin
TAE	Tris acetate EDTA
TEMED	Tetra-methylethylene-diamine
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
TIMP	Tissue inhibitor of matrix metalloproteinase
TNF-α	Tumour necrosis factor alpha
TWEAK	TNF-related weak inducer of apoptosis
TZPs	Transzonal projections
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
Angptl4	Angiopoietin like 4
Zp	Zona pellucida protein

Appendix 3: Reference list

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