INTER-FOLLICULAR COMMUNICATION AND THE REGULATION OF FOLLICLE DOMINANCE

Stuart James Baker

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Declaration

I declare that this thesis has been composed by myself and has not been submitted for any previous degree. The work described herein is my own and all work of other authors is duly acknowledged. I also acknowledge all assistance given to me during the course of these studies.

Stuart James Baker

Abstract

The growth and development of an ovarian follicle is a long and complex process during which time the few successful follicles that are permitted to ovulate must pass through several checkpoints and selection mechanisms. The final process of selection, achieving the appropriate ovulatory number, is referred to as 'dominance'. To date, most of the research into follicular dominance has concentrated on its endocrine regulation. While endocrine control of follicle dominance can explain much of the later processes that occur (such as ensuring that subordinate follicles ultimately undergo atresia), intra-ovarian interactions are also involved in its regulation. Follicle-follicle interactions have various possible roles both at early stages when follicles are being selected from amongst a cohort or cluster of follicles and/or later, when dominant follicles are 'holding back' challengers. Furthermore, intra-follicular processes also mediate the response of a follicle to endocrine changes. These intra-ovarian processes have received less attention than endocrine changes, perhaps because they are less amenable to investigation. Further understanding of intra-ovarian interactions will help us to determine how each species selects the correct number of follicles for continued development during an ovulatory cycle.

In an attempt to begin to extend our knowledge in this area, this thesis had two principle goals: **I. To establish if direct contact between follicles may play a part in the selection of the ovulatory follicle(s) *in vivo***, and if this appeared to be the case, **II. To investigate the precise nature of direct follicle-follicle communication *in vivo* and *in vitro*.** **I.** To investigate how follicles are positioned in respect to one another *in vivo,* histological sections from a diverse range of mammalian species were examined, which clearly demonstrated that follicles are closely positioned in the ovary, making direct follicle-follicle contact mediated selection feasible. Furthermore, a numerical analysis of mouse ovaries was undertaken and revealed that pre-antral follicle 'clusters' are the norm in young mice. **II.** Investigations branched at this point. **a)** Culture experiments that regulated follicle-follicle contact determined that there was not an absolute requirement for contact to establish dominance, providing evidence for a very

locally acting diffusible factor. **b)** A molecular approach was taken in an attempt to identify possible genes involved in juxtacrine communication between follicles. **c)** Other experiments examined the role of FSH and LH as survival factors for follicles at different stages of development, using apoptotic laddering of genomic DNA as a sensitive assay for atresia. Early antral follicles were unable to utilise LH and became apoptotic in low levels of FSH, in contrast with the more mature antral follicles that remained healthy when provided with LH in these low FSH concentrations. These observations have implications for follicle selection, with the possibility that inter-follicular communication could result in a subordinate follicle being held in a retarded stage of development rendering it vulnerable to decreased systemic FSH.

In conclusion this thesis has demonstrated that geography-dependent follicle-follicle communication is feasible *in vivo*. Culture experiments demonstrated that it has a diffusible component and can be effected by the gonadotrophin environment of the follicle. Additionally the *Notch* family of neuro-genes were shown to be present in the ovary, although their expression and function remains to be clarified.

Publications arising, or containing work from this thesis

Published papers

Baker, S.J. and Spears, N.

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In preparation

Murray, A.A., Molinek, M.D., Baker, S.J., Hillier, S.G. and Spears, N.

Ascorbic acid supports survival of murine ovarian follicle in vitro

Spears, N., Baker, S.J., Lapping, R., Mullan, J., Sršeň, V., Nelson, R.J. and Allison, V.

Mouse ovarian follicles secrete factors affecting the growth and development of other ovarian follicles in vitro

Abstracts of oral presentations

Baker, S.J., Cameron, H.I. and Spears, N.

The role of mammalian neuro-genes in follicle development

Proceedings of the 4th National Ovarian Workshop 1999; Abstract 7

Baker, S.J. and Spears, N.

Follicle Stimulating Hormone inhibits apoptosis in pre- and early antral murine follicles in vitro

J. Reprod. Fert. Abstract Series 1997 19:40

Abstracts of poster presentations

Baker, S.J. and Spears, N.

The examination of pairs of co-cultured murine ovarian follicles

Abstracts of Serono XIth Ovarian Workshop 1996

Assistance given throughout this investigation

All the work detailed throughout this thesis was conducted solely by myself with the following exceptions:

Chapter 3: Vivian Allison cut the feline and marmoset histology sections.

Chapter 4: Vivian Allison assisted me in the preparation of polycarbonate membrane inserts and acted as an unbiased observer when making decisions whether or not follicle pairs where juxtapositioned according to criteria specified by myself.

Chapter 7: Helen Cameron (laboratory technician) assisted me in optomising and performing RT-PCR reactions, under my supervision. Theresa Grey (undergraduate student) performed the Notch 2 *in situ* hybridisation reaction illustrated in Figure 7.12, under my supervision and according to protocols optomised by myself.

I was assisted in setting up large follicle cultures by various members of the laboratory. They were: Vivian Allison, Alison Murray, Michael Molinek, Rowena Smith and Vlastimil Sršeň.

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For Emma

1971-1995

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Abbreviations

BCIP	5-bromo-4-chloro-3-indoyl phosphate
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytisine 5'-triphosphate
DEPC	diethylpyrocarbonate
dGTP	2'-deoxyguanosine 5'-triphosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTPs	deoxyribonucleoside triphosphates
dsDNA	double strand DNA
dTTP	2'-deoxythymidine 5'-triphosphate
dUTP	2'-deoxyuracil 5'-triphosphate
E_2	oestradiol 17β
En	embryonic day n
ECM	extracellular matrix
EDTA	ethelenediaminetetraacetic acid
FSH	follicle stimulating hormone
GCIF	granulosa cell inhibitory factor
GCMP	granulosa cell mitostatic protein
GDF-9	growth and differentiation factor 9
gDNA	genomic DNA
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IU	international unit
IVF	*in vitro* fertilisation
kb	kilobase

LB	Luria-Bertani
LH	luteinising hormone
MEM	Mimimal Essential Medium
MMP	matrix metalloproteinase
mRNA	messenger RNA
NBT	nitroblue tetrazolium chloride
OSE	ovarian surface epithelium
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGC	primordial germ cell
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
RNAse	ribonuclease
RT-PCR	reverse-transcriptase polymersase-chain reaction
SDS	sodium dodecyl sulphate
S.E.M.	scanning elctron microscopy
S.E.M.	standard error of the mean
SSC	saline sodium citrate buffer
ssDNA	single strand DNA
STWS	scotch tap water substitute
TBS	tris buffered saline
TE	tris-EDTA
TEA	triethanolamine
TIMP	tissue inhibitor of matrix metalloproteinases
T_{MAX}	maximum temperature
Tris	tris(hydroxymethyl)amine-methane
UV	ultraviolet

Chapter 1

General Introduction

1.1. FORMATION OF THE OVARY

1.1.1. Migration of the primordial germ cells to colonize the gonad

Primordial germ cells (PGCs) can be seen outside the embryo in the epithelium of the yolk sac on embryonic day 8 (E8) in the mouse and at about 3 weeks post-fertilization in the human. From here they migrate through the dorsal mesentery of the hind gut, reaching the genital ridge (mouse: E11-12; human: ~E30) and colonizing the indifferent gonad (Snow and Monk, 1983). The PGCs have defined pseudopodia and migrate by amoeboid movement, possibly directed by the secretion of a chemotactic substance from the genital ridge. It is the mesenchymal tissue of the embryonic indifferent gonad that gives rise to the somatic components of the ovarian follicle.

1.1.2. Proliferation and quiescence

Following invasion of the indifferent gonad, the PGCs lose their motility (Donovan *et al.*, 1986) and both they and the somatic cells undergo rapid proliferation (mouse: Snow and Monk, 1983). The mitotically active PGCs are now termed oogonia and continue to proliferate until shortly before birth when influenced by meiosis initiation factor, mitosis terminates and the oogonia enter the first meiotic division thereby becoming primary oocytes. During the first meiotic prophase the primary oocytes become surrounded by clusters of somatic mesenchymal or pre-granulosa cells (Gondos, 1970) that secrete a basal lamina thus forming the primordial follicles. The timing of this process differs between species and has been reported to occur both pre-partum (human: Gillman, 1948; rat: Mauleon, 1978) and post-partum (mouse and rabbit: Peters, 1978; rat: Ueno *et al.*, 1989). At this stage meiosis is arrested in diplotene of the first meiotic prophase (Bacharova, 1985), the chromosomes being enclosed in a nuclear membrane and referred to as the germinal vesicle. The majority of oocytes found within the adult ovary are housed in these primordial follicles (mouse: Peters *et al.*, 1973a; human: Forabosco *et al.*, 1991) and can remain in this quiescent state for the duration of a females' reproductive life span.

1.1.3. Early attrition of PGCs and primordial follicles

During both mitosis and meiosis vast numbers of PGCs are lost by atresia resulting in less than a third of potential follicles being formed (rat: Beaumont and Mandl, 1962; reviewed: Hirshfield, 1991a). During the formation of primordial follicles those PGCs that are not surrounded by somatic cells (50-70% in the rat) will also degenerate (Ohno and Smith, 1964). This loss of oocytes continues with the attrition of primordial follicles in early post-natal life but is rarer in older animals (mouse: Edwards *et al.*, 1977). The molecular, biochemical and cellular characteristics of the atretic process will be considered below.

1.2. GENERAL MORPHOLOGY OF THE OVARY

Histological studies of the bovine ovary (which is similar to that of the human) show that the ovary contains at least five zones (Vigne *et al.*, 1994; van Wezel and Rodgers, 1996). Zone 1, the ovarian surface, is covered by epithelial cells (ovarian surface epithelium, OSE) which are separated from the ovarian stromal cells by a dense basal lamina. Both the OSE and the stromal cells appear to contribute to the production of this extra-cellular matrix (Nicosia and Nicosia, 1988). Encapsulated by this continuous basal lamina, zone 2 contains a sparse population of ovarian surface stroma cells (the inner tunica albuginea), frequently spindle shaped and parallel to the epithelium (van Wezel and Rodgers, 1996). Zone 3 cells (the outer tunica albuginea) tend to be more rounded and have a less regimented orientation. Both zones 2 and 3 are rich in matrix components. Similarly, zone 4 is rich in collagen fibrils, but unlike the more cortical areas, has a denser stroma cell population. Large numbers of primordial and primary follicles are found in this region. Zone 5 contains less densely arranged stroma cells. Large antral follicles are observed in this region (van Wezel and Rodgers, 1996). Analogous zones have not been identified in the mouse ovary.

1.3. FOLLICLE STRUCTURE

As detailed previously, the majority of follicles found within the ovary are at the primordial stage and consist of an oocyte arrested in prophase I of the first meiotic division, surrounded by flattened pre-granulosa cells and a basal lamina (Figure 1.1). In the young mouse these follicles are found in closely packed clusters, at synchronized stages of development and frequently connected to each other by inter-follicular bridges (Zamboni and Merchant, 1973). An observation also found in the cat ovary (J. Mullan, personal communication. Figure 1.2). The oocytes in these follicles are approximately

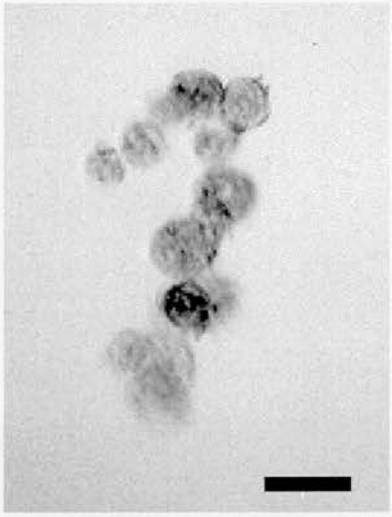


Figure 1.2 Photomicrograph of a string of cat primordial/ primary follicles. Scale bar represents 50 μm. Kindly supplied by J. Mullan.

15μm in diameter in the mouse and 30μm in humans (Gosden and Telfer, 1987). Thecal cells are generally regarded as being indistinguishable until the follicle attains a multi-laminar stage, although recent work has indicated that pre-thecal cells associated with a specific follicle may also be present from the outset of follicular growth, at least in the rat (Hirshfield, 1991b). A steady trickle of primordial follicles become activated and

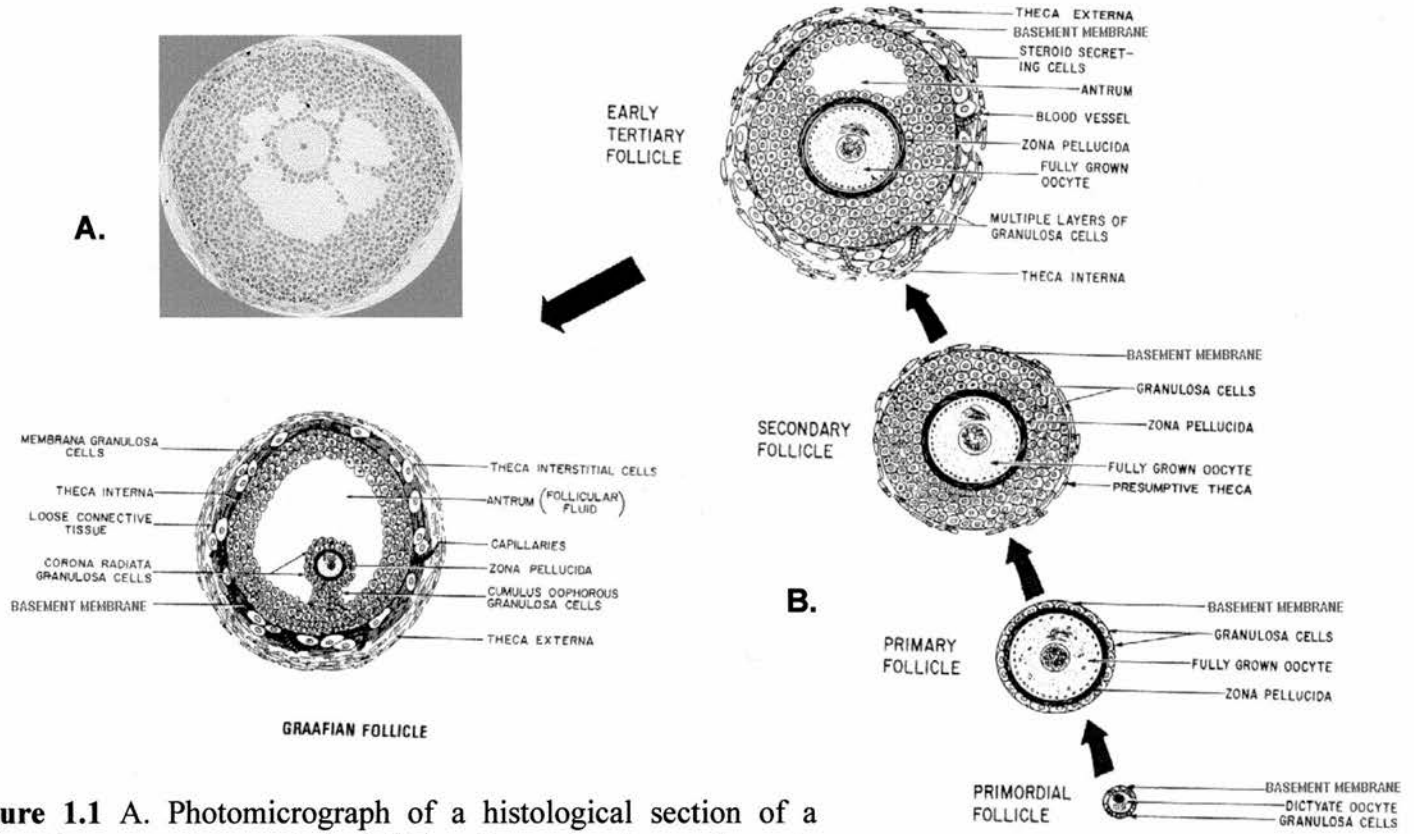


Figure 1.1 A. Photomicrograph of a histological section of a cultured mouse late antral follicle (haematoxylin and eosin stained). B. The classification and structure of ovarian follicles throughout their growth and development, according to Erickson *et al.* (1985).

leave the resting pool, forming primary follicles. Due to the avascular nature of zone 4 where the primordial follicles are located, locally produced growth factors are more likely to regulate this process than systemic factors (Greenwald and Terranova, 1988; van Wezel and Rodgers, 1996; Hirshfield, 1991a).

1.3.1. The oocyte and granulosa cells

During pre-antral development, the oocyte enlarges and the zona pellucida is formed between the oocyte and the granulosa cells (Figure 1.1). At this time the granulosa cells become cuboidal and are the site of rapid synthesis of matrix components, including the basal laminae. As the follicle continues its growth it acquires a fluid-filled antral cavity. Antral formation begins when the granulosa cell population numbers around 2000 cells for all species studied (Gosden *et al.*, 1993). It is during antral development that granulosa cells differentiate to form two major populations, the mural granulosa cells which are proximal to the basal lamina, and the cumulus granulosa cells which surround the oocyte (cells in the stalk may possess characteristics of both). The granulosa cells regulate oocyte development, for example, 85% of oocyte metabolites are of granulosa cell origin (Heller *et al.,* 1981). Conversely the mural granulosa cells are the recipients of instruction from the oocyte, e.g. GDF-9 an oocyte-secreted factor that is involved in granulosa cell development (Dong *et al.,* 1996).

1.3.2. Theca cells

The mature follicle has many associated theca cells (Figure 1.1). The highly vascularised theca interna, the layer most closely associated with the convex surface of the basal lamina, is readily identifiable, the cells containing prominent lipid droplets and being more rounded than the theca externa (O'Shea, 1971). There is a marked change in extra-cellular matrix composition at the interface between the interna and externa. Distinguishing between theca externa and stroma interstitial cells is hampered by the presence of an intermediate population of cells bearing both thecal and stromal characteristics (O'Shea, 1971). Whether these cells constitute a distinct population or are in the process of differentiating from stroma to theca cell types is unclear. In general, the

theca externa consists of several layers of fusiform cells, some of which posses cytoplasmic processes. Tight junctions are found between the cells. Examining the theca/stroma interface at a molecular level shows a clearer division of cell type. Long term *in vivo* infusion of [^{3}H]thymidine into rats resulted in a distinct boundary between cell compartments, the theca cells being considerably more active than their neighbours (Hirshfield, 1991b). Whether the intermediate population identified by O'Shea (1971) are labelled or not is unclear. Similarly, topical autoradiograph localization studies can show distinct compartmentalization of the theca externa (e.g. IGF-1 binding sites, Eckery *et al.*, 1997).

1.4. THE OVARIAN EXTRA-CELLULAR MATRIX

In the ovary, as in all other tissues, the extra-cellular matrix (ECM) provides the architectural framework that supports and compartmentalizes the different cell types. Increasingly, research is highlighting the additional role of the ECM in regulating cell behaviour in all aspects of development and maintenance. This seems to be particularly true of the ovary, a highly dynamic organ which exhibits rapid tissue remodelling throughout reproductive life (Luck, 1994).

1.4.1. Composition of the ECM

The ECM consists of two principal classes of extra-cellular macromolecules, glycosaminoglycans usually protein linked to form proteogylcans, and fibrous proteins (Alberts *et al.*; 1994, Luck, 1994).

1.4.2. Location of ECM in the ovary

The basal lamina separating the thecal and granulosa cell compartments contains collagen type IV, laminin, fibronectin and heparan sulphate proteoglycan. During follicular growth and expansion there is a rapid production of basal lamina, the regulation of which is unclear. Granulosa cells are capable of manufacturing and assembling the components of a basal lamina *in vitro* (Rodgers *et al.*, 1995). Recent evidence shows that there are subtypes of granulosa cells, differing in their abilities to produce ECM, making the mechanism of basal lamina expansion complex. This may involve an increase in the number of ECM-secreting granulosa cells, rather than increased synthesis by each cell (Rodgers *et al.*, 1995; 1996). Despite being less closely associated with the ECM, the role of possible thecal contribution to the basal lamina should not be dismissed, as these cells also produce basal lamina components such as collagen type IV, although these components could be destined for the thecal vasculature (Zhao and Luck, 1995). During follicle growth, basal lamina undergoes a 30×10^3 fold increase in the mouse and a 600×10^6 increase in the human (Gosden *et al.*, 1993).

The follicular theca contains laminin and collagen fibrils types I, III and IV (reviewed by Luck, 1994) and fibronectin in some species (e.g. sheep: Huet *et al.*, 1997; rat: Bagavandos *et al.,* 1983). This ECM used to be thought of as primarily supportive, but is now also considered to act as a binding and storage site for many factors that regulate the growth, development and function of follicular cells (Armstrong & Webb, 1997; McIntush & Smith, 1998).

In the periphery of the ovary, the ovarian capsule and the ovarian surface epithelium consist of collagen types I, III and IV, with type V also found in the ovarian surface epithelium (Luck, 1994). Other specialized ECMs in the ovary include the zona pellucida, the follicular fluid and Call Exner Bodies found in the granulosa cells of some species and sites of glycosaminoglycan production.

1.4.3. Extra-cellular proteases

ECM is constantly remodeled by the action of extra-cellular proteases, mainly matrix metalloproteinases (MMPs) (such as collagenase and gelatinase) and the plasminogen activator/plasmin family (Luck, 1994; McIntush & Smith, 1998). Around the follicles, degradation of the ECM results in release of sequestered ovarian factors. Inhibition of these proteases, such as through the action of TIMPs (tissue inhibitors of metalloproteinases), maintains the ECM and hence favours retention of growth factors and cytokines. This site of storage and release may represent a fundamental control mechanism of follicle development.

1.5. STEROIDOGENESIS

The ovary is the principal site of oestrogen production in the female body, with up to 90% of systemic oestrogen at times being produced by the one, dominant follicle in the human (Hillier *et al.*, 1994). The principal oestrogens, oestradiol 17β (E_2) and oestrone, have crucial roles in reproductive function, including stimulation of the secondary sexual characteristics during puberty and the selection of follicles destined to ovulate each ovulatory cycle. Oestradiol is produced in the ovarian follicles (Figure 1.3) by an interaction between granulosa and theca cells known as the 2-cell, 2-gonadotrophin theory (Armstrong and Dorrington, 1979), as described below.

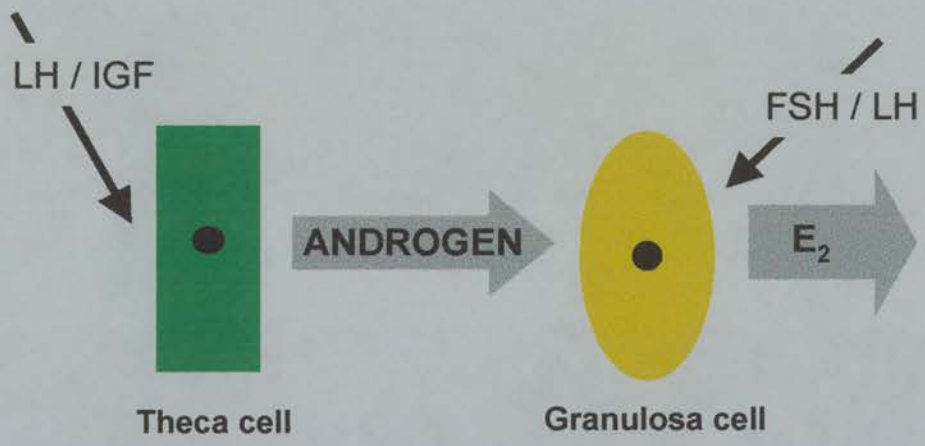


Figure 1.3 The two-cell two-gonadotrophin theory for oestrogen (E_2) synthesis. LH/IGF stimulates androgen synthesis in the theca interna. The androgen moves to the granulosa cells which convert it to oestrogen, stimulated by FSH and later LH (after Hillier 1991).

1.5.1. Production of androgen in the theca cells

The vascularised theca interna is able to convert systemic acetate and cholesterol to androgen (Gwynne and Strauss, 1982). C_{21} steroids (progestagens) are converted to C_{19} steroids (androgens) in the theca cells by the cleavage of the C17-C20 bond by cytochrome P450 ($P450_{C17}$), expressed by the CYP17 gene (Nebert *et al.*, 1991). This is the rate-limiting step in androgen synthesis. $P450_{C17}$ is regulated by LH, via its binding

to LH receptors located on the theca cells, during antral development (Erickson *et al.*, 1985; Richards *et al.*, 1987). This binding activates cAMP / protein kinase A mediated signalling, ultimately catalysing the steroidogenic conversion. *In vitro* experiments have demonstrated that IGF-1 can enhance both basal and LH stimulated androgen production (rat: Cara and Rosenfield, 1988; human: Hillier *et al.*, 1991) acting via IGF-1 receptors (Bergh *et al.*, 1993). The resultant androgens either enter the systemic circulation following drainage into the venous effluent or pass through the granulosa cell layer towards the antral cavity.

1.5.2. Production of oestradiol in the granulosa cells

As granulosa cells lack $P450_{C17,}$ they are unable to synthesize androgens themselves, however, if provided with an androgen substrate (i.e. from the neighbouring theca cells) they are suitably equipped to metabolize it further (Tamura *et al.* 1992). Another cytochrome P450, aromatase ($P450_{arom}$), is expressed exclusively in the granulosa cell compartment (particularly the mural cells) by the CYP19 gene (Nebert *et al.*, 1991), where it is regulated by FSH (Hickey *et al.*, 1988; Whitelaw, 1992). Following activation, $P450_{arom}$ converts androstenedione into oestradiol.

1.6. ATRESIA, AN APOPTOTIC PROCESS

Greater than 99% of follicles that are laid down in the developing ovary are destined to an atretic fate (Byskov 1978). This is an essential process that ensures that only the correct number of oocytes reach maturity and are ovulated in each cycle. While much debate and research attempts to gain an understanding of what regulatory signals and mechanisms determine which follicles will become atretic, and conversely which will ovulate, it is well established that atresia itself is an apoptotic event (Tilly *et al.*, 1991; reviewed: Hurwitz and Adashi, 1993; Hsueh *et al.*, 1994; Tilly, 1996; Tilly 1998). Apoptosis is a clearly defined process of physiological cell death, the mechanisms of which show conservation across all species in the animal kingdom. Since the term was first coined and the differences between apoptotic and necrotic cell death distinguished (Kerr *et al.*, 1972), there have been many comprehensive reviews detailing the apoptotic process and its various roles in tissue regulation (e.g. central nervous system: Naruse and Keino, 1995; developing limb bud: Hurle *et al.*, 1996). The 'classic' biochemical and morphological markers of a cell undergoing apoptosis are detailed in Table 1.1.

Phase I	Internucleosomal cleavage of nuclear DNA
	Nuclear and cytoplasmic condensation
	Membrane 'blebbing'
	Formation of apoptotic bodies
Phase II	Release of apoptotic bodies
	Phagocytosis by neighbouring cells / macrophages

Table 1.1 Markers of apoptotic cell death

Long before the documentation of the apoptotic process and its subsequent widespread 'discovery' in virtually all mammalian tissue, detailed morphological and histological examination of ovaries revealed characteristics that we would now attribute to apoptosis. One of the first morphological markers of a follicle undergoing atresia is the appearance

of pyknotic nuclei in the granulosa cells most adjacent to the antral cavity. This is due to the condensation of nuclear DNA into caps at the nuclear periphery, and as such represents one of the key steps during Phase I of apoptosis. A further atretic process, karyorrhexis, has been reported (Freeman, 1988) which is equivalent to nuclear disruption and subsequent formation of apoptotic bodies (Hirshfield, 1989). In the atretic follicle these are released into the antral follicular fluid where they are phagocytosed by macrophages or macrophage-like granulosa cells (Hay *et al.*, 1976), an homologous process to apoptotic Phase II. More recently, investigators have examined ovarian tissue with the specific aim of looking for apoptosis. An early biochemical marker of this process is the generation of $(185)_n$ base pair oligomers from the nuclear DNA (Figure 1.4). This occurs as a result of Ca^{++}/Mg^{++}-dependent endonuclease, DNase I (Boone *et al.*, 1995; Boone and Tsang, 1997), cleaving the DNA duplex in the vulnerable linker

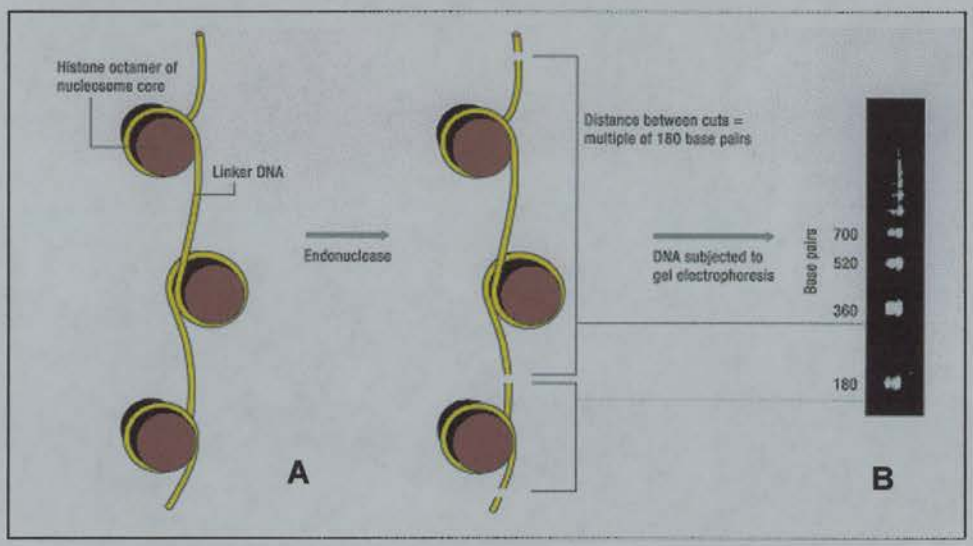


Figure 1.4 Laddering of nuclear DNA A. Vulnerable linker regions of DNA either side of nucleosomal cores are cleaved by DNase I. B. The digested DNA can be extracted and run by agarose gel electrophoresis and viewed with a suitable visualisation protocol.

regions either side of nucleosomal cores comprising of histone proteins (Wyllie *et al* 1986). Where the duplex is in contact with the histone proteins it is afforded protection from the endonucleases, sparing approximately 185 base pair (bp) sections of DNA.

When DNA from apoptotic tissue is extracted and run by electrophoresis on an agarose gel a characteristic 'laddering' pattern is seen when the DNA is visualised, each 'rung' of the ladder representing a stretch of protected DNA, i.e. 185 bp, 370 bp, 555 bp, etc. It is thought that Ca^{++}/Mg^{++} endonuclease activity is inhibited by Zn^{++}(e.g. Giannakis *et al.*, 1991) so it is likely that the intra-cellular ratio of Ca^{++}/Mg^{++} to Zn^{++} may be the determinate as to whether a cell undergoes apoptosis or not. This fine balance of intra-cellular ions may represent a conserved mechanism of apoptotic control common to all cells, with proceeding signal transduction cascades varying between different cell types and physiological environments.

1.7. DEVELOPMENT OF FOLLICLES: CHECKPOINTS AND SELECTION

1.7.1. Resumption of follicle growth

A constant trickle of quiescent follicles from the primordial resting pool continually resume growth and development. The factors that control this process are unknown but it has been shown to be independent of gonadotrophic support (Peters *et al.*, 1973b). Upon the resumption of growth the granulosa cells begin to proliferate and 'round up' to form a cuboidal shape, and the oocyte exhibits a dramatic increase in cytoplasmic volume and nuclear content (Lintern-Moore and Moore, 1979).

1.7.2. Reaching the late pre-antral / early antral stage: temporal selection

Most follicles will develop at least until the acutely FSH-dependent early antral stage. At that point, if FSH levels are low the follicles will undergo atresia (Hirshfield, 1991a). Conversely, if FSH levels are suitably elevated at that time (in response to the decline in oestrogen production from the regressing corpus luteum of the previous cycle: Le Nestour *et al.*, 1993), a certain number of follicles in the cohort will continue development to the later antral stages. This is probably the first process of selection which follicles undergo after leaving the resting pool and it is temporally regulated, i.e. if a follicle reaching the FSH-dependent stage finds itself in the 'window' when FSH is elevated it can proceed to the next developmental stage. Although temporally selecting follicles for a relatively discreet period of the oestrous cycle, work on follicle recruitment in the pig has shown that there can be considerable heterogeneity in terms of morphology and biochemical activity among the recruited cohort (Grant *et al.*, 1989). The implications of this work are that there may already be a degree of selection amongst the cohort at this stage of the developmental pathway. Despite significantly reducing the number of contenders, the number of follicles in the cohort that continue to develop is greater than the desired ovulatory number, so a further process of reduction occurs.

1.7.3. Antral development and the emergence of dominance

The second phase of selection involves the emergence of dominant follicles amongst the growing cohort. Confusion frequently arises when making comparisons between multi- and mono- ovular species although the processes involved are broadly similar. The key difference is the extent of the final stage of selection: the emergence of the dominant follicle(s). In cattle and humans the final selection process results in just one follicle attaining dominance in comparison to 6-8 in mice and rats. Henceforth I shall refer to this second selection process as **follicular dominance**. Dominant follicles continue to the final stages of development while the remaining subordinate follicles in the cohort ultimately undergo atresia and regress.

The emergence of dominant and subordinate follicles is the result of complex interplay between a range of factors, and our understanding of many of these is still poor. I have defined follicular dominance as having two principle components: indirect endocrine actions and direct intra-ovarian regulation. The latter can modulate endocrine regulation of dominance **within** a follicle (**intra**-follicular) or can initiate or exacerbate differences **between** follicles (**inter**-follicular).

1.7.3.1. Endocrine action via the hypothalamic-pituitary system

This is the aspect of follicular dominance into which most research has been conducted to date. The larger follicles in a cohort indirectly cause the cessation of growth and development in subordinate members of the same cohort by releasing increasing levels of oestradiol and inhibin into systemic circulation (Zeleznik and Hillier, 1984; Gibbons *et al.*, 1997). These act on the hypothalamic-pituitary system to decrease FSH concentrations to levels that will not support the continued growth and development of the highly FSH-dependent, less-developed subordinate follicles (Brown, 1978). The slightly more mature follicles that initiated the fall in FSH will withstand this decline in trophic support due to an up-regulation in functional LH receptors (Webb and England, 1982; Ireland and Roche, 1983) coupled to the aromatase systems in granulosa cells, and a possible increase in FSH receptor levels (Ireland and Roche, 1983; Zeleznik and Hillier, 1984). The ovary is also the site of extremely high levels of angiogenesis (for

recent review, see Redmer & Reynolds, 1996), and the dominant follicles acquire more vascular theca perhaps due to an increase in bFGF, positively correlated with oestradiol levels (Schams *et al.,* 1996). This allows dominant follicles to obtain an increased uptake of serum gonadotrophins (Zeleznik *et al.*, 1981). The dominant follicles have, therefore, several mechanisms for sequestering more of the available gonadotrophins and surviving the decline in circulating FSH levels. In contrast, subordinate follicles are highly susceptible to a decline in circulating gonadotrophins: granulosa cells undergo apoptosis, and follicular atresia results (Hughes and Gorospe, 1991; Hsueh *et al.*, 1994; Tilly, 1996; Tilly, 1998).

It seems likely that the FSH decline is the major endocrine selection mechanism by which the ovulatory quota is determined, with dominant follicles proceeding to ovulation and subordinate follicles being forced down the atretic pathway. However, I believe that this mechanism alone fails to adequately explain how the appropriate number of follicles first emerges as dominant.

1.7.3.2. Endocrine action between ovaries

Ovulation had been considered to occur from alternating ovaries in mono-ovular species since original observations by Rühl (1925), an opinion bolstered by appearances in seminal reproductive texts (e.g. Knobil and Neill, 1988). If this is the case, it could be due to a locally suppressive effect of the corpus luteum from the previous cycle, or alternatively it is possible that some as yet undefined communication mechanism is acting between the bilateral ovaries to regulate this 'turn about' process. Similarly in multi-ovular species this ovary-to-ovary 'talk' could divide up the total number of ovulatory follicles ensuring that each uterine horn receives equal numbers of fertilized embryos. Evidence in the literature regarding consecutive ovulation sites is conflicting. Support for the contra-lateral theory comes from a histological study of ovaries obtained from 25 women by Gougeon and Lefèvre (1984) in which corpora lutea were identified and assigned an age on the basis of morphology. A chronological order of ovulations was then calculated which suggested that ovulation occurred in a turn about manner.

Other studies in the human (Marinho *et al.,* 1982) and non-human primate (Dukelaw, 1977; Hodgen, 1982) also implicate contra-lateral ovulation. In contrast, Werlin *et al.* (1986) suggests that ipsi-lateral ovulations are the norm in women.

A third possibility is that selection of the ovary containing the dominant follicle is random. Considerable weight was lent to this hypothesis by the sonographic data of Check *et al.* (1991), due to its large sample size. Obtained from a study of 572 cycles in 92 women, they demonstrated a 52.4% incidence of ipsi-lateral ovulation and a 47.6% incidence of contra-lateral ovulation which is a non-significant difference. This study supported an earlier finding by Wallach *et al.* (1973) in the rhesus monkey. Doubt has also been cast on the incidence of contra-lateral ovulation by the observation that ovulation in the primate appears to be more common from the right ovary, than from the left. Thus, Morse and van Wagenen (1936) report a bias towards ovulation in the right ovary of 60% in a study of eight rhesus monkeys, and the results of Potashnik *et al.* (1987) support this finding in humans. Even studies that do not demonstrate a statistical bias toward the right ovary report a slight trend towards that side (e.g. Check *et al.*, 1991; Fukuda *et al.,* 1996). In all the literature examined detailing side of ovulation in the primate, I found no trend, statistically significant or otherwise, toward the left ovary. In some species, the trend towards one or other ovary is taken to an extreme with one ovary becoming totally inactive (e.g. the mountain viscacha: Pearson, 1949) , or even regressing (as in the domestic hen: Gilbert, 1979). There seems to be little discussion as to why there should be a bias towards one ovary, although anatomical asymmetries possibly bought about by genes like Pitx2 (Ryan *et al.,* 1998), such as the origin and drainage of vasculature and development of other organs such as the kidneys (and adrenal glands) may effect the development and function of the ovaries, favouring one side.

The main body of evidence would seem to suggest that, despite what is written in text books, contra-lateral ovulation is not the physiological norm in mono-ovulatory species. The detailed histological examination conducted by Gougeon and Lefèvre (1984) presents the strongest argument in support of this proposal, although this would appear to conflict with the larger clinical investigations of Potashnik *et al.* (1987) and

Check *et al.* (1991). Difficulties in accurately aging the corpora lutea and consequently determining the sequence of ovulations may offer an explanation of these different findings. Transient increases in local progesterone concentration of the ovary most recently bearing the ovulatory follicle only appear to affect the choice of subsequent ovary when cycle length is short (Wallach *et al.*, 1973). It still remains unclear whether choice of ovary housing the next dominant follicle in the primate is a truly random event or if there is a bias towards the ipsi-lateral or right handed side, the last two proposals being mutually compatible. Even less clear is an understanding of how dominance is established **between** ovaries if ovulation does not occur in a turn about manner driven by the intra-ovarian environment. Interestingly from a clinical viewpoint, whether ovulation is from the contra- or ipsi- lateral ovary may have implications for subsequent oocyte retrieval, fertilization, cleavage and embryo transfer during assisted fertility treatments: Fukuda *et al.* (1996) demonstrated that the success rate of all these procedures was significantly higher if ovulation was on the contra-lateral side. Whatever the mechanism(s) at work, extrapolating these findings to non-primate species, particularly to multi-ovulatory species, may be harder. The ability of embryos to migrate along the uterine horns in some species means that bilateral ovulation is not an absolute requirement for evenly-distributed embryonic implantation. However, it seems unlikely that the majority of the oocytes released in multi-ovular species originate from one ovary. Instead it seems more probable that both ovaries contribute to a similar degree with perhaps a marginal bias towards one side, depending on species.

1.7.3.3. Intra-ovarian regulation

It would seem essential that intra-ovarian communication is involved in selection of the dominant follicle(s) from a growing cohort. This could occur via three possible pathways: **paracrine regulation**, the 'talk' between different cells, involving the local diffusion of a chemical messenger produced in one cell to another 'target' cell; **autocrine regulation**, a self-regulatory mechanism whereby a certain cell type produces factors that act back on the cell of origin; and **juxtacrine regulation**, communication between cells as the result of direct cell-cell or cell-matrix contact, allowing cell- or

matrix surface-associated molecules to interact with one another. All of these types of communication may subsequently lead to signal transduction cascades within the cell, giving rise to functional alterations.

The endocrine regulation of follicular dominance, discussed briefly above, results in a lowering of FSH levels. The response of a follicle to that drop in FSH is dependent on its dominant or subordinate status, with differential alterations in FSH-dependent growth factor and hormone levels directing ultimate follicle fate (Mihm *et al.*, 1997). Dominant follicles continue to grow and produce oestradiol in an environment of decreased FSH (Ireland and Roche, 1983; Sunderland, 1994) whereas subordinate follicles exhibit markedly reduced oestradiol production. That the dominant follicle continues to grow and increase its steroidogenic output is thought to be due to the increased bioavailability of the insulin-like growth factors (IGF-1 and 2) (Spicer *et al.*, 1988; Mihm *et al.*, 1997; Gong *et al.*, 1993) following enhanced secretion of this peptide and a decrease in IGF binding protein (IGFBP) production (Echternkamp *et al.*, 1994). During this period of selection, the subordinate follicles exhibit increased IGFBP production reducing the concentration of available IGF-1 (Mihm *et al.*, 1997). As a result of these differential responses to the reduced levels of FSH, the dominant follicles continue to grow and develop whilst the subordinate follicles undergo follicular atresia and die. The role of such intra-follicular factors on follicle dominance has been the subject of several comprehensive reviews, e.g. Adashi and Rohan (1992); Erickson and Danforth (1995); Campbell and McNeilly (1996) and Armstrong & Webb (1997).

There is, however, an additional method of intra-ovarian regulation of follicular dominance, namely through interactions **between** follicles. Such interactions could enhance the effect of endocrine regulation of follicular dominance, with the dominant follicle also producing factors that will directly affect the development of subordinate ones. For example, inter-follicular interactions may have a role in maintaining dominance once it has been established, by 'holding back' challengers. This could explain the phenomenon of follicular waves exhibited by some species. If the dominant follicle found itself in a hostile environment upon reaching the ovulatory stage and consequently regressed, the inhibitory influence would be removed and the follicles that

had been held in check could resume development and contend for the dominant position (Matton *et al.*, 1981; Ko *et al.*, 1991). Alternatively, follicle-follicle interactions could initiate differences between follicles upon which endocrine action can subsequently act. These possibilities are explored more fully later.

1.8. INTRA-OVARIAN INTERACTIONS AND FOLLICLE FATE

I believe that selection of the correct number of follicles for ovulation cannot easily be achieved by endocrine mechanisms alone. Variations in systemic concentrations of follicular trophic factors do not seem subtle enough, or targeted in any way, making it hard to envisage how they could account for such precise and regimented control of ovulatory number. If this is the case, it seems essential that intra-ovarian factors play a role in regulating the development of the correct number of follicles. These factors could be acting in two ways, predisposing certain follicles for successful growth culminating in ovulation and/or condemning the unsuccessful contenders to an atretic pathway. Once follicle dominance has been established, intra-ovarian factors could also 'hold back' any challengers (as discussed in 1.7.3.3).

Such follicle-follicle interactions could either establish differences between a group of initially equivalent follicles or, at a later stage, allow a follicle to influence the fate of another non-equivalent (subordinate) follicle group. These processes bear striking resemblance to methods of **cell** fate determination commonly described in developmental biology, namely those of inductive signalling and of lateral specification.

1.8.1. Inductive signalling and lateral specification

Inductive signalling is a method of communication between adjacent, non-equivalent cell populations, whereby one cell type influences the fate of another, and can thus generate new cell types. Lateral specification (also called lateral inhibition) is the short range cell-cell 'talk' between initially equivalent cells, an example of juxtacrine communication. This cell-cell dialogue may give rise to signal transduction cascades within the cells causing functional alterations. Thus, from an initially equivalent and equipotent group of cells, interactions between these cells leads to the generation of two distinct cell fates.

Examples of inductive signalling can be found in many developing systems in a diverse range of organisms that includes plants, invertebrates and mammals. One example recently reviewed by Horster *et al.* (1997) describes the cell-cell interactions

that bring about the formation of the mammalian metanephric kidney. Two types of tissue with distinct embryological origins, the metanephric mesenchymal blastema and the ureteric bud, come into contact with one another at the site of the future kidney. The mesenchymal cells aggregate around the branching ureteric bud tip, allowing the two cell types to communicate with each other via inductive signalling (Figure 1.5). The signalling between the different cell populations is successful as the two cell types express ligands and receptors in a complementary pattern (Birchmeier and Birchmeier, 1993). Interactions with the ureteric bud tip cause the mesenchymal cells in contact with the ureteric bud to adopt epithelial morphology and function. These newly created epithelial cells subsequently differentiate into the variety of cell populations that comprise the nephron.

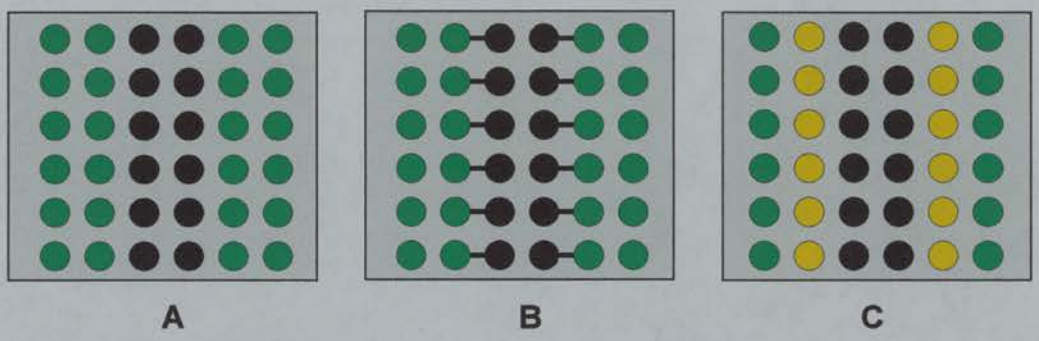


Figure 1.5 Inductive signalling A Two different cell types are adjacent to each other. B Cells from one population in immediate contact with the cells from the other population communicate with each other. C Cells at the population border are pushed down a different developmental pathway (e.g. to become a third cell type, or to undergo programmed cell death).

Lateral specification is involved in the patterning of differentiated cell types. An often-cited example of lateral specification can be found in the developing *Drosophila* bristles where evenly spaced sensory mother cells are created from an initial population of equivalent proneural ectodermal cells. These cells would all differentiate to become sensory mother cells unless prevented from doing so. As the ectodermal cells begin down the pathway to sensory differentiation they send an inhibitory signal to their neighbours. A 'battle' is then fought as each cell attempts to suppress the differentiation

of the adjacent cells and gain a slight developmental edge, allowing it to escape the inhibitory influence. Consequently this eminence is enhanced as the cell becomes more differentiated and produces a stronger inhibitory signal, preventing the contacting cells themselves from becoming sensory mothers and producing an inhibitory signal (Figure 1.6). These 'weak' cells consequently develop into epidermal cells (Hartenstein and Posakony, 1990; Heitzler and Simpson, 1991).

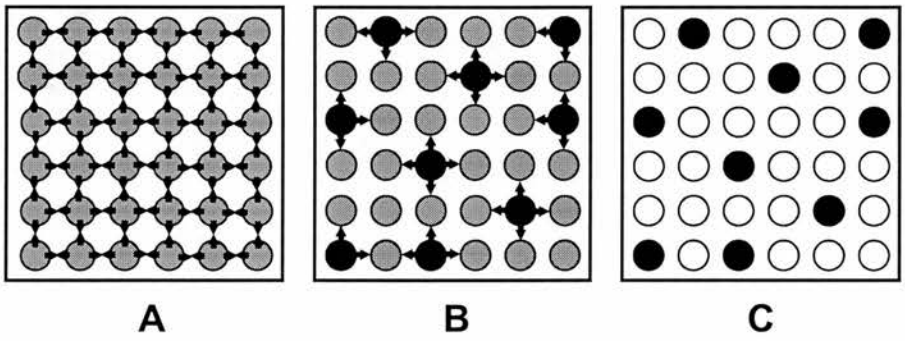


Figure 1.6 Lateral specification A. An initially equivalent population of cells all possessing the ability to differentiate and simultaneously send an inhibitory signal to the neighbouring cells. B. A few cells have been able to differentiate to a greater degree than their neighbours. They produce a stronger inhibitory signal preventing the further differentiation of the surrounding cells which in turn lose the ability to inhibit the dominant cell. C. Two distinct cell types have arisen from an initially equipotential population. A regular 'pattern' has been generated.

Some of the genes regulating local cell interactions necessary for inductive signalling and lateral specification are known, more so for the latter process. These will be discussed further in Chapter 7.

1.8.2. 'Inductive signalling' and follicular dominance

The traditional view of inter-follicular regulation of follicular dominance is that an already dominant follicle secretes some factor(s) that inhibits the development of its subordinate neighbours. As this is one population of cells affecting the fate of another, non-equivalent population of cells, it is analogous to inductive signalling, and the factors produced by the dominant follicles can be thought of as 'inductive signalling' molecules.

Several putative 'dominance' factors have been reported. Di Zerega *et al.* (1982) identified a protein with a molecular weight between 14 000 and 18 000 kDa secreted by the dominant follicle in humans which suppressed the follicular response to gonadotrophins. Cahill *et al.*, (1985) demonstrated that ovine follicular fluid inhibited the development of follicles greater than 2mm in diameter in the ovary and reduced the mitotic index of the granulosa cells of follicles <2mm. Later, substances with molecular weights of 180 000 and <10 000 kDa were identified in ovine follicular fluid that inhibited the mitotic activity of murine embryonic lung fibroblasts (Carson, 1988). Campbell *et al.*, (1991) report a substance in the ovine pre-ovulatory follicle that is atresia-inducing and results in a loss of steroidogenic activity following injection into the cycling sheep. How a follicular fluid derived factor would act on neighbouring follicles was called into question when Driancourt (1994) failed to demonstrate inter-follicular interactions in the Booroola sheep. Using aromatase activity as a measure of follicle development, no positive role of the largest atretic follicle or negative role of the dominant follicle was found on other follicles following his experiments using ovarian serum or conditioned media. However, he substantiated the previous literature reporting the presence of an inhibitory factor in the follicular fluid, which was shown to significantly reduce the activity of aromatase in large follicle pieces, as compared to cultures with serum or conditioned medium. Perhaps then his failure to observe either an inhibitory or stimulatory effect was an *in vitro* artefact, the mechanisms by which the follicular fluid factors are transported from the antrum being absent or disabled *in vitro*. Another possibility is that such factors were indeed present but not at sufficient concentrations in culture to exert an effect.

Most recently, work on the bovine follicle resulted in the characterisation of granulosa cell-inhibitory factor (GCIF) which was shown to inhibit the proliferation of small and medium follicles (Hynes *et al.*, 1996 a, b). Steroid-free bovine follicular fluid was separated into high and low molecular weight fractions and purified. A factor with a molecular weight <5kDa was shown to inhibit granulosa cell proliferation *in vitro*, inhibit the proliferation of granulosa cells taken from small and medium follicles but not large follicles, and following systemic administration to cycling rats, inhibit the

formation of large follicles and increase the number of small follicles. In a further study, it was demonstrated that immunization of rats against GCIF increased the number of large follicles and decreased the number of small follicles. Immunization of sheep against GCIF resulted in a significant increase in ovulation rate (Hynes *et al.*, 1999). The authors report similarities between GCIF and factors found in porcine (Kigawa *et al.*, 1986) and rat follicular fluid (granulosa cell mitostatic protein GCMP, Chakrovorty *et al.*, 1993). Gore *et al.* (1997) note the disappearance of 'challenger' follicles from around dominant follicles and speculate that oestrogen may be responsible for this phenomenon. They cite the studies of Dierchke *et al.* (1985) and Koering *et al.* (1994) which demonstrated a detrimental role of oestrogen on follicles *in vivo*.

1.8.3. 'Lateral specification' and early determination of follicular fate

While a process analogous to that of inductive signalling can help explain how follicle dominance is maintained once established, it does not address the issue of how dominance has arisen (with the correct, species-specific number of follicles continuing to develop). Based on morphological criteria, the emergence of dominant and subordinate follicles would seem to arise from a cohort of initially equipotential follicles. Whilst we cannot discount the possibility that differences are established at the time of gonadal formation, marking the follicles destined to ovulate, this must at the very least be a readily reversible designation, as the number of ovulating follicles can be manipulated with ease. Experiments where the dominant follicle is ablated (e.g. Matton *et al.*, 1981; Ko *et al.*, 1991) show that a new dominant follicle rapidly emerges from the cohort of antral follicles. Presumably, had the original dominant follicle been allowed to ovulate, the 'new' replacement dominant follicle would have become atretic. Similarly, superovulation (e.g. Baird, 1987) results in a far greater number of follicles than would be expected in a normal cycle, suggesting that at least some, if not the majority of follicles have been deflected from an atretic fate. In a recent review article (Baker and Spears, 1999), we propose that differences in follicle development may have arisen through a process early in follicle development equivalent to lateral specification.

At the onset of follicle development, a cohort of primordial follicles enters the growth phase. At this point they are most likely equipotential. As they start to grow, there are presumably fluctuations in the production of signalling molecules setting up transient differences between neighbouring follicles. These differences could become magnified as selected 'stronger' follicles inhibit development of their immediate neighbours. In this manner, a pattern of selected and non-selected follicles, or later, of dominant and subordinate follicles would emerge (Figure 1.7): the endocrine loop would then act on those differences.

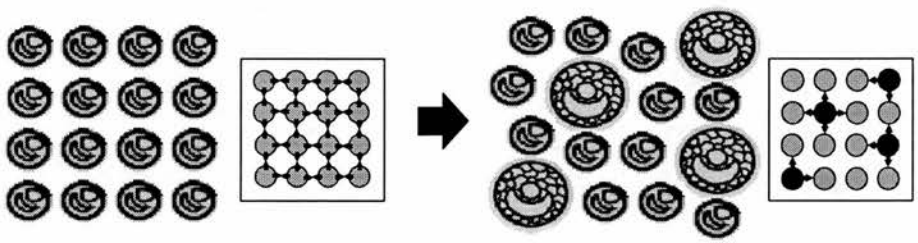


Figure 1.7 Lateral specification and determination of follicle fate Follicles are selected for continued growth from a larger cluster of pre-antral follicles in a manner analogous to lateral specification.

Recent work has highlighted a contact-mediated mechanism whereby 'dominance' is established between co-cultured murine follicles *in vitro* (Spears *et al.*, 1996), although this may be more analogous to the process of selection *in vivo*, particularly in large mammals. Using a whole follicle culture system which allows the growth of follicles from the pre-antral to the Graafian stage (as detailed in Chapter 2), experiments were conducted to investigate the influence of follicle-follicle interactions on growth and development. It was found that when pairs of follicles were co-cultured in contact, one follicle invariably became dominant over its partner (Figure 1.8). When follicles were cultured in identical conditions but placed slightly apart (Figure 1.9), this phenomenon was not seen, implying a requirement for follicle-follicle contact. It would seem plausible that this observation may be an example of 'lateral specification' between neighbouring follicles, resulting in the initially equivalent follicles adopting different

fates and only the successful follicle being selected for further development and maturation.

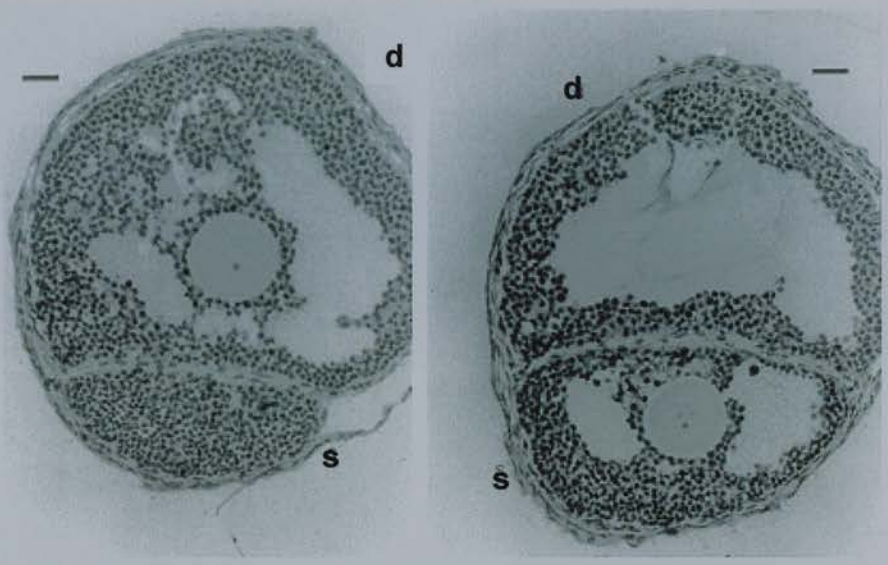


Figure 1.8 Photomicrograph of sections through follicles co-cultured with contact d: dominant follicle; s: subordinate follicle. 2 μm plastic sections, haematoxylin and eosin stained. Scale bars represent 40 μm. From Spears *et al.*, 1996.

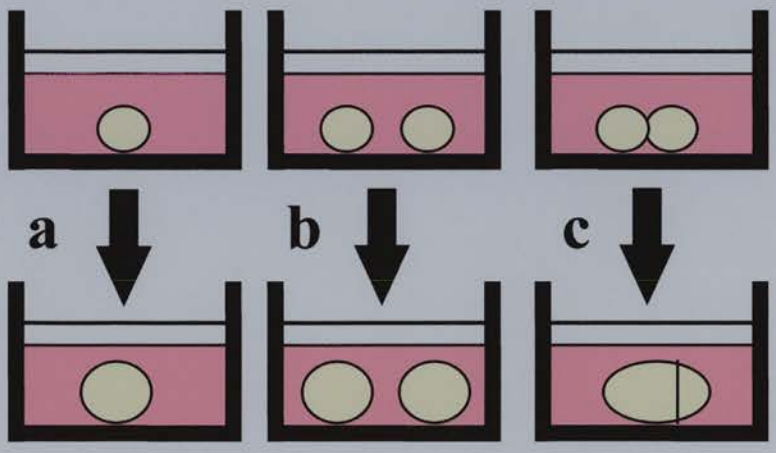


Figure 1.9 Whole follicle co-culture experiments (Spears *et al.*, 1996). Co-cultured follicles not in contact both attain sizes comparable with single controls. When cultured in contact one follicle becomes dominant over its partner.

1.9. OVULATION AND BEYOND

With increasing levels of E_2 being produced by late antral follicles a positive feedback loop leads to the generation of an LH surge. Approximately three days prior to the LH surge in the rat the dominant follicles prepare for ovulation (Hirshfield, 1991a). The systemic concentration of oestradiol rises dramatically as the steroidogenic output of the dominant follicles increases. This elevated oestradiol concentration acts on the hypothalamic pituitary system and causes a surge of LH secretion. 24 hours prior to the ovulatory LH surge, granulosa cells acquire LH receptors. The bolus of LH terminates granulosa cell proliferation (Yong *et al.*, 1992), inhibits oestradiol production, initiates the ovulatory process, induces luteinisation and the production of progesterone. The ovulated granulosa-oocyte complex is then guided into the oviduct by the fimbrae where the oocyte meets its ultimate fate: fertilization or death.

1.10. RESEARCH AIMS

The growth and development of an ovarian follicle is a long and complex process. During this time, the few successful follicles that are permitted to ovulate must pass through several checkpoints and selection mechanisms. Despite having a relatively comprehensive picture of the large scale, endocrine role in follicle selection, less is known about the vital local mechanisms which underpin this process. Whilst current knowledge can explain how local events can modulate follicular response to systemic factors, we are still in a position of speculation as to how differences initially arise between equivalent follicles, i.e. how does a follicle emerge as dominant? The aim of this thesis was to investigate this local selection process. To this end I had two principle goals:

I. To establish if direct contact between follicles plays a part in the selection of the ovulatory follicle(s), and if this appeared to be the case,
II. To investigate the precise nature of direct follicle-follicle communication.

These goals were addressed by a series of experiments that aimed:

1. To investigate how follicles are positioned in respect to one another *in vivo*, indicating if direct, follicle-follicle contact-mediated selection is feasible (Chapter 3).

2. To investigate the nature of communication between 'contacting' follicles *in vitro* and determine if absolute physical contact is a requirement for the dominance effect (Chapter 4).

3. To determine the effects of declining FSH on follicles at different developmental stages (Chapter 5), and then demonstrate how an FSH decline could bring about follicle selection by acting on follicular differences already established by contact-mediated mechanisms (Chapter 6).

4. To take leads from developmental biology and investigate the expression in the ovary of genes known to be involved in lateral specification. The aim of this work was to determine if these genes could be involved in the regulation of follicle selection (Chapter 7).

Chapter 2

General Materials and Methods

2.1. FOLLICLE ISOLATION

2.1.1. Dissection medium

Leibovitz L-15 dissection medium (Gibco) supplemented with 0.3% B.S.A. (fraction V, tissue culture grade. Sigma) was used for all bench top manipulation of ovarian material. Unused medium from freshly opened 100 ml bottles was discarded after 5 days. The osmolarity of each bottle opened was measured by an osmometer (Roebling) and adjusted to 285-292 mOsm/kgH_2O by the addition of sterile water (Phoenix Pharmaceuticals Ltd.). Following the addition of B.S.A., the medium was filter sterilised (syringes: Becton Dickinson and Co.; 0.25 μm filters: Iwaki) into sterile flasks (Iwaki) and heated to 37°C.

2.1.1. Gross dissection

Three-week old mice (CBA / C57 Black crosses, referred to as F_1 mice henceforward) were killed by cervical dislocation and the ovaries removed following a ventral incision and displacement of the abdominal viscera. This and all subsequent procedures were performed in a laminar airflow hood (Astecair). The ovaries were transferred to pre-warmed dissection medium in embryo dishes and 'cleaned up' under magnification on a heated stage (Linkam) using insulin syringes (Sherwood) to remove pieces of non-ovarian tissue such as oviduct and fat.

2.1.3. Micro-dissection

Using a dissecting microscope (Zeiss) fitted with a 37°C heated stage (Linkam), base illumination and a graticule eyepiece, pre-antral follicles were dissected from halves of ovaries using insulin syringes (Sherwood) and acupuncture needles (Acumedic) mounted in steel holders. Ovary halves were dissected in pre-warmed embryo dishes containing dissection medium. Tissue was worked on for a maximum of 30 minutes *post mortem*, with isolated follicles being placed in culture within 45 minutes.

2.2. STANDARD FOLLICLE CULTURE

2.2.1. Standard Culture Medium

α-Minimum Essential Medium (α-MEM) (Gibco BRL) was supplemented with 5% serum taken from mature F_1 mice. rhFSH was added at a concentration of 1 IU ml^{-1}. Medium was then filtered (as for dissection medium, above), 30 μl pipetted into U bottomed 96-well plates, overlaid with 75 μl silicon fluid and allowed to equilibrate in a 37°C, 5% CO_2 atmosphere, humidified incubator. At the start of this research project this medium was sufficient to routinely facilitate follicle growth from the pre-antral to Graafian stages over a six day culture period, however at the start of later experiments ascorbic acid was also added, as detailed below.

2.2.1.1. The role of ascorbic acid in culture

Since its development, this culture technique would suffer from unexpected 'down' periods, during which time it would not be possible to sustain the growth of follicles much beyond the early antral stage. Follicular growth and cellular proliferation would appear to proceed as normal until approximately 72 hours of culture when bursting would occur in virtually all follicles. This appeared to be due to loss of basement membrane integrity characterised by a 'fuzzy' appearance around the follicle's periphery. This phenomenon was prevented by the addition of ascorbic acid into the culture medium at a concentration of 50 μg ml^{-1} (Murray *et al.*, in preparation). The growth rate of cultured follicles was not effected in these experiments. In addition to its role in preventing rupture, I found that ascorbic acid added at this concentration has a protective effect against a strongly apoptotic stimulus (absence of serum), as determined by analysis of DNA fragments (Figure 2.1) Methodology and discussion of this technique are detailed in Chapter 5. Following these discoveries, ascorbic acid was added to culture media used for any new experiments. Investigations that had commenced before this time were completed without this addition. This meant that culture numbers were increased to compensate for a higher rate of follicle loss.

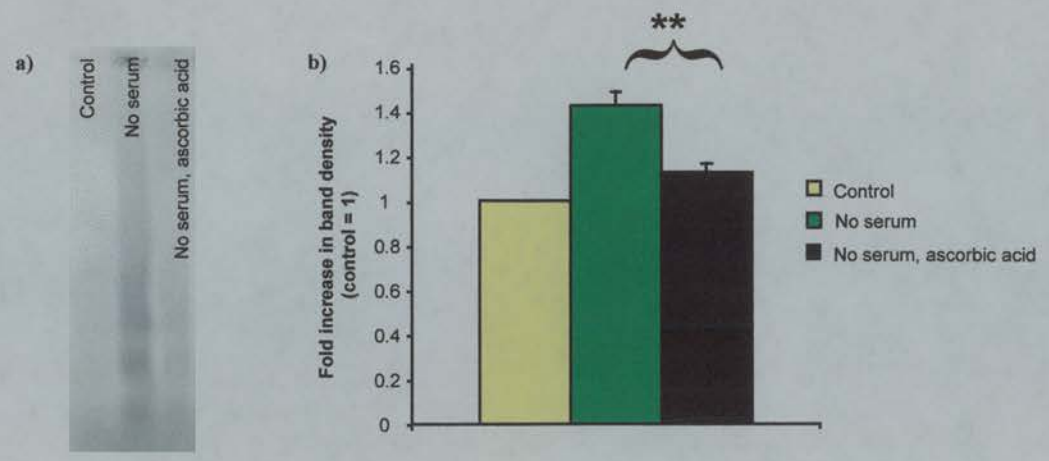


Figure 2.1. Apoptotic fragmentation in follicles cultured without serum, +/- ascorbic acid. a) 'Laddering' of nuclear DNA into ~185 base pair multiples. b) Relative densities of apoptotic bands between culture groups. Ascorbic acid significantly reduces DNA fragmentation in the absence of serum, a strong apoptotic stimulus ($p<0.01$). Data normalised so that control = 1.

2.2.2. Follicle incubation

Following isolation healthy follicles were transferred into the equilibrated culture medium using fine drawn pipettes coated with B.S.A. to avoid sticking to the pipette barrel. Caution was taken to ensure that the culture trays were maintained at 37°C whilst out of the incubator and bench top time was kept at a minimum. Culture trays were returned to the incubator (Forma Scientific) and left undisturbed for 24 hours.

2.2.3. Media changes and assessment of follicle morphology and growth

Follicles were moved everyday using a glass pipette into a new well containing fresh medium. Daily measurements of follicle diameter and developmental stage were taken using a graticule eyepiece. In addition any abnormalities were noted, such as an unusually thick theca layer. In all experiments conducted, at least one control group of single follicles was included. If these failed to develop as expected the entire experiment was abandoned.

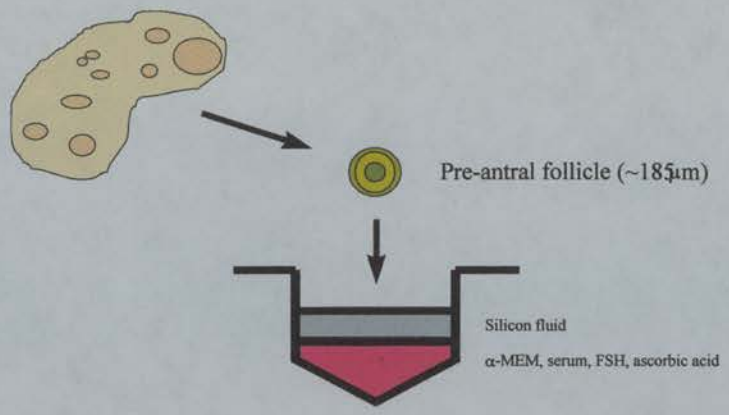


Figure 2.2. Culture of pre-antral follicles. Following isolation from three week old ovaries, pre-antral follicles are incubated in culture medium at 37°C, 5% CO_2 atmosphere.

2.3. HISTOLOGY

2.3.1. Fixation of follicles

If histological examination was required, follicles were placed in 4% paraformaldehyde (BDH) at 4°C for ~24 hours, then transferred into 70% ethanol for storage until processing.

2.3.2. Processing of follicles for morphological assessment

2.3.2.1. Processing of samples

Follicles where dehydrated by being moved through a series of ethanols (70%; 90%; 95%; 100%; 100%, 30 minutes each). Material was then left overnight at room temperature in LR White (TAAB). The following day the follicles were transferred to gelatine capsules containing LR White and incubated overnight at 60°C. Once the resin had hardened the gelatine capsule was dissolved in running hot water for ~4 hours and the resin block mounted on a wooden chuck.

2.3.2.2. Sectioning and mounting

Blocks were cut using a plastic-section microtome (Reichert-Jung), floated out on a 50°C water bath and transferred to gelatine coated slides (see Appendix B). Sections were then dried on a hotplate overnight at 60°C.

2.3.2.3. Staining

The dried sections were stained in haematoxylin for 5 minutes, rinsed in running tap water then placed into Scotch Tap Water Substitute (STWS) for 2 minutes; running water for 2 minutes; eosin for 1 minute; dipped in running tap water; potassium alum for 1 minute; rinsed in tap water before being left to air dry. Once dry the sections were cover-slipped with DPX.

2.3.3. Fixation of whole ovaries

Whole ovaries were placed in freshly made 4% paraformaldehyde (BDH) for 3-5 hours for processing for *in situ* hybridisation experiments, or Bouin's fixative for periods of time ranging from 24-72 hours depending on tissue size. Ovaries were then transferred to 70% ethanol for storage until processing.

2.3.4. Processing of whole ovaries for morphological assessment

2.3.4.1 Processing of samples

Ovaries where dehydrated by being moved through a series of ethanols of increasing purity up to absolute alcohol (70%; 90%; 95%; 100%; 100%, one hour in each), before clearing in toluene (~2 hours). Tissue was then placed in plastic moulds containing molten wax and allowed to impregnate at 60°C for 3-5 hours before being orientated for sectioning and the wax allowed to set.

2.3.4.2. Sectioning and mounting

Wax blocks containing the tissue to be sectioned were melted onto metal chucks and quickly hardened under running water. The blocks were then cut on wax-section microtomes (Reichert-Jung) and floated out onto a 50°C water bath before transfer to gelatine coated slides. Sections were then heated overnight at 37°C.

2.3.4.3. Staining

Sections were dewaxed in xylene, re-hydrated in descending alcohol concentrations to water before staining for 5 minutes in haomatoxylin. Sections were then acidified in acid alcohol, washed, moved into STWS, washed again, stained with eosin for two minutes, fixed with potassium alum, washed, dehydrated through an alcohol series then placed in xylene prior to cover-slipping with DPX.

2.3.5. Processing whole ovaries for *in situ* hybridisation

Paraformaldehyde fixed ovaries were processed, sectioned and heated as for morphological assessment, with the following exceptions. Care was taken to ensure a

clean, RNase free environment including the use of latex gloves and fresh dH_2O in alcohol-cleaned water baths. All equipment that came into contact with the material, including the microtome blade, was thouroughly swabbed with alcohol. Sections were floated onto TESPA-coated slides (see Appendix B).

Chapter 3

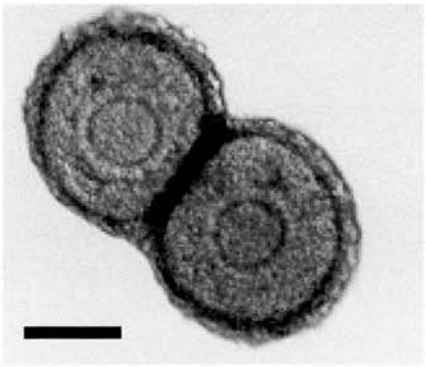
Histological evidence for clustering of like-sized follicles *in vivo*

3.1. INTRODUCTION

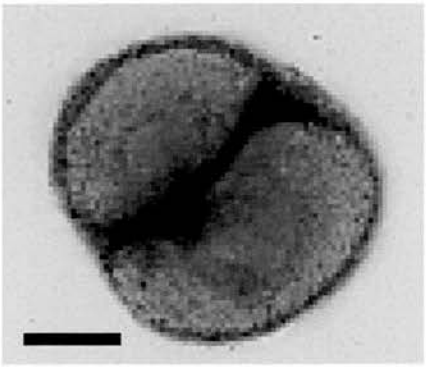
Chapter 1 detailed recent work that highlighted a contact-mediated mechanism whereby 'dominance' is established between co-cultured murine follicles *in vitro* (Spears *et al.*, 1996). In this study, an intact-follicle culture system was used to investigate the influence of follicle-follicle interactions on growth and development. It was found that when pairs of follicles were co-cultured in contact, one follicle invariably became dominant over its partner (Figure 3.1). When follicles were cultured in identical conditions but placed slightly apart, this phenomenon was not seen, implying a requirement for follicle-follicle contact. The authors hypothesise that this *in vitro* contact-dependent process may represent a paradigm for a selection mechanism *in vivo*, with 'lateral specification' between adjacent follicles resulting in initially equivalent follicles adopting different developmental fates. For this *in vitro* observation and proposed hypothesis to be relevant *in vivo*, there would be a requirement for follicles to be in direct contact with others at the same stage for at least part of their development.

Whilst reports in the literature describe the location in the ovary of follicles at different developmental stages (briefly discussed in 1.2), there tends to be little discussion of the geographical relationship **between** follicles. Merchant and Zamboni (1972) describe interconnections between primordial follicles in newborn animals, which Hirshfield (1991) suggests may be a result of incomplete fragmentation of germ cell 'cords' at an earlier developmental stage. These interconnections may persist into adulthood with the follicles appearing to be arranged into cord-like patterns, although no detail of the developmental stage of these follicles is given (Merchant and Zamboni, 1972). I was unable to find any reference that discussed the precise position of further developed follicles in relation to each other. To address this, I undertook an histological examination of the ovaries from a diverse range of species and identified the location of pre-antral follicles and their proximity to neighbouring follicles at similar stages of development. If follicles are geographically intimate (with directly contacting basal laminae or theca cells), it is feasible that lateral specification is involved in intra-ovarian follicle selection. Conversely, if developmentally similar follicles do not assume these close

a) start of culture



b) 24 hours of culture.



c) 48 hours of culture.

Figure 3.1. Contacting co-cultured follicles at various intervals of culure. Scale bars represent 100 μm.

relationships *in vivo*, it would seem unlikely that lateral specification or some such similar process has a significant role in follicle selection at that developmental stage.

3.2. MATERIALS AND METHODS

3.2.1. Source of material

When possible, fresh ovaries were obtained and processed (mouse; rabbit, and cat), although some archived material was also used (kitten and marmoset).

3.2.2. Processing of tissue: mouse and other mammals

Tissue specimens were fixed, wax embedded, sectioned and stained according to the protocols outlined in Chapter 2.

3.2.3. Computer capture of histological images: mouse

Using a video camera attached to a microscope, sequential images were captured of serially sectioned (6μm) ovaries taken from 3 three-week-old mice. Every fourth section through the ovary was imaged in this way. The images were processed on a computer (Apple MacIntosh) using NIH Image 1.49 software, and then printed out to make an ovary 'atlas' (Figure 3.2 A and B).

3.2.4. Numerical analysis of mouse ovarian sections

Each ovary section in the atlas was looked at in detail and all follicles that were between 150 – 250 μm were identified and marked (Figure 3.2 C). This size range was chosen as it represents follicles from the late pre-antral stage to the beginnings of antral development. Greatest diameter was measured across the centre of the follicle which, for the purposes of this investigation, was taken to be where the nucleus of the oocyte could be seen. It was assumed that the follicles were spherical. Having identified a suitably sized follicle, its location was marked in all preceding and subsequent sections. In this way it was possible to map the whereabouts of all the follicles in the ovary that fell into the chosen size range. From this 3-dimensional map, the numbers of like-sized follicles that were directly in contact with one another and the total cluster size of all connecting follicles were calculated.

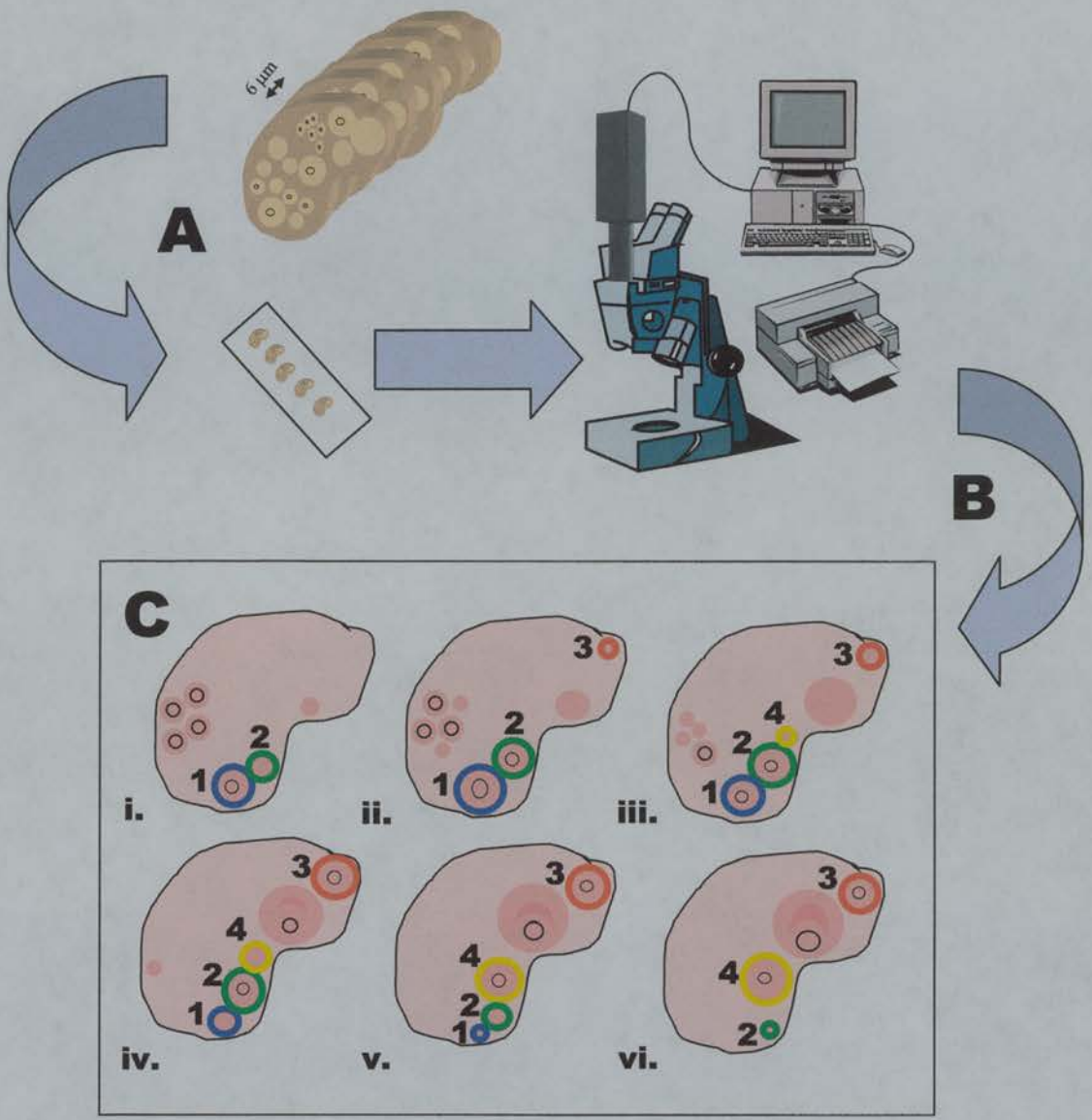


Figure 3.2 Numerical analysis of mouse ovary sections. (A) Ovaries from 3-week-old mice were fixed, processed and sectioned. (B) Using a video camera attached to microscope sequential sections were 'captured' and printed out in order to make an entire ovary atlas. (C) Every follicle in the atlas was measured and marked if found to be between 150 - 250 µm at its greatest diameter. Identified follicles were then traced backward and forward through all of the sections and marked (Each follicle has been given a different colour in C). All identified follicles were then numbered and the numbers of contacting, neighbouring follicles recorded, e.g. in section vi. follicle 4 appears to have no contacting neighbours, however, looking through preceding sections it can be seen that it makes contact with follicle 2.

3.3. RESULTS

3.3.1. Photomicrographs of contacting pre-antral follicles *in vivo*

Histological examination of sections from a range of mammalian species (mouse: Figures 3.3-3.6; rabbit: Figures 3.7 and 3.8; cat: Figures 3.9-3.11, and marmoset: Figures 3.12 and 3.13) reveals that pre-antral follicles are frequently found in close contact with each other, often with a very thin dividing theca layer.

3.3.2. Numerical analysis of contacting follicles in the young mouse ovary

Detailed analyses of serial sections from three 3-week-old mice revealed that most pre-antral/early antral follicles are found in clusters of 10-50 follicles (Figure 3.14), frequently in contact with two or more like-sized follicles (Figure 3.15).

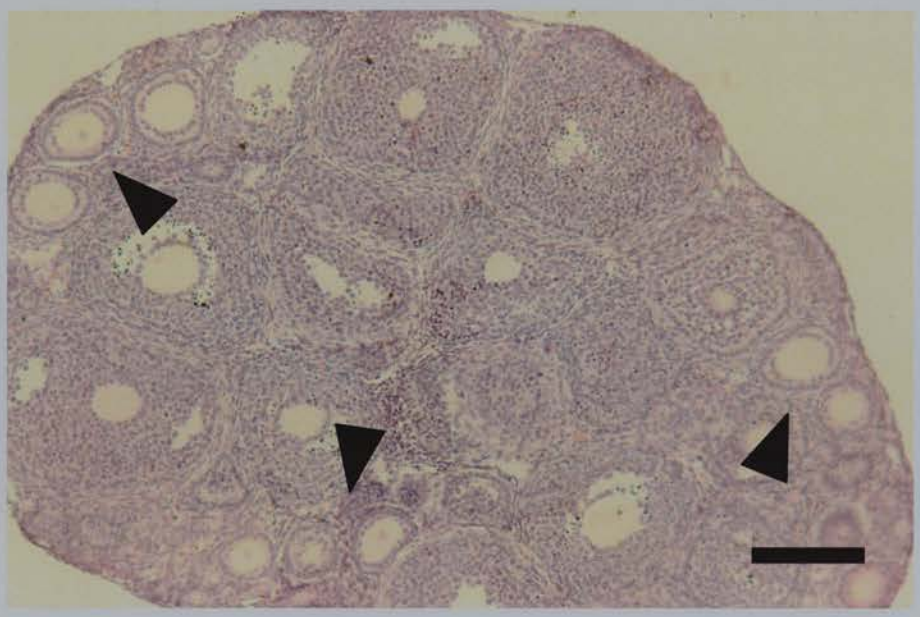


Figure 3.3. Photomicrograph of a wax section through a 3-week-old mouse ovary (haematoxylin and eosin stained). Scale bar represents 100 μm. The arrows show clusters of pre-antral follicles.

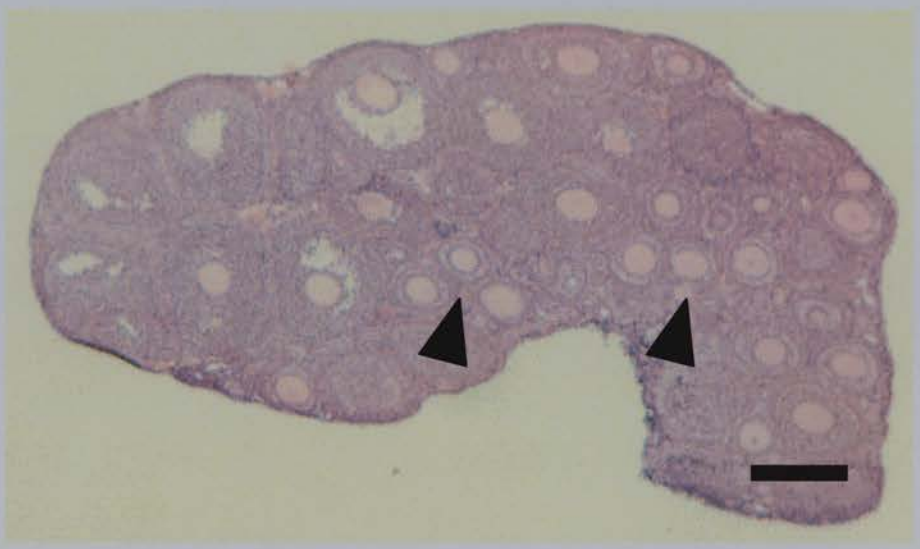


Figure 3.4. Photomicrograph of a wax section through a 3-week-old mouse ovary, haematoxylin and eosin stained. Scale bar represents 100 μm. The arrows show clusters of pre-antral follicles.

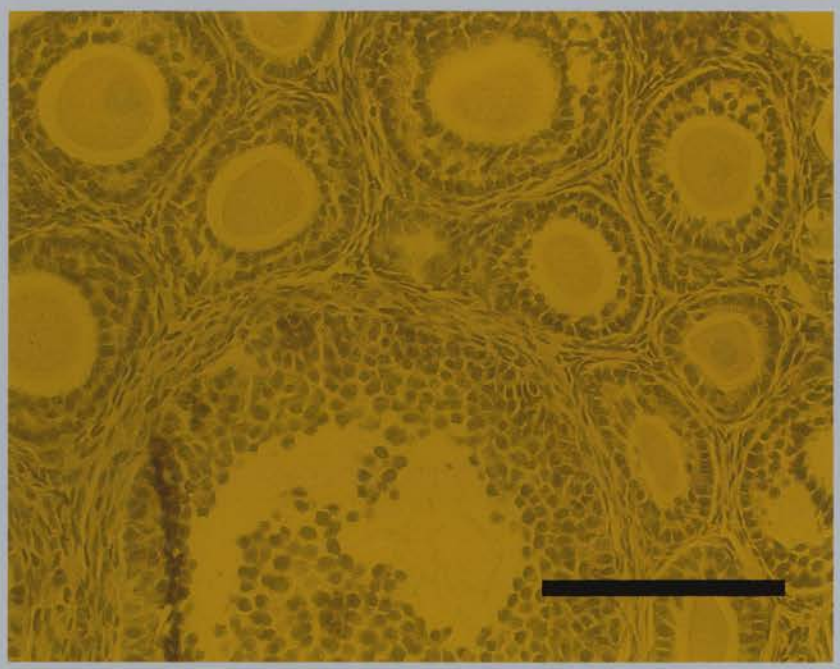


Figure 3.5. Photomicrograph of a wax section through a 3-week-old mouse ovary. Contacting pre-antral follicles clustered around a mid-antral follicle. Scale bar represents 100 μm.

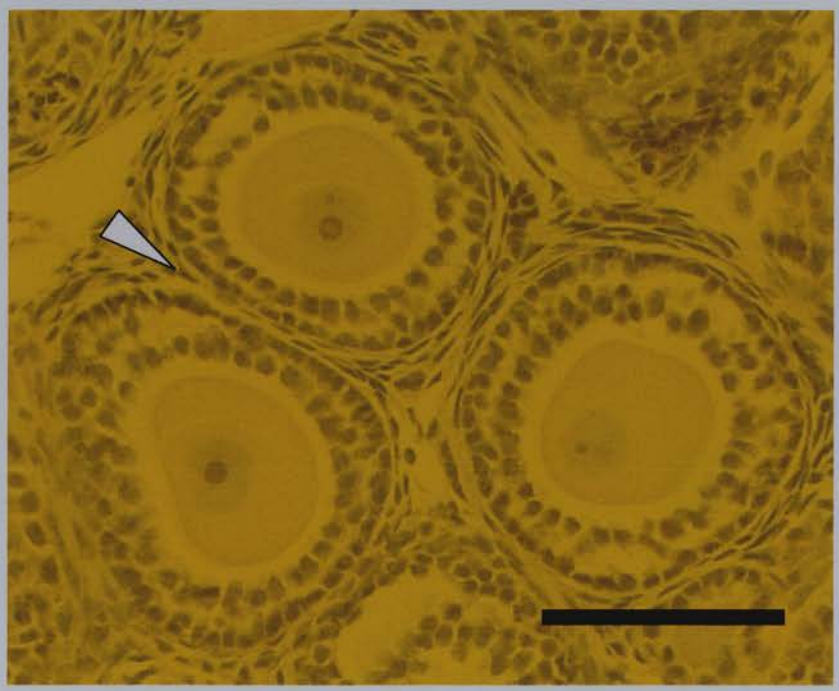


Figure 3.6. Photomicrograph of a wax section through a 3-week-old mouse ovary. Contacting pre-antral follicles. Scale bar represents 50 μm. The arrow shows the thin, shared theca layer between follicles.

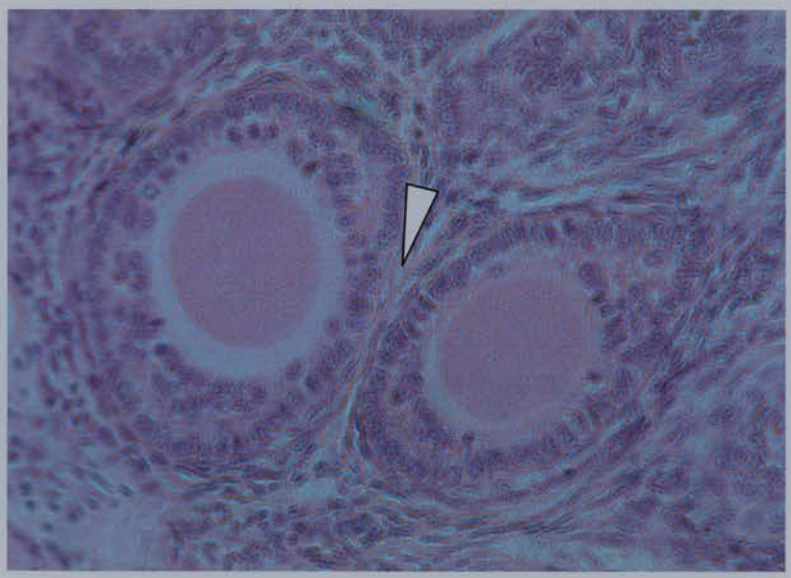


Figure 3. 7. Photomicrograph of a wax section taken from a rabbit ovary (haematoxylin and eosin stained). Scale bar represents 50 μm. The arrow shows the thin theca layer between contacting pre-antral follicles.

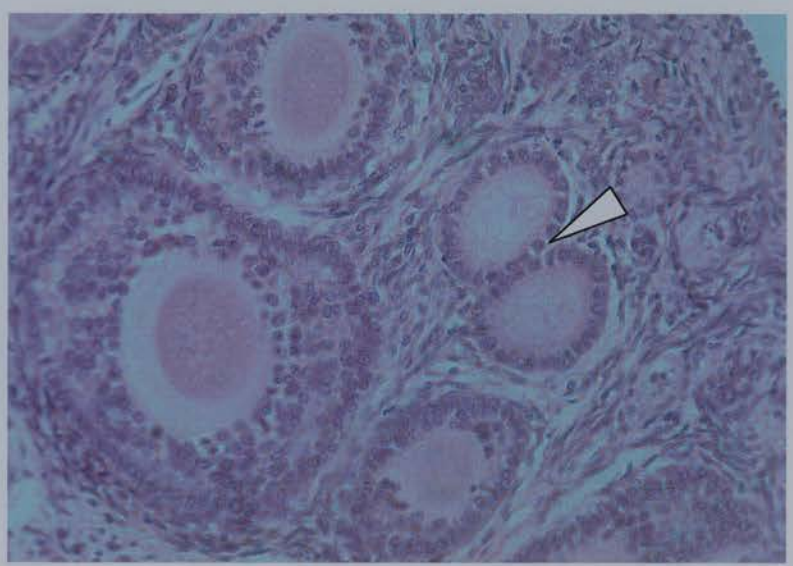


Figure 3. 8. Photomicrograph of a wax section taken from a rabbit ovary (haematoxylin and eosin stained). Scale bar represents 100 μm. The arrow shows the basal lamina between contacting primary follicles.

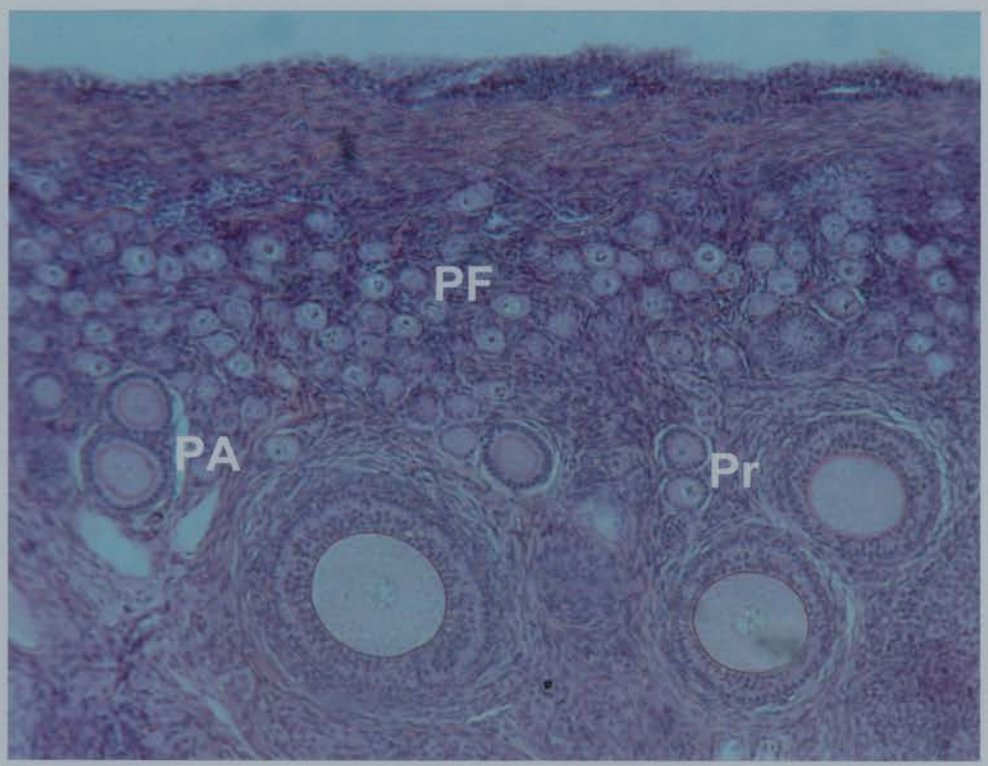


Figure 3.9. Photomicrograph of a wax section of a kitten ovary (haematoxylin and eosin stained). Scale bar represents 200 μm. The cat ovary is a very organised organ with primordial follicles (PF) being located in tightly packed clusters towards the periphery. Moving inwards, primary (Pr) and early pre-antral follicles (PA) tend to be found next to these clusters. Antral follicles are located more centrally in the ovary (not shown).

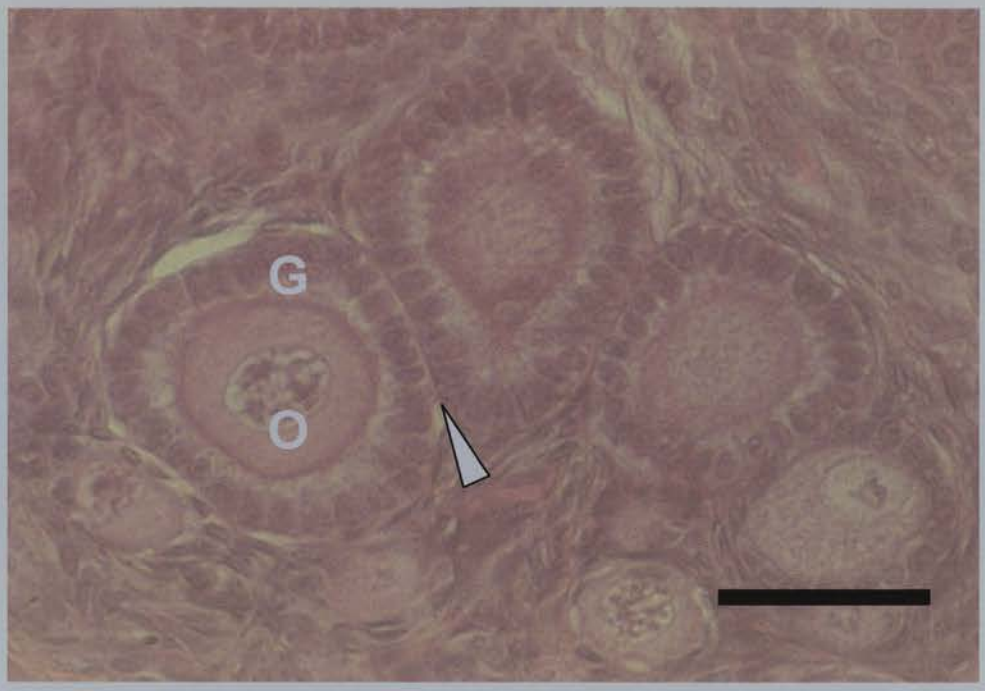


Figure 3.10. Photomicrograph of a wax section taken from a kitten ovary (haematoxylin and eosin stained). Scale bar represents 50 μm. Granulosa cells (G) and the oocyte (O) are marked. The arrow shows the basal lamina between contacting pre-antral follicles.

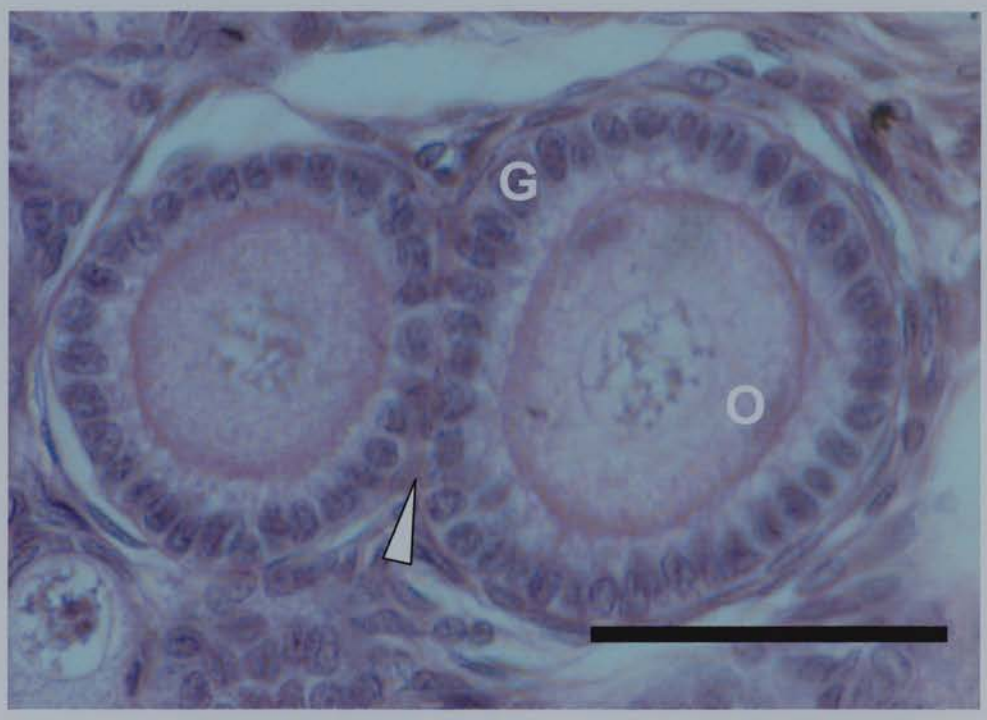


Figure 3.11. Photomicrograph of a wax section taken from a kitten ovary (haematoxylin and eosin stained). Scale bar represents 50 μm. Granulosa cells (G) and the oocyte (O) are marked. The arrow shows the basal lamina between contacting pre-antral follicles.

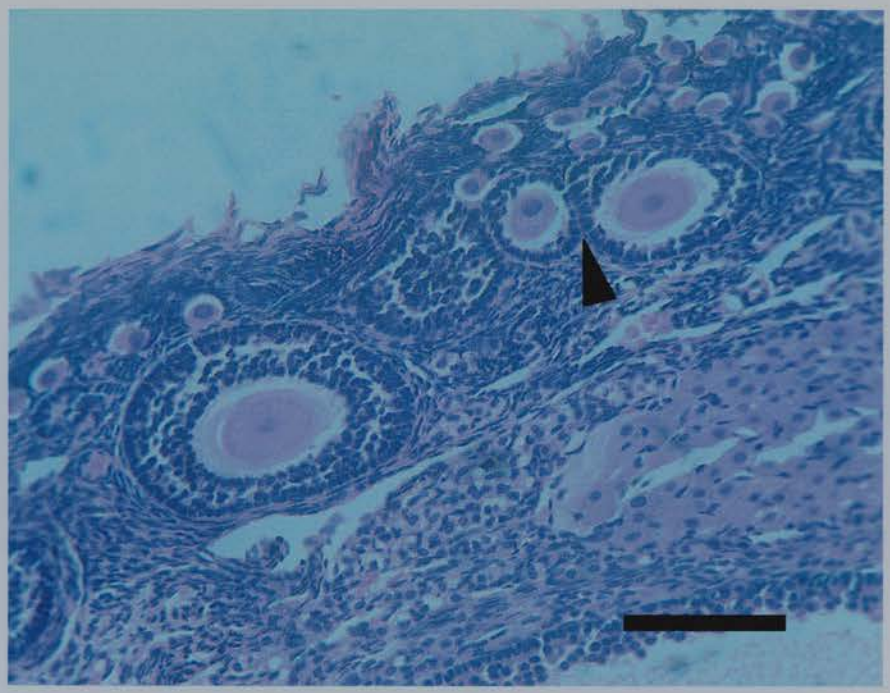


Figure 3.12. Photomicrograph of a wax section (haematoxylin and eosin stained), taken from a marmoset ovary. Scale bar represents 100 μm. The arrow shows contacting pre-antral follicles.

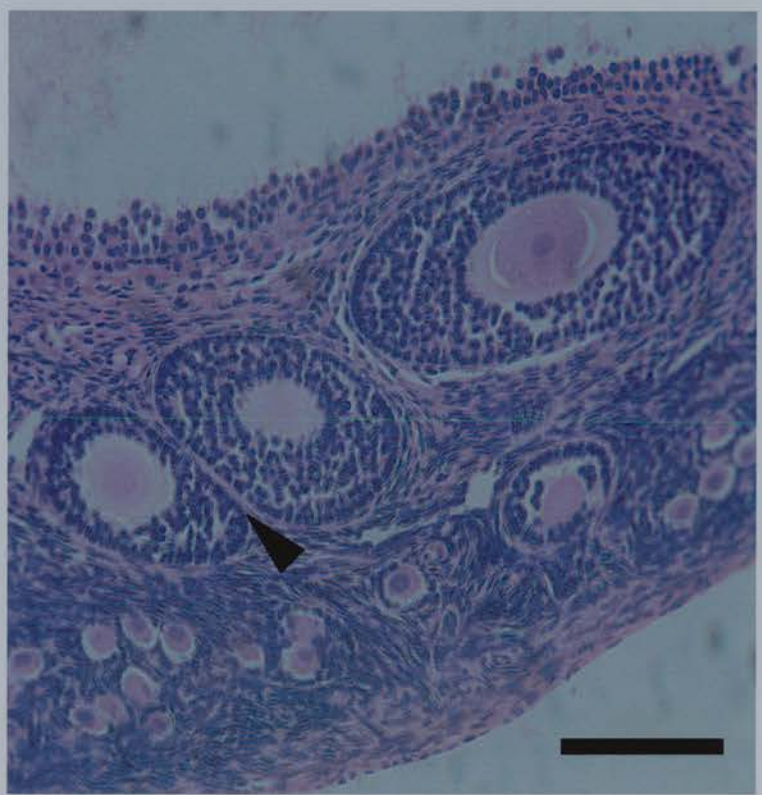


Figure 3.13. Photomicrograph of a wax section (haematoxylin and eosin stained), taken from a marmoset ovary. Scale bar represents 100 μm. The arrow shows contacting pre-/early antral follicles.

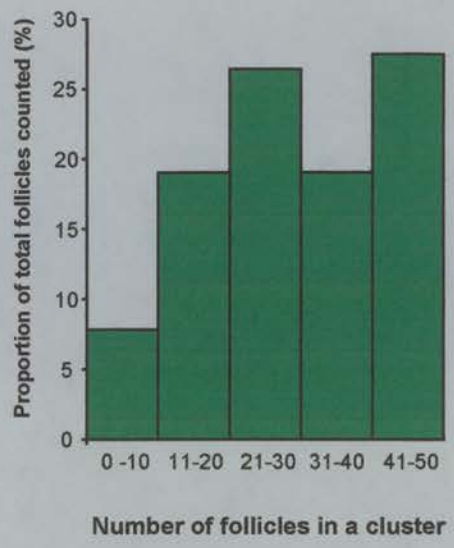


Figure 3.14 Cluster size of contacting follicles, 150-250 μm in diameter. The ovaries from three 3-week-old mice were analysed. Follicles were deemed to be contacting if they had either an absent, or thin shared theca layer.

Figure 3.15 Incidence of directly contacting like-sized follicles, 150-250 μm in diameter. The ovaries from three 3-week-old mice were analysed. Follicles were deemed to be contacting if they had either an absent, or thin shared theca layer.

3.4. DISCUSSION

The ovary sections clearly illustrate the degree of closeness that neighbouring follicles assume. These follicles often have a very thin, or occasionally absent, dividing theca layer. This raises the interesting question of whether follicles have their 'own' theca cells or whether these cells are recruited by the follicle from the interstitial cell population. Work by Hirshfield (1991b) has suggested that theca cells may be associated with a specific follicle from the outset, possibly giving rise to the theca population that comprises the theca interna. How this hypothesis would fit with the histological observations made earlier in this chapter is unclear. Using Figure 3.6 as an example, it can be clearly seen that two of the pre-antral follicles pictured have a theca mono-layer between them (indicated by the white arrow). In this instance which follicle 'owns' that theca layer? Or is the theca population in that region contributed to by both follicles? The upper follicle has a two-cell theca layer around the remainder of it's periphery. This would suggest that either some of the theca layer has moved away from the follicle-follicle junction, or that it has failed to develop in this region. Interestingly, this thin shared theca layer is also observed in co-cultured follicles (Figure 1.8). That this phenomenon is seen *in vitro* in the intact-follicle culture system may be important as it could offer a relatively simple paradigm to investigate the issue of theca 'ownership' further. This is an important question to address as it has implications for several areas of follicle research. Presumably, if 'lateral specification' is involved in follicle regulation at any stage of development, this must occur at the follicle boundary, wherever that it delimited. It is possible that this changes as the follicle progresses developmentally, initially being at the basal lamina, then later at the edge of the theca interna. It seems unlikely that the theca externa would provide a clear enough border to present a unified follicle 'front', as various studies have reported a 'blending' of theca and stroma cells with an intermediate theca/stroma population bearing some characteristics of both cell types (e.g. O'Shea 1971). A second area of research that will require knowledge of follicle boundaries is that of follicle migration. If a follicle migrates within the ovary which structures move with the core granulosa-oocyte unit? Further discussion will be devoted to this process in Chapter 4.

Zamboni and Merchant (1973) report that bi- and tri-laminar follicles are found interconnected by granulosa cell projections in young mice, presumably as a result of persisting intercellular bridges between primordial germ cells. Connected 'strings' of primordial/primary follicles have also been reported in young cats (J. Mullan, personal communication; Figure 1.2). It would be interesting to see if these 'strings' of follicles resume growth as a unit, giving rise to closely contacting pre-antral follicles at equivalent stages of development. Our understanding about the initiation of primordial/primary follicle growth is in its infancy.

A recent paper discusses the possible role of activin, secreted by secondary follicles, in maintaining primary follicle quiescence in the mouse (Mizunuma *et al.*, 1999). Co-culture experiments revealed that the presence of a secondary follicle (300-350 μm) inhibited the response of a smaller pre-antral follicle to FSH, but that this inhibition was removed when the secondary follicle was taken away. The addition of follistatin, an activin binding protein, resulted in both the small and large follicles responding to FSH, with both groups showing a significant increase in follicle diameter. The authors conclude that although the initiation of follicle growth is not dependent on FSH, early pre-antral follicles are susceptible to FSH and GH and that these factors are potent stimulators that enhance the first stages of follicle development. However, not all early pre-antral follicles are able to respond to these factors due to inhibitory control by activin released by secondary follicles. They continue by suggesting that this presents a mechanism that could regulate the number of follicles leaving the resting pool to enter the growing cohort. Atresia of the secondary follicles would remove the source of activin in a very localised area and permit those primordial/primary follicles that were ready to continue development to proceed. Combining this hypothesis with the observations of Zamboni and Merchant (1973) it seems highly feasible that a string of developmentally similar follicles, by default geographically restricted, would all be under the same paracrine influence from a larger follicle. In this way, the demise of a secondary follicle producing activin would permit a whole string of follicles to resume development at the same time. Even if interconnections between the follicles had broken down, they would still be very closely associated. This would result in a cluster of follicles growing

together, presumably in concert, until such time as additional developmental influences, such as lateral specification, come into play.

The numerical analysis of the three 3-week-old mouse ovaries indicates just how common close contact between late pre-antral follicles is (at least at that age), with greater than 90% being found associated with a cluster of 10 or more like-sized follicles. These findings, together with the pictorial evidence from the histological study clearly show that pre-antral follicles do develop in contact with one another. This fact makes it at least feasible that lateral specification (or an equivalent process) could influence follicular fate, resulting in only certain follicles from within each cluster proceeding on to further stages of follicular development. For the purposes of this thesis and given the time constraints that were imposed, I was satisfied that this evidence was strong enough to proceed with the investigations detailed in subsequent chapters. In the future I would like to see this work consolidated with a more comprehensive analysis of mouse ovaries at a variety of ages, and extended to examine differences in the organisation of ovaries from different species. This thesis has been concerned with follicle-follicle interactions at a temporally discreet period of development. It would seem likely that contact-dependent mechanisms have roles to play at all stages of a follicles' life span. Cursory examination of ovary sections shows not only primordial and late pre-antral follicles in contact with one another, but antral follicles at all stages including pre-ovulatory follicles also being very closely associated.

I believe that there are important anatomical data to be obtained from earlier studies of the ovary: publications from the beginning of this century hold many examples of meticulously recorded observations. A re-examination of this rich data-base, with the benefit of contemporary knowledge, may offer a short-cut to understanding how anatomically dependent relationships influence ovarian function.

Chapter 4

The nature of communication between co-cultured follicles

4.1. INTRODUCTION

Previous experiments have shown that contacting co-cultured follicles establish a dominant-subordinate relationship (Spears *et al.*, 1996). This relationship is not seen when same-sized follicles are co-cultured slightly apart implying that a) the 'dominance' factor is not a diffusible factor, and b) there is therefore a requirement for direct physical contact for this dominance mechanism to be seen. It is important to be cautious when interpreting these observations. As the authors state, it is possible that the volume of culture medium used resulted in a diffusible factor becoming so dilute that it was unable to exert an effect over even a short distance. This problem would be overcome if follicles are cultured in contact, as any diffusible factor released by one follicle would be able to pass to the neighbouring follicle without having to diffuse through the surrounding medium. One problem of the original paradigm is that the contacting co-cultured follicles form such a close and extensive bond that they assume an abnormal morphology (Figure 3.1). Ideally a culture system used to investigate follicle-follicle interactions would maintain normal morphology as much as possible, removing the possible influence of abnormal surface area to volume ratios on follicle health.

To further investigate the dominance effect, I conducted a series of experiments utilizing an adapted version of the whole-follicle culture system detailed in Chapter 2.

Previous work on other physiological systems has demonstrated that polycarbonate membranes can be used effectively to examine the nature of communication between co-cultured tissue (e.g. Saxen and Lehtonen, 1978, Slack, 1992). These membranes are thin (7μm) and have pores of known size and number which directly traverse the membrane, unlike standard cellulose membranes which have very winding pores. It has been shown that pore sizes as low as 0.2 μm permit cell-cell contact via cellular processes. Any size lower than this should prevent direct contact but allow the passage of diffusible factors. This is ideal for the investigation of contact-mediated follicle dominance. Two follicles can be juxtapositioned either side of these membranes and cultured as normal. By varying the pore size is possible to either allow direct cell-cell contact between follicles or permit two follicles to be very close to each

other without actually touching. This use of polycarbonate membranes in culture has the additional advantage of preserving the spherical shape of the follicle as the co-cultured follicles are unable to 'mould' together. My aim was simply to establish whether or not there is an absolute requirement for contact for the development of co-cultured follicles to be influenced by one another.

4.2. MATERIALS AND METHODS

4.2.1. Scanning electron microscopy (S.E.M.) and light microscopy

Polycarbonate membranes ('Isopore' from Millipore) are supplied coated with a wetting agent, polyvinylpyrrolidone (PVP), which was found to encourage adhesion of cultured follicles resulting in spreading, distortion and finally rupture. Boiling these membranes in de-ionised water for >1 hour successfully removes the PVP coating and consequently abolishes abnormal follicle growth. Once treated to remove the PVP coating, membranes were wrapped individually in aluminium foil and autoclaved, then allowed to dry.

It is well documented that 0.1 μm pores will prohibit the growth of cellular processes across the membrane and conversely that 10 μm pore membranes should permit this cellular growth. It is possible that incomplete removal of the PVP wetting agent might result in the pores becoming blocked. To ensure that the PVP removal protocol is adequate the undersides of 10 μm pored membranes were examined by scanning electron microscopy (S.E.M.) for evidence of cellular protrusions following the culture of pre-antral follicles for 48 hours on these membranes. The intact membrane-follicle complex was fixed and sent to the Electron Microscope Unit at the Royal (Dick) School of Veterinary Studies, University of Edinburgh, which provides an S.E.M. commercial service. Once the material had been processed and was in the S.E.M. I was assisted by Mr. Stephen Mitchell in scanning the tissue and taking photomicrographs. Co-cultured follicle-membrane-follicle complexes were also fixed, plastic embedded, sectioned and haematoxylin and eosin stained for light microscopy according to protocols outlined in Chapter 2.

4.2.2. Polycarbonate membrane experiments

4.2.2.1. Making membrane constructs

Under sterile conditions, membranes with 0.1 μm and 10 μm pore-sizes were cut into rectangles approximately 2 mm x 4 mm. In both groups, 50% of these were folded in half using forceps, ensuring that a crisp crease was formed. The folded membrane pieces

were stuck to unfolded rectangles of the same membrane type using a surgical adhesive (Histoacryl Blue, B. Braun Melsungen AG). The two-piece assembly could then be stuck into flat-bottomed 96-well plates (Corning). A silicon based sealant (RTV 32, Dow Corning) was used after trials showed that it presented no toxicity problems for follicle growth. Once a row of wells had been filled with either 0.1 μm or 10 μm inserts the tray was left for at least 24 hours at 37 °C to allow the sealant to cure. As a final assurance of sterility, the trays were UV irradiated for ~15 minutes immediately prior to the addition of culture medium.

4.2.2.2. Follicle culture

Standard culture medium containing ascorbic acid (see Chapter 2 for materials and methods) was used for all of these membrane culture experiments. 150 μl of medium was pipetted into each well and overlaid with silicon fluid (Corning) to prevent evaporation. The medium was equilibrated for an hour in a humidified incubator, 5% CO_2, before follicles were added.

A typical experiment contained equal numbers of pairings over 0.1 μm and 10 μm membrane constructions and a tray of single control follicles. Data was also obtained from paired control follicles cultured in separate wells. Like-sized pre-antral follicles ~185 μm were paired and placed either side of the membrane upright (Figure 4.1). After three hours the wells were checked to ensure that the follicles were still positioned opposite one another. If they had moved away from their starting position they were replaced using a bent acupuncture needle then returned to the incubator and left undisturbed until the following day. After 24 hours, the pairs were examined by an unbiased observer who assessed if they were still suitably juxtapositioned. Measurements of growth were recorded and any pairs that had moved apart repositioned. Following a further 24 hours of culture the follicle pairs were examined again by an unbiased observer and measurements taken if they were still opposite. In wells that contained opposing follicle pairs, 50% of the medium was carefully extracted using an angled gel-loading pipette, and replaced with the same volume of previously equilibrated medium. Any follicles that did not remain in contact after the first 48 hours

were excluded. The culture continued for a further 48 hours at which time all follicles still included in the study were transferred to a watch glass for a more accurate assessment of growth and measured.

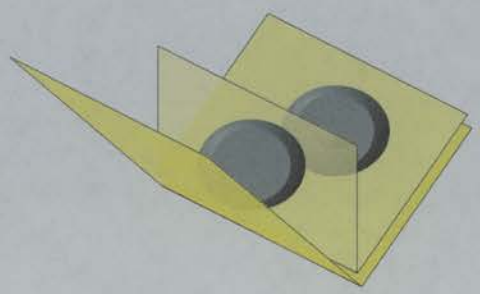


Figure 4.1 Diagram of follicles placed on either side of the polycarbonate membrane upright.

4.2.3 Statistics

For each experimental group the mean final size of the follicles was calculated together with the S.E.M. To determine if there was an effect of co-culture on growth a Chi-squared test was performed, comparing each experimental group with controls. The student's t-test was used to establish if there was a bimodal population present by comparing the difference is size between co-cultured, paired follicles in the same well, and paired follicles grown in separate wells. Dominant and subordinate follicle final size was compared to singly cultured control follicles using the student's t-test. A Chi-squared test was a used to assess if there was any difference between experimental groups.

4.3. RESULTS

4.3.1. Scanning electron microscopy (S.E.M.) and light microscopy

Figure 4.2 clearly shows that cellular processes can pass through the 10 μm pores in the polycarbonate membranes. It is unclear from these photomicrographs if these process originate from theca or granulosa cells, as Figure 4.3 shows that in some cases the theca cells either draw back, or are absent from the area of the follicle that makes contact with the membrane. This is reminiscent of the theca mono-layer seen between some pre-antral follicles (Chapter 3, Figure 3.6).

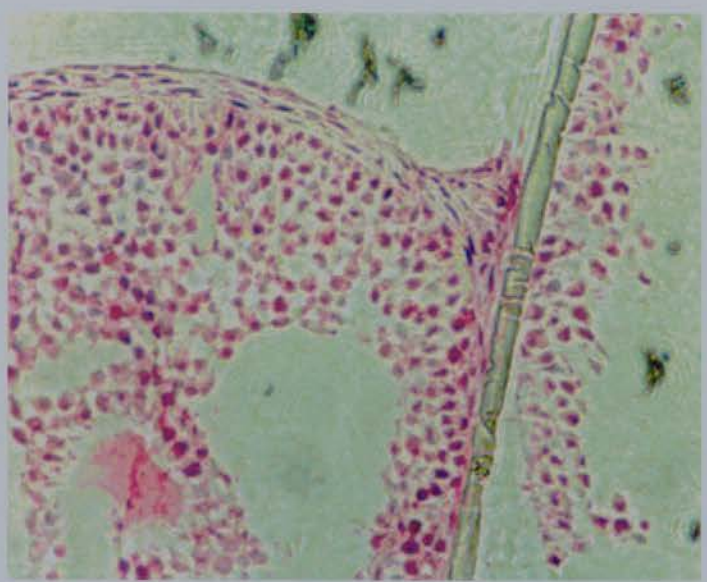
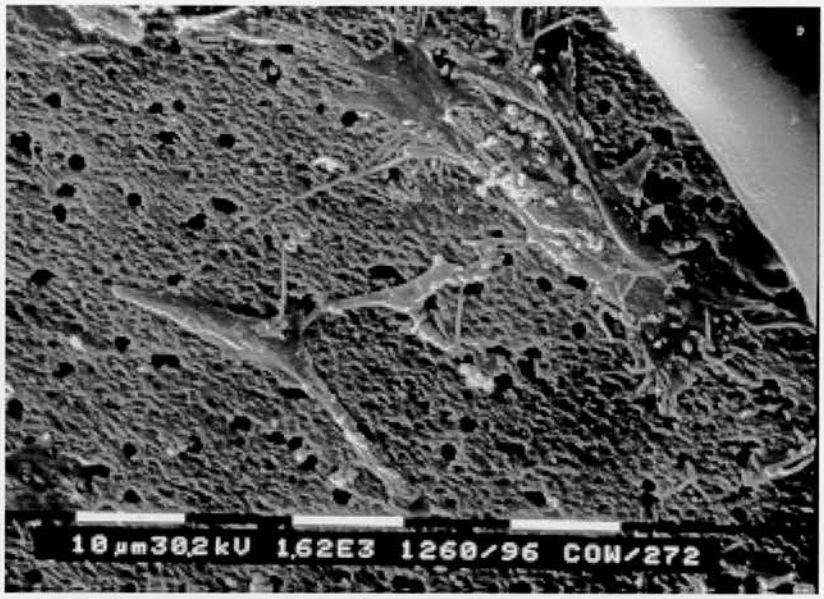
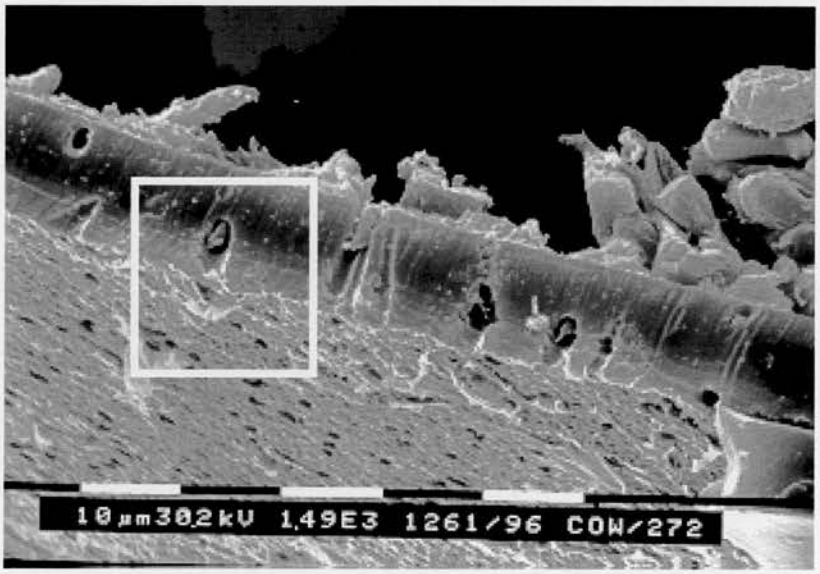


Figure 4.3 A Haematoxylin and eosin stained follicle-membrane-follicle complex One follicle has become partially detached during processing. Cellular processes are growing through the membrane pores, allowing the two follicles to make direct contact throughout the culture. The theca layer is absent or very thin where it comes into contact with the membrane. This is similar to the theca mono-layers seen between pre-antral follicles *in vivo* (see chapter 3).





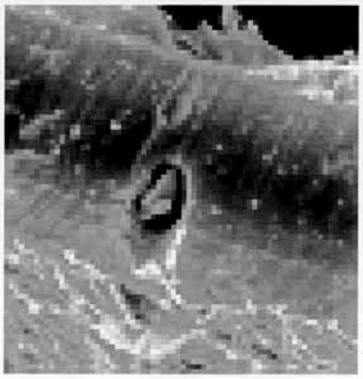


Figure 4.2 Scanning electron photomicrograph of follicle growth on 10 µm pore membranes A. The underside of the membrane after follicle culture. A cell mono-layer has grown on the underside. B. 1.5x 10^3 magnification showing cellular processes growing through the membrane pores. The follicle has fallen off during processing. C. Enlargement of cellular protrusion.

4.3.2. Polycarbonate membrane experiments

This investigation was greatly hampered by the slow acquisition of results due to follicle movement. The 'success' rate, i.e. follicles pairs that stayed juxtapositioned until the end of the culture period, was less than 6%. As preparing the membrane inserts was time consuming in itself the number of cultures that could be realistically accomplished was limited to one a week (assisted by Mrs. Vivian Allison in culture tray preparation, dissection and follicle maintenance). Over 500 follicle pairs were set up, yielding just 28 valid results, making this a very labour intensive study.

4.3.2.1. 10 μm pore experiments

A total of 16 follicle pairs met the criteria to be included in the final analysis of growth. Figure 4.4A shows the final size of all follicles (both subordinate and dominant) versus frequency. The raw data from these membrane experiments suggests that a dominant-subordinate relationship is established within each pair. Given that the starting size of all follicles cultured is approximately the same, it follows that if a dominance phenomenon is exerting an effect it will 'sort' the follicles into larger (dominant) and smaller (subordinate) groups. This should be seen as two distinct peaks on the frequency versus size graph (Figure 4.4A), which is the case. The single control follicles have been plotted in the same manner and are shown by the orange line.

Figure 4.5A shows a graph of dominant and subordinate follicle growth within each co-cultured pair. The average difference in size between the co-cultured pair is 63.8 μm S.E.M. ± 11.1. The two populations touch at 360 μm: this is the largest size recorded for a subordinate follicle, and the smallest size recorded for a dominant follicle. It is interesting to note that the distribution of the subordinate follicles is within a very narrow range. The differences in size between paired, singly cultured controls is shown in Figure 4.6. The average difference in size between pairs is 18 μm S.E.M. ± 4.1. The data from these experiments are also illustrated as a box and whisker plot (Figure 4.7) which allows an easy visual comparison of the different experimental groups. Again, it can be clearly seen that the range of final size values that the subordinate follicles in the

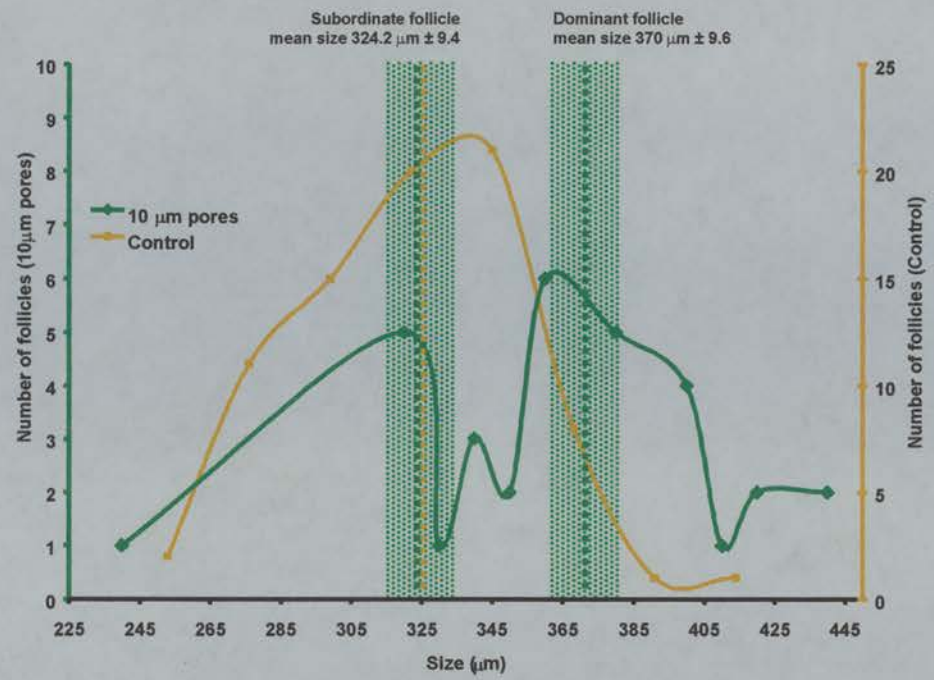


Figure 4.4A Final growth sizes of all follicles co-cultured in pairs across 10 μm pore polycarbonate membranes, as compared to single control follicles. The vertical orange line shows the mean size of controls (326.4 μm ± 0.2). Shading denotes S.E.M. Growth of co-cultured follicles is significantly different from controls ($p<0.001$).

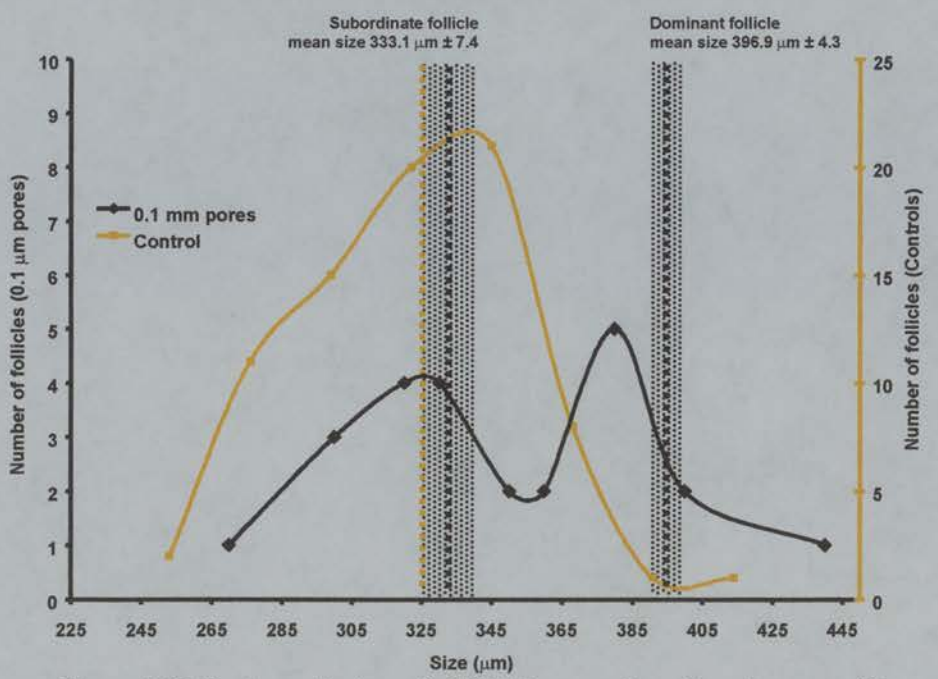


Figure 4.4B Final growth sizes of all follicles co-cultured in pairs across 0.1 μm pore polycarbonate membranes, as compared to single control follicles. The vertical orange line shows the mean size of controls (326.4 μm ± 0.2). Shading denotes S.E.M. Growth of co-cultured follicles is significantly different from controls ($p<0.01$).

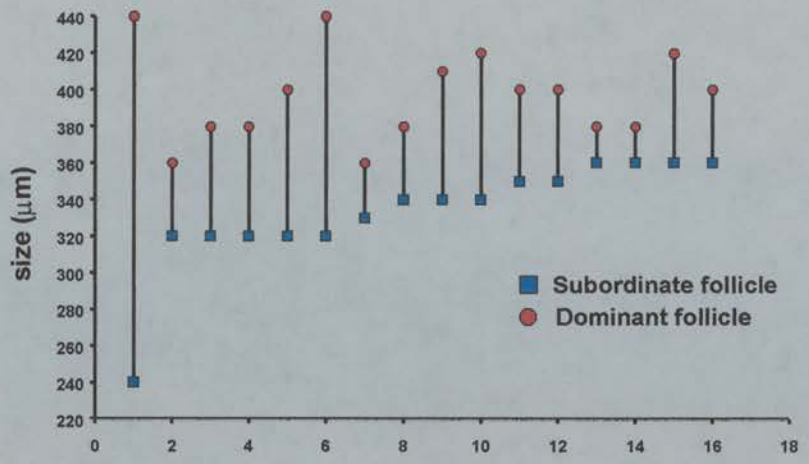


Figure 4.5A Graph showing the final sizes of dominant and subordinate follicles within a pair co-cultured across a polycarbonate membrane with 10 µm pores.

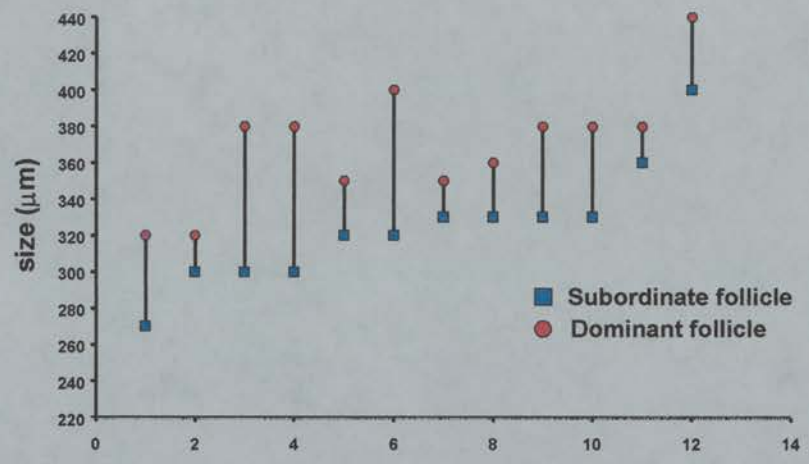


Figure 4.5B Graph showing the final sizes of dominant and subordinate follicles within a pair co-cultured across a polycarbonate membrane with 0.1 µm pores.

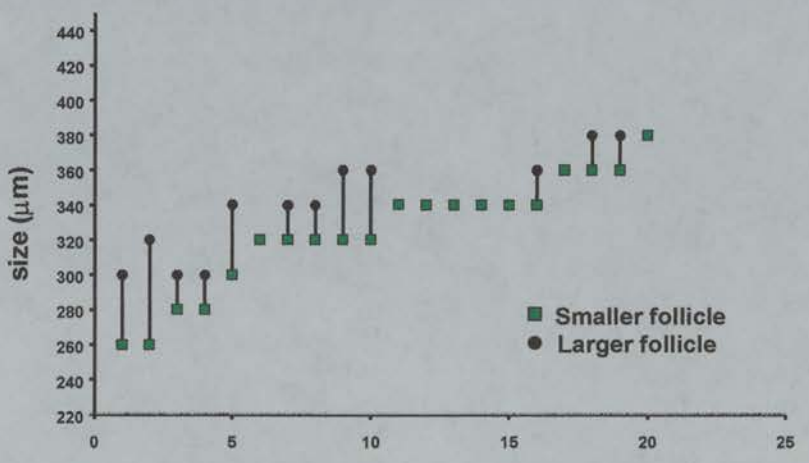


Figure 4.6 Graph showing the final sizes of paired, singly cultured control follicles.

10 μm membrane group span is very limited (320-360 μm, excluding outlier) as compared to controls.

4.3.2.1.1. Further analysis

Statistical analysis of this type of culture is hampered by the fact it is difficult to establish which follicle is affecting which (e.g. experiments that use one follicle to condition medium then move a second follicle into the vacated well rather than having both follicles in the same well at the same time, have a clearly defined experimental follicle. This allows a student's t-test to be used to assess differences in growth). To determine if the growth of co-cultured follicles is significantly different from the controls (thereby illustrating an effect of co-culture on growth) a Chi-squared test was performed. Single control follicle size at the end of culture was divided into bins so that approximately equal numbers of follicles fell into each bin. These were: <285 μm; 286-310 μm; 311-335 μm; 336-355 μm and >356 μm. (e.g. in the control group 20 follicles from a total of 79 fell into the 311-335 μm bin). The Chi-squared test returned a highly significant difference ($p<0.001$, degrees of freedom = 4). This test shows that co-culture does have an effect on growth, but cannot confirm that a bimodal population is present. To ascertain if there are distinct dominant and subordinate populations a student's t-test was performed, comparing the differences in size between dominant and subordinate follicles within each pair in the experimental group with the difference in size between paired, singly cultured controls. This returned a highly significant difference between the two groups ($p<0.001$) and shows that there is a bimodal distribution. When both dominant and subordinate follicle final size was compared to that obtained by single controls, the subordinate follicles showed no significant difference (subordinate: 324.2 μm S.E.M. ± 9.4; control: 326.4 μm S.E.M. ± 0.2), however, the dominant follicles were significantly larger (dominant: 370 μm S.E.M. ± 9.6; control: 326.4 μm S.E.M. ± 0.2, $p<0.001$).

4.3.2.2. 0.1 μm pore experiments

In these experiments 12 follicle pairs (all of which were assessed by an unbiased observer to be juxtapositioned during each day of culture) were included in the final growth analysis. Figure 4.4B shows the final size of the total co-cultured follicle population versus frequency. Figure 4.5B shows a graph of the final sizes attained by dominant and subordinate follicles within each pair. Unlike the 10 μm pore experiments there was a considerable overlap between the dominant and subordinate populations, between 330-440 μm. Although this only represents 29.4% of the entire growth range, 66.7% (16 of 24) of the combined follicle population fall into this band. The average size difference within a co-cultured pair is 45.8 μm S.E.M. ± 6.8. This data is also represented as a box and whisker plot in Figure 4.7.

4.3.2.2.1. Further analysis

Statistical analysis was conducted as described previously for the 10 μm pore experiments. Again, the Chi-squared test returned a highly significant result ($p<0.01$) when co-cultured follicle growth was compared to single controls. When difference in size between paired control and experimental follicles was compared, the student's t-test returns a highly significant difference between the populations ($p<0.001$) again illustrating that there is a bimodal distribution. Comparing dominant and subordinate follicle growth to controls, the subordinate follicles show no significant difference (subordinate: 333.1 μm S.E.M. ± 7.4; control: 326.4 μm S.E.M. ± 0.2), however, the dominant follicles are significantly larger (dominant: 396 μm S.E.M. ± 4.3; control: 326.4 μm S.E.M. ± 0.2, $p<0.001$).

4.3.2.3. Comparison of 0.1 μm and 10 μm pore co-cultures

A Chi-squared test for contingency tables was performed to establish if there was an overall statistical difference between the two experimental groups or not. This test failed to find any significant difference between the groups.

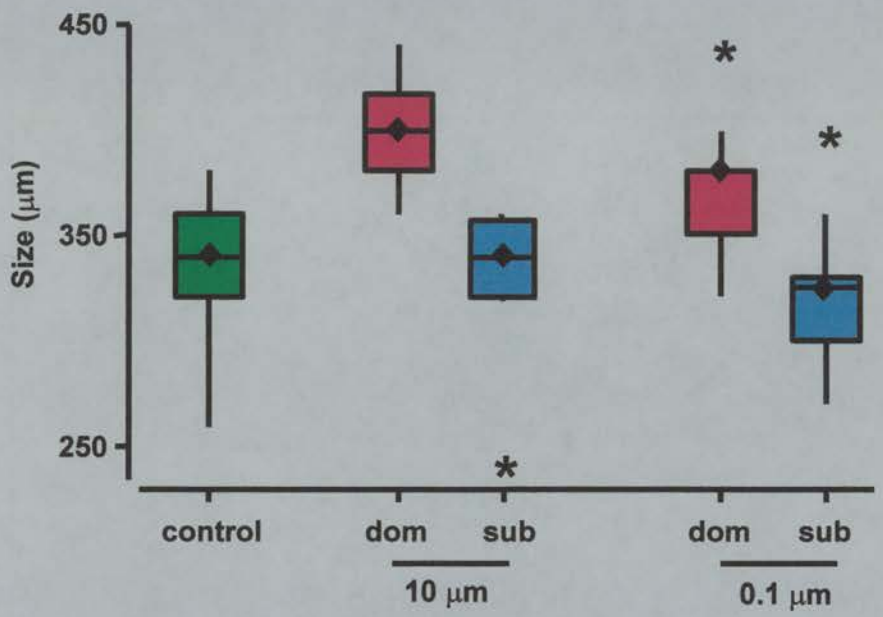


Figure 4.7 A box and whisker plot showing the final sizes of follicles co-cultured across membranes and controls. Coloured boxes represent the first to third inter-quartile ranges (Q1-Q3). The median follicle size is denoted by the diamonds. The distribution of sizes within each group are represented by the whiskers. Outlying follicles (upper limit defined as Q3 -1.5{Q1-Q3}, lower limit Q1-1.5{Q3-Q1}) are shown by asterisks. It can be clearly seen that the growth of dominant follicles in both the 0.1μm and 10 μm membrane groups is greater than controls. It is interesting to note that the final size range of the subordinate follicles in the 10 μm is very tightly distributed (from 320-360 μm, excluding outlyer).

4.4. DISCUSSION

4.4.1. Follicle dominance

These experiments have shown that co-culturing same-sized follicles has an effect on follicle growth when follicles are able to make direct physical contact **and** when physical contact is prevented. The 10 μm experiments, which were in essence a repeat of the original co-culture experiments (Spears *et al.*, 1996) with spherical follicle morphology maintained throughout the culture period, show a dominance effect with dominant and subordinate follicle populations being clearly distinct. Interestingly, the subordinate follicles grew as well as controls and the dominant follicles were significantly larger than controls. This observation suggests that contact has a positive effect on growth for the dominant follicle, but no negative effect on the subordinate follicle. This finding is at odds with earlier observations. The Spears *et al.* cultures (1996) clearly show a suppression of growth of both the dominant and subordinate follicles compared to controls, with subordinate growth being so retarded that there was little increase over the culture period. The most likely explanation for these different findings is follicle morphology. When co-cultured follicles are allowed to make contact freely, with no membrane barrier, they become so closely associated that as much as 50% of their surface area is in contact with the neighbouring follicle (probably more for the subordinate follicle once differences in size become pronounced). This represents a significant loss of area available for diffusion of nutrients in, and metabolites out, of the culture medium. Given this it seems likely that in the original experiments the loss of available surface area significantly compromised follicle growth. The dominance effect is caused by a growth **enhancing** factor that the dominant follicle is receptive to – this would increase the growth of the dominant follicle in the original experiments, causing a significant difference in growth compared to the subordinate follicle, however, the dominant follicle would be unable to realize it's full potential (equal to, or greater than controls) as it is nutritionally compromised.

Again contrasting with the earlier experiments, the 0.1 μm pore co-cultures also produced dominant and subordinate follicles, providing strong evidence for a diffusible

dominance factor. As with the 10 μm pore experiments, the subordinate follicles were not significantly different in size compared to the controls, but dominant follicle growth was significantly enhanced. These experiments have provided evidence that pre-antral follicles produce a diffusible factor that enhances growth (this could be directly, or by modulating response to other factors such as FSH). One effect that co-culturing in contact does appear to have is the synchronising of subordinate follicle growth, with the subordinate follicles in the 10 μm pore experiments distributed across a very narrow size range. Unfortunately it is impossible to know if this is coincidence or a direct result of contact, due to the small sample size.

The results and conclusions from these membrane experiments have been based on the small number (less than 6%) of paired follicles that stayed together. While I have made the assumption that these 'static' follicle pairs are normal and that conclusions made following the analysis of their growth are true for all cultured murine follicles it is important to remember that the majority of follicles **did not** stay together. In many experiments it would be the 94%+ that were chosen as representative of growing follicles. It is impossible to know if the follicles I have included in this experiment are 'normal', however other factors, such as healthy morphology and growth rates suggest that these follicles are typical of others at the same developmental stage. I have made the assumption that when adjacent follicles are allowed to make contact through the large pored membranes that they form connections similar to those *in vivo*. Further investigations could examine this, perhaps by looking at the expression of proteins such as the connexin family (Wright *et al.*, 1997) in co-cultured follicles in direct contact, across membranes and *in vivo* and make comparisons between these.

This experimental design has served its purpose and given us an insight into the nature of the dominance mechanism, however, the accumulation of these data has been laborious due to the technical difficulties associated with preparing the membrane constructs and the need to perform so many experiments to obtain reasonable numbers. For these reasons, further work to investigate this diffusible factor has utilised other experimental techniques. Recent investigations by others in this laboratory have used follicle-conditioned medium in an attempt to characterise growth-affecting substances

produced by the follicle, also finding evidence of diffusible factors released by follicles which affect the development of others.

The membrane co-culture results are novel in that they are the first to demonstrate a diffusible factor(s) secreted by late pre-antral follicles that is stable in culture and can influence the growth of like-follicles. It is reasonable to speculate that this factor(s) is involved in the establishment of differences between growing follicles within a cohort. It is possible that the same factor(s) also has a role in the maintenance of these developmental differences once established and as such may have been characterised previously (see chapter1, 1.8.2 for discussion of identified inductive signalling factors).

4.4.2. Follicle migration *in vitro*

The fact that greater than 94% of co-cultured follicle pairs were excluded from this study due to movement is an interesting observation in itself. From the outset great care was taken to ensure that mechanical trauma to the culture trays was avoided. The trays were housed in incubators that were left undisturbed for long periods of time and movement of the trays for follicle assessment was slow and fluid. Despite this, follicles were still found to move from were they had been positioned. After several successive cultures I was satisfied that the observed movement was not due to mechanical perturbation and concluded that the follicles themselves were actively moving on the polycarbonate membrane. This movement was not confined to the horizontal: the follicles also frequently moved vertically, adhering to the upright portion of the membrane construct. A search of the literature in an attempt to find documentation of follicle migration found no specific references, although one likely candidate for follicle migration is the horse. Equids have an ovulation fossa, into which ovulatory follicles are always released (Carnevale *et al.*, 1988). This requires that the ovulatory follicle must make its way to the fossa to release the oocyte, regardless of its site of development. Tracks of connective tissue can be clearly seen in ovarian sections (Prickett, 1962), radiating across the ovary towards the fossa.

There are two possible ways that follicles could move to an appropriate sight for ovulation, a) follicles could expand sufficiently during the late antral stages to ensure that they reach the ovary's edge, or b) follicles could migrate to ensure that they are at the correct location (the ovarian periphery) for oocyte release. Follicle migration could be either active, with the follicle directing its own movement, or passive, with the follicle being pushed to the correct location by other movement within the ovary. In either case there would be a need for radical remodeling of the extracellular matrix, a point highlighted by Song *et al.* (1999) working on equine tissue. Their work implicates the production of gelatinases and TIMPs by stromal cells as permitting this re-arrangement of follicle geography. Histological examination of the ovaries from a variety of species for the studies detailed in Chapter 3, showed that large follicles can be fully contained within the ovary, making no contact with the surface, despite their size. It would seem feasible therefore, that these follicles may have a migratory requirement if they are to release their oocytes outside the ovary. If follicles do migrate, this poses the question of which cells do they take with them? Does the theca externa delimit the follicle boundary and everything contained within those cells moves? Or does the basal lamina mark the edge of the 'core' follicle that moves? Although too time consuming to be pursued further during the course of this thesis investigation, this topic is currently receiving further attention from others in this laboratory. Investigations are underway to document the movement of mouse follicles *in vitro* (my previous observations had indicated that this occurred most noticeably on culture day 3, typically the time that antrum formation is first seen). Investigative strategies include attempts to identify follicle chemo-attractants and record patterns of follicle movement. A molecular approach can also be taken to investigate the role of genes known to be involved in migration in other developmental systems (an area that may overlap with the genes being investigated for possible roles in follicle selection detailed in Chapter 7).

Chapter 5

The effect of FSH concentration on follicles at different stages of development, *in vitro*

5.1. INTRODUCTION

As discussed in Chapter 1, section 1.7.1.1., the major force involved in follicle selection is the systemic concentration of FSH. Governed by negative feedback acting on the hypothalamic-pituitary system, the amount of FSH released into circulation is dependent on the level of oestradiol and inhibin produced by the ovary (Zeleznik and Hillier, 1984; Gibbons *et al.*, 1997). This provides a relatively straight-forward model for follicle selection. As certain follicles become more advanced in their development, the amount of oestradiol and inhibin that they produce increases, resulting in an elevated systemic concentration of these factors. They act on the pituitary-hypothalamic axis resulting in a decreasing amount of FSH released by the pituitary gland. In this way circulating levels of FSH decline to a concentration that is insufficient to support the growth of most gonadotropin dependent follicles (Brown, 1978), other than those late antral follicles that are able to survive by sequestering what FSH there is (Zeleznik *et al.*, 1981) and transferring dependence to LH, via up-regulated LH receptors on the granulosa cell layer (Webb and England, 1982; Ireland and Roche, 1983). The lesser developed follicles are pushed down the atretic pathway, reducing the number of follicles in the growing cohort (possibly to the desired ovulatory number in the mouse and other multi-parous mammals). This mechanism is likely to form the broad framework by which the ovulatory number is regulated, with intra-ovarian processes under-pinning this process by locally selecting the follicles which become most developed within the growing cohort. It is feasible that the dominance mechanism under investigation during the course of work for this thesis is involved early on in the selection 'cascade' by establishing differences between equivalent follicles. Other processes may then maintain these differences (e.g. Campbell *et al.*, 1991), either independently or in adjunct with this mechanism, holding non-selected follicles in a retarded stage of development until the declining FSH concentration pushes them down the atretic pathway.

The central tenet of the endocrine mechanism for follicle selection is that mature (late antral) follicles are able to withstand low concentrations of FSH by utilising available LH, but less developed counter-parts do not posses this ability. To test that this

hypothesis holds true for mouse follicles *in vitro* I conducted experiments to investigate very early and later antral follicle FSH dependency. Follicles were cultured in a range of FSH concentrations with a constant level of LH, at two different stages of development. At the end of the culture period these follicles were examined for evidence of apoptosis, by examining gel electrophoresis separated labelled DNA, a sensitive assay for the early detection of atresia (discussed in Chapter 1).

While it has been widely held that antral follicles are highly susceptible to undergo atresia *in vivo* (Hirshfield and Midgley, 1978; Hirshfield, 1988; Hirshfield, 1991a), most likely via an apoptotic process (Hsueh *et al.*, 1994; Tilly *et al.*, 1991; Hughes and Gorospe, 1991) also demonstrated *in vitro* (Chun *et al.*, 1996), it is less clear to what extent pre- and early antral follicles are vulnerable to apoptosis *in vivo*. Using *in situ* techniques for the localisation of apoptotic cells, Billig *et al.* (1994) and Palumbo and Yeh (1994) report granulosa cell apoptosis in pre-antral follicles, however this method can generate false positive results as other forms of DNA damage can be labelled. In the rat, it has been shown by DNA fragment analysis that pre-antral follicles undergo apoptosis in serum free conditions (McGee *et al.*, 1997). It is possible that mouse pre-antral follicles are not developmentally competent to undergo atresia via an apoptotic pathway. In addition to examining the effect of low FSH concentrations of cultured murine follicles these experiments should offer an insight into whether or not cultured mouse pre- /early antral follicles are competent to undergo apoptosis.

5.2 MATERIALS AND METHODS

5.2.1. Follicle cultures

A whole follicle culture technique was used, as detailed in Chapter 2. The culture medium used was α-Minimum essential medium supplemented with 5% mature F_1 mouse serum and 0.01 IU ml^{-1} of rLH. rFSH was also added at the following concentrations:

Tray 1: 0 FSH
Tray 2: 0.1 IU ml^{-1} FSH
Tray 3: 0.25 IU ml^{-1} FSH
Tray 4: 0.5 IU ml^{-1} FSH
Tray 5: 1 IU ml^{-1} FSH (standard control concentration).

In the first part of the experiment, which examined the effect of FSH concentration on the growth and health of small follicles, pre-antral follicles ~185 μm in diameter were cultured for two days in the five different FSH levels. In the second part of the experiment, all pre-antral follicles were cultured for three days with the standard concentration of FSH (1 IU ml^{-1}) then randomly assigned to the experimental concentrations. This experimental design allowed the follicles to reach antral stages of development before being affected by the decrease in trophic FSH. At the end of the culture period the follicles were examined under the microscope and discarded if they displayed any obvious morphological signs of damage or abnormal development. A sample size of 10 antral follicles was sufficient to extract enough DNA for the subsequent procedures. All experiments were repeated at least four times.

5.2.2. Detection of DNA ladders in cultured follicles

To assess the effect of differing FSH concentrations on follicles at different stages of development, genomic DNA was examined for evidence of apoptosis.

5.2.2.1. Extraction of genomic DNA

After removal from the culture medium, follicles were snap-frozen on dry ice in eppendorf micro-centrifuge tubes to halt all cell function (thereby reducing the risk of artificially inducing apoptosis). Samples were then lysed with 100 μg ml^{-1} Proteinase K in 400 μl of mouse tail solution (0.5% SDS, 0.1M NaCl, 0.05M tris, pH 8.0, 2.4mM EDTA) at 55°C for 5-6 hours. After this process, samples could be stored indefinitely at –70 °C if required. To extract DNA from the digest, 75 μl 8M Potassium Acetate and 500 μl chloroform was added, vortexed briefly (so as not to shear the DNA), frozen at –20 °C for 1 hour then centrifuged for eight minutes at 10 000 g (4 °C). The aqueous layer was then removed and transferred to a clean micro-centrifuge tube, 500 μl isopropanol added then precipitated at –70 °C for two hours. Centrifugation for 30 minutes at 14 000 g (4 °C) resulted in a small DNA pellet collecting at the bottom of the tube. Working on ice, the isopropanol was removed and the pellet washed with 80% ethanol prior centrifugation at 14 000g for ten minutes. The ethanol was then removed and the pellet allowed to air dry for ~15 minutes before resuspension in ddH_2O. A spectrophotometer was used to estimate the amount of DNA extracted.

5.2.2.2. DIG 3'-end labeling of extracted DNA

A digoxygenin (DIG) 3'-end labelling kit was used to tag the extracted DNA (Boehringer Mannheim). Equivalent amounts of DNA were end-labelled within each experimental group, made up to a final volume of 10 μl with ddH_2O, e.g. if sample A had 40 ng ml^{-1} DNA, and sample B had 20 ng ml^{-1} DNA, 10 μl of undiluted sample B would be taken for end-labelling and 5 μl of sample A (made up to 10 μl with ddH_2O).

To the 10 μl DNA sample, 4 μl of 5x reaction buffer; 4 μl of $CoCl_2$; 1 μl of terminal transferase and 1 μl of 1mM DIG-ddUTP was added. This was incubated at 37 °C for ~3 hours. The reaction was stopped with 5μl of 0.25mM EDTA. 2 μl of glycogen was added (this acted as a carrier for the low weight DNA) and the DNA precipitated with 5 μl 4M LiCl and 150 μl absolute ethanol at –70 °C for 1 hour. Centrifugation at 13 000g for 15 minutes produced a visible DNA/glycogen pellet which was washed with

70% cold ethanol, centrifuged at 13 000g for five minutes and allowed to air dry after removal of the ethanol. The dried pellet was re-suspended in 50 µl ddH_2O.

5.2.2.3. Agarose gel electrophoresis

A 75 ml, 2% agarose gel was prepared by dissolving 1.5g of agarose in TAE. The gel was poured taking care to remove any air bubbles, and allowed to set for 30 minutes prior to use. 15 µl of the labelled-DNA solution was loaded onto the gel, mixed with 5 µl of gel loading buffer (200 µl bromophenol blue-xylene cyanole dye mixture, Sigma), 300 µl glycerol, 10 µl 0.5M EDTA made up to 1 ml with ddH_2O). The gel was run at 75 volts until adequate separation of the DNA bands was achieved.

5.2.2.4. Southern blotting

The separated DNA was transferred from the agarose gel onto a positively charged nylon membrane (Boehringer Mannheim) by capillary action. The gel was placed on a 20x SSC saturated wick (QuickDraw™ blotting paper, Sigma) that covered a gel support. The edges of the wick sat in a 20x SSC reservoir. The nylon membrane was placed on top of the gel and any air bubbles caught between the two layers removed with gentle pressure. Further sheets of blotting paper were placed on top of the membrane and finally the whole stack weighed down (~500g) and left overnight. The following day the stack was dismantled and the membrane baked at 120 °C for 30 minutes to adhere the DNA.

5.2.2.5. Detection of DNA bands

The following buffers were prepared:

Buffer 1 (100mM maleic acid and 150mM NaCl).

Washing buffer (49.5 ml Buffer 1, 0.5 ml 3% Tween 20).

Blocking solution (3 ml 10% blocking reagent (Boehringer Mannheim) in 27 ml washing buffer).

Detection solution (3 ml 1M NaCl; 3 ml 1M Tris-HCl pH 9.5, and 1.5 ml 1M

$MgCl_2$ made up to 30 ml with ddH_2O).

Baked membranes were placed in heat-sealing plastic bags and washed in 10 ml washing buffer then incubated in 20 ml blocking solution for 30 minutes with shaking. To the remaining 10 ml blocking solution Anti-DIG AP was added at a concentration of 1:5000 (2 µl). The membrane containing bags were emptied and replaced with the Anti-DIG solution and shaken for 30 minutes, before 3 x 10 minute washes in the remaining washing buffer. The membrane was then equilibrated briefly in 20 ml detection buffer while 200 µl NBT/BCIP (Boehringer Mannheim) was added to the remaining 10 ml. The membranes were incubated in this colour reagent solution for 2-24 hours at 37 °C until the DNA bands were visible to the naked eye. The reaction was stopped with copious washing in TE pH 8.0 and the plastic bags sealed.

5.2.2.6. Computer analysis of band intensity (degree of apoptosis)

The colour-reacted membrane was scanned using an optical densitometer (Bio-Rad) and the band density calculated using the Molecular Analyst (Bio-Rad) software. The density of the bands in each experimental group was calculated relative to the control group (see Figure 5.1).

5.2.2.7. Statistical analysis

All data was normalized so that the control results equaled 1. The student's t-test was used to compare experimental groups with the appropriate control group (1 IU FSH ml^{-1} throughout).

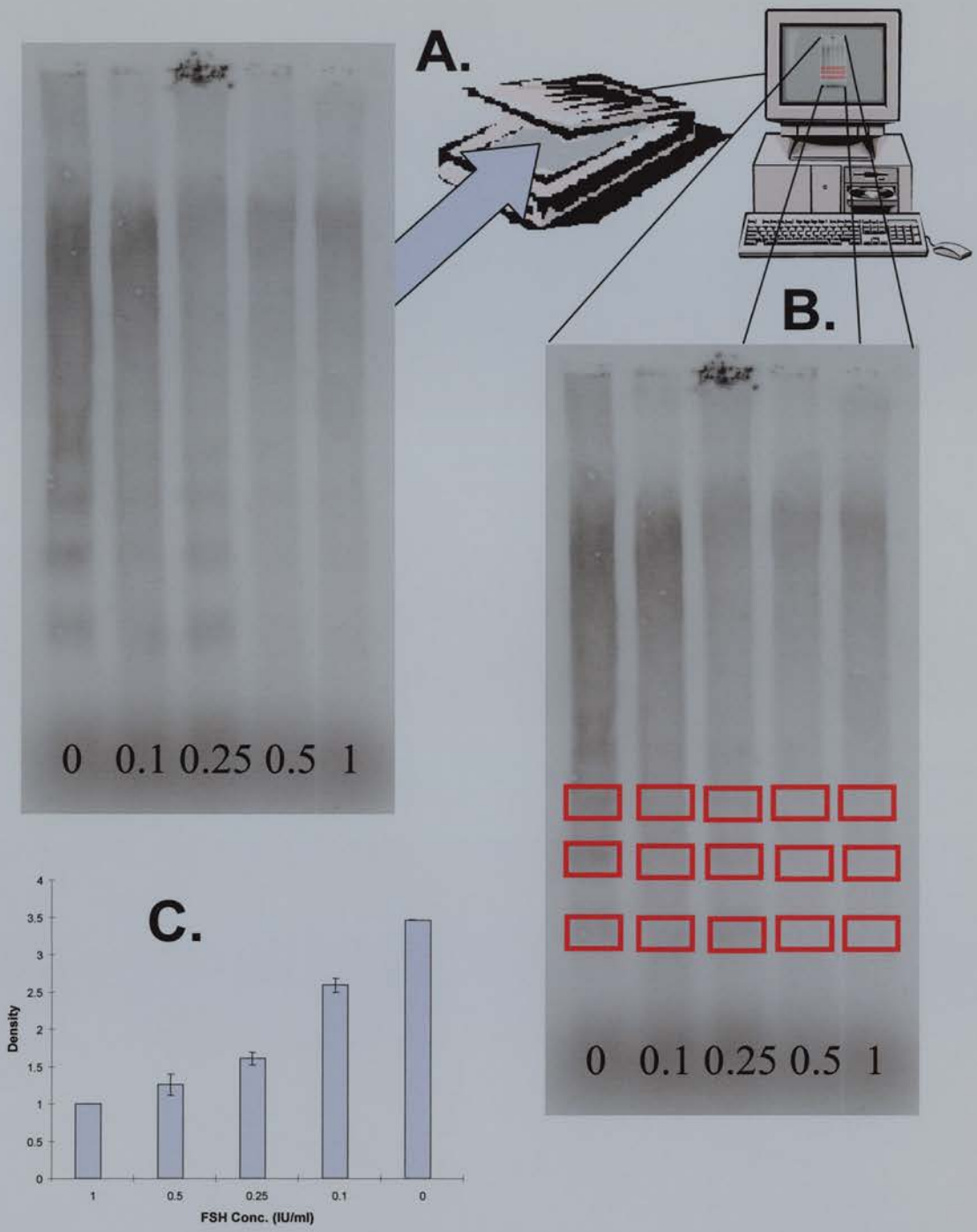


Figure 5.1 A. The colour developed membranes are scanned into a computer. **B.** The apoptotic bands are manually highlighted (illustrated with the red boxes) and a value calculated for the density of each band. The density value for the 370 bp, 550 bp and 735 bp bands are amalgamated for each treatment (different concentration of FSH as denoted at the bottom of the membrane in IU/ml) to give one overall value. **C.** This data can be shown on a histogram to give a clear representation of the differences between groups.

5.3. RESULTS

5.3.1. Follicle culture growth

Two day cultures (follicles cultured in experimental levels of FSH from the outset).

No record of growth was taken for these short-term cultures. At the end of the culture period, follicles from all the groups appeared to be healthy when examined by light microscopy.

Five day cultures (follicles cultured for three days in 1 IU ml^{-1} FSH, then in experimental FSH levels for a further two days).

Figure 5.2 shows the growth of the cultured follicles. By the end of the culture period the follicles cultured in the absence of FSH, or with only 0.1 IU ml^{-1} had grown significantly less than the control (1 IU ml^{-1}) follicles ($p<0.05$ and $p<0.001$, respectively). There was no significant difference in antral development between the groups (see table 5.1).

5.3.2. Detection of DNA ladders in cultured follicles

5.3.2.1. Two day cultures (follicles cultured in experimental levels of FSH from the outset).

When the degree of apoptosis within each experimental group (different levels of FSH) is displayed graphically (Figure 5.3) it can be clearly seen that apoptosis increases as FSH concentration decreases. These data are displayed numerically as the percentage increase relative to controls in Table 5.2, e.g. there was a 312% increase in the degree of laddering in the 0 IU ml^{-1} group. When each experimental concentration was compared to controls (1 IU ml^{-1}) using the student's t-test, the 0.1 IU and 0 IU ml^{-1} groups show significantly higher levels of apoptosis ($p<0.05$ and $p<0.01$, respectively).

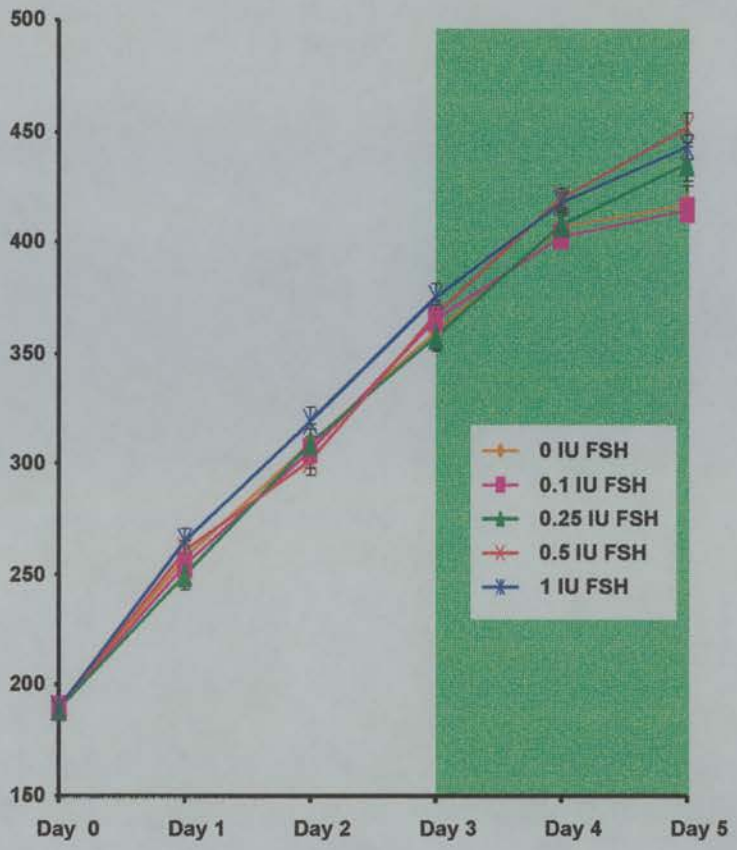


Figure 5.2 Growth of follicles over the culture period. The shaded area illustrates when follicles were moved into experimental levels of FSH. The 0.1 IU ml^{-1} and zero FSH groups grew significantly less than control follicles ($P<0.001$ and $p<0.05$, respectively).

	Early antral (%)	Mid antral (%)	Graafian (%)
0 IU FSH	17.2	44.8	37.9
0.1 IU FSH	16.1	54.8	29.0
0.25 IU FSH	16.7	56.7	26.7
0.5 IU FSH	3.4	48.3	48.3
1 IU FSH	14.3	31.4	54.3

Table 5.1 Antral development of follicles cultured in 1 IU ml^{-1} FSH for three days then experimental FSH levels for a further three days. Chi-squared analysis failed to find any statistical differences between control follicles and experimental groups.

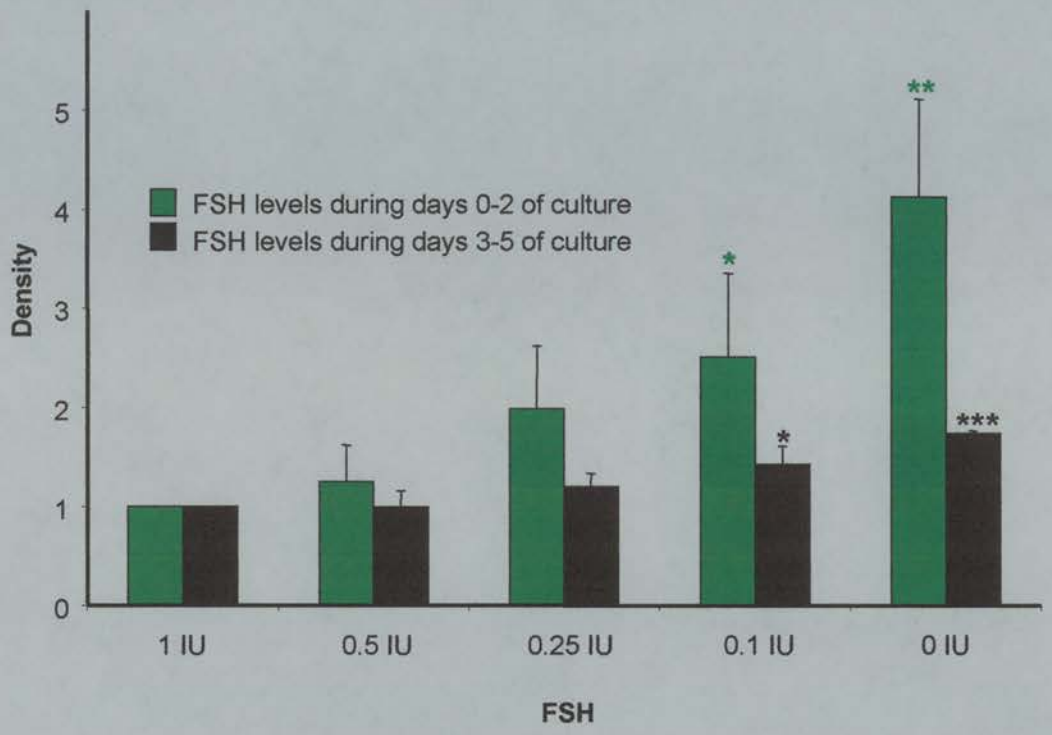


Figure 5.3 The density of apoptotic fragment ladders following extraction from cultured follicles, DIG 3'-end labelling, agarose gel electrophoresis, Southern blotting and DIG visualisation. Data is normalised so that the density of bands from the 1 IU ml^{-1} FSH cultures are assigned a value of 1. *=$p<0.05$; **=$p<0.01$; ***=$p<0.001$

	1 IU FSH (control)	0.5 IU FSH	0.25 IU FSH	0.1 IU FSH	0 IU FSH
2 day cultures	-	25.6	99.1	150.9	312.5
5 day cultures	-	-0.7	19.9	42.3	73.4

Table 5.2 Percentage increase (relative to controls) of band density in each treatment.

5.3.2.2. Five day cultures (follicles cultured for three days in 1 IU ml^{-1} FSH, then in experimental FSH levels for a further two days).

As for the shorter cultures, the degree of apoptosis within each group is displayed graphically (Figure 5.3) and numerically (Table 5.2). In these experiments there was a less marked increase in the degree of apoptosis as FSH levels were decreased, e.g. in these experiments the 0 IU ml^{-1} group showed a 73% increase in laddering intensity. When each experimental concentration was compared to controls (1 IU ml^{-1}) using the student's t-test, the 0.1 IU and 0 IU ml^{-1} groups show significantly higher levels of apoptosis ($p<0.05$ and $p<0.001$, respectively).

5.3.2.3. Comparison of two day and five day cultures.

When the data is normalised so that the 1 IU ml^{-1} groups in both experiments are assigned the value of one it appears that the follicles in the five day cultures are more tolerant of an FSH drop (comparing the 0 IU ml^{-1} groups there is a 73% increase in the degree of apoptotic laddering in the longer cultures compared to 312% in the short cultures). While it is in keeping with the proposed hypothesis that the more mature follicles are able to utilise the available LH and so protect themselves against the FSH decline it is not possible to directly compare the two cultures. The end point of the experiment is different for the two cultures, with the follicles taken for molecular analysis being at different developmental stages. It is possible that the extent of laddering in the control group is a lot higher in one of the experiments (this could be viewed as the base-line). Thus although it may appear that there is a relatively small increase in laddering in one of the experimental treatments, it is not possible to know whether or not this represents a significant increase if compared to the control in the parallel experiment.

5.4. DISCUSSION

The results from these experiments clearly show that late pre-antral/early antral follicles *in vitro* do have a requirement for FSH as a survival factor and are competent to undergo apoptosis in insufficient concentrations of this gonadotropin, despite the presence of LH. Apoptosis has previously been detected in mouse pre-antral follicles taken from 12 day-old animals and grown *in vitro* (Robertson and Telfer, 1994). This finding is in accordance with an earlier report by the same authors who observed apoptosis *in vivo* in primordial and pre-antral follicles taken from mice up to 20 days of age (Telfer and Robertson, 1993). Follicles taken from older mice did not display signs of apoptosis (as characterised by laddering). The culture of more developed antral follicles in different concentrations of FSH (the five day cultures) support the role of LH in maintaining antral follicle health. These follicles also have a requirement for FSH and become increasingly apoptotic as it is withdrawn. However, they display a markedly lower increase in apoptosis relative to their controls than do the less-developed pre-/early antral follicles. This is presumably a result of their ability to respond to LH via granulosa LH receptors. These findings support previous work which demonstrated that FSH and LH are effective inhibitors of apoptosis in cultured rat antral follicles (Tilly *et al.*, 1993). This inhibition of apoptosis is likely to be as a result of intra-cellular signalling involving the adenyl cyclase-cAMP pathway (Tilly *et al.*, 1993) and IGF-1 (Tilly and Furuta, 1993). Given the hypothesis that LH dependence is the principle way in which antral follicles are able to withstand the FSH decline *in vivo* it is perhaps suprising that there is any detectable increase in apoptosis in these more developed follicles. There are two main reasons that could explain this. Firstly, the DIG 3'-end labelling assay for apoptosis is very sensitive and so is able to detect very small changes in DNA laddering. There is less than a two-fold increase in the degree of apoptosis in the zero FSH group as compared to controls. This could be an increase from 10 to 20 apoptotic cells in a total population of many hundreds – which may represent a significant increase in apoptosis but have no serious ramifications for overall follicle, or perhaps most importantly, oocyte health. Secondly, the follicles in these cultures were

transferred into the experimental FSH concentrations after three days in culture with 1 IU ml^{-1}. These follicles would have been in the early-mid stages of antral development. Although LH receptors should start to be manufactured on granulosa cells at this developmental stage (Peng *et al.*, 1991) they might not be in sufficiently high numbers to allow the follicles to transfer their dependence to LH. Additionally, at least ten follicles are pooled for each ladder analysed which may be at different stages of maturity. It would only take one less developed, more acutely FSH-dependent follicle to increase the combined ladder intensity for the whole group.

Together, this series of experiments has shown that follicles are developmentally competent to undergo apoptosis-mediated cell death from the pre-/early antral stage onwards, at least *in vitro*. Furthermore, the addition of LH into the culture medium provides a survival factor that follicles are able to respond to, dependent on their developmental stage. These findings tie-in with the gonadotrophin hypothesis for follicle selection. What these results are unable to tell us is the follicle compartment(s) that are undergoing apoptosis. The genomic DNA extractions from the cultured follicles are from theca and granulosa cells, and the oocyte. It is entirely possible that as a follicle matures the cell population's susceptibility to apoptotic death changes. It has been reported that pre-antral follicle oocyte degeneration preceeds atresia in the rest of the follicle (Hakuno *et al.*, 1996). It is possible therefore that laddering seen from the pre-antral cultures might be contributed to predominantly from the oocyte. Co-culturing oocytes with granulosa cells results in an increase in the incidence of apoptosis in the granulosa cell population possibly as a result of activation of Fas on the granulosa cells following expression of Fas ligand on the oocyte surface (Quirk *et al.*, 1995; Hakuno *et al.*, 1996). This would imply that atresia in these follicles starts at the oocyte and moves outwards to the follicle periphery. Conversely, studies of cell death in antral follicles show that apoptosis originates in the peripheral areas first, moving inwards from the mural granulosa cells and finally the oocyte, a reversal of observations in pre-antral follicles. Despite the possibility that these experiments have encompassed different patterns of cell death, I believe it is justifiable to compare the two developmental stages as the end point is presumably the same (atresia of the entire follicle). It is entirely

however, that the two follicle populations undergo apoptosis initially in different cell compartments, possibly stimulated by different extra- and intra-cellular messengers. While it is possible to separate mouse follicles into individual cell populations and analyse them separately, their size would make this a difficult and time consuming pursuit. Other possibilities include *in situ* DNA fragment labelling of follicle sections, although again size makes this approach problematic, and whole follicle TUNEL labelling and visualisation using confocal microscopy, an approach taken throughout the experiments detailed in Chapter 6. This has the advantage of relatively easy processing (once a follicle permeablisation and fixation protocol has been constructed) and localisation of fragmented DNA within individual cells. As with all *in vitro* work, it is important to consider that cultured follicles may behave differently *in vivo*, although I believe that the whole follicle culture system is as near to physiological as is possible. *In situ* analysis of whole ovaries, fixed immediately *post mortem* looking specifically at pre- and early antral follicle health would indicate whether apoptosis in these follicles is a physiological event.

Chapter 6

The effect of co-culture on follicles in different concentrations of FSH

6.1 INTRODUCTION

Chapter 5 discussed the major endocrine component of follicle selection *in vivo*. In essence, circulating levels of FSH decline to a concentration that is inadequate to support the growth of FSH-dependent follicles (Brown, 1978). Some late antral follicles are able to survive by being better equipped to use what FSH there is (Zeleznik *et al.*, 1981) and by transferring dependence to LH, via up-regulated LH receptors on the granulosa cell layer (Webb and England, 1982; Ireland and Roche, 1983). The lesser developed follicles have not developed these survival mechanisms and are pushed down the atretic pathway. In this way the number of follicles in the growing cohort is reduced. It is likely that intra-ovarian processes, possibly including the dominance mechanism under investigation, under-pin this process by locally selecting the follicles which become most developed within the growing cohort.

The cornerstone of the hypothesis for endocrine follicle selection is that mature follicles are able to withstand low concentrations of FSH by utilising available LH, an ability lacking in less developed follicles. I have hypothesised that the dominant-subordinate relationship established and maintained when follicles are co-cultured in contact is responsible for holding non-selected follicles in a retarded state of development so that the systemic FSH decline has a clearly defined 'target' population of less healthy follicles. To test this hypothesis I exposed co-cultured follicles to different concentrations of FSH during the culture period and observed the effect on dominant (selected) and subordinate (non-selected) follicle health.

Follicle pairs were chosen with an initial size difference as this meant that culture time could be reduced, circumventing problems of bursting due to the absence of ascorbic acid addition to the culture medium. Ascorbic acid was not added to these cultures as earlier experiments showed it to be a powerful inhibitor of apoptosis even in strongly atretogenic environments (Chapter 2, 2.2.1.1). Previous work from this laboratory has shown that co-culturing follicles with an initial size difference is a reasonable strategy for bypassing the initial setting up of 'dominance'.

In Chapter 5, apoptotic-associated cleavage of genomic DNA (laddering) was used as an assay to determine relative follicle health in the single follicle cultures. To gain a clearer picture of where apoptosis was occurring in the co-cultured follicles a different protocol was adopted. The co-cultures were terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelled (TUNEL) using a commercially available kit. The follicles could then be looked at using a confocal microscope as the TUNEL kit puts a fluorescent tag onto genomic DNA strand breaks (ladders). This apoptosis assay is in keeping with previous experiments (detailed in Chapter 5) which also quantified the amount of genomic DNA laddering present in follicles. Furthermore, it has three clear advantages. Firstly, it is possible to look at individual intact follicles enabling clear identification of the cells most affected by FSH deprivation. Secondly, the spectrum of follicle response to FSH withdrawal can be seen: differences between follicles in the same treatment are unidentifiable when DNA is extracted from several follicles. Finally, there is no danger of DNA from the other co-cultured follicle contaminating the DNA preparation from the first follicle and hence leading to false results. To individually assay dominant and subordinate follicles that have been co-cultured using the total DNA extraction protocols means that the follicle pair has to be separated. This is technically difficult and even meticulous separation is likely to result in some mixing of each follicles' peripheral cell population. One negative aspect of using the TUNEL protocol is that results reflect fewer follicles due to the fact that analysis of each follicle is very time consuming.

Before embarking on the co-culture experiments a small-scale repeat of the experiments detailed in Chapter 5 was carried out. This enabled comparisons between the two assay techniques to be made.

6.2 MATERIALS AND METHODS

6.2.1. Follicle cultures

Follicles were dissected from the ovaries of three-week-old mice according to the protocols outlined in Chapter 2. Before co-culture experiments were started small scale cultures (6 follicles in each group) were set up as described previously (Chapter 5, 5.2.1). These were single follicles either cultured for 48 hours in different concentrations of FSH or single follicles cultured for three days in 1 IU ml^{-1} FSH then transferred to experimental concentrations of FSH. For the co-culture experiments, freshly dissected follicles were accurately measured and 160 μm and 220 μm follicles paired. These pairs were then cultured in standard medium (see Chapter 2) containing 1 IU ml^{-1} FSH, without ascorbic acid, for three days (media was changed after the first 48 hours). After this time half of the pairs were moved into medium containing either 0 IU ml^{-1} FSH whilst the other half remained in medium containing 1 IU ml^{-1} FSH for a further two days. A tray of single control follicles that remained in 1 IU ml^{-1} FSH for the duration of the culture period was also included in these experiments. At the end of culture, all the follicles were fixed, permeabilised and stained for confocal microscopy according to the protocols detailed below.

6.2.2. Staining for confocal microscopy

6.2.2.1. Permeabilisation and fixation

At the end of culture, follicles were washed for 10 minutes in PBS, pH 7.2-7.4 at 37 °C before being transferred into 0.5% Triton X-100 and 0.25% paraformaldehyde in PBS for 30-40 minutes. The follicles were then fixed in 4% paraformaldehyde for 30 minutes, washed twice for 10 minutes in PBS. At this point they could be stored overnight (or longer if required) in PBS with 0.02 % sodium azide at 4 °C. After removal from overnight storage the follicles were washed in PBS for 10 minutes and transferred into 17.1 μg ml^{-1} Proteinase K for 25 minutes at 37 °C. They were then washed in PBS with

0.01% Triton X-100 for 10 minutes and fixed in 3% paraformaldehyde at room temperature for 30 minutes. Follicles were then washed twice in PBS for 20 minutes.

6.2.2.2. TUNEL and propidium iodide staining

Following 10 minutes pre-incubation in 50 µl of terminal deoxynucleotidyl transferase (TdT) buffer (30 mM Tris-HCl pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride), each follicle/double follicle unit was placed in ~15 µl of a commercially available terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) reaction mixture (Roche) for 2.5 hours at 37 °C. TdT catalyses polymerization of nucleotides to free 3'-OH DNA ends with incorporated fluorescein labels which can be seen using fluorescence microscopy in the green channel. Following a 10 minute wash in PBS the follicles were moved into RNase buffer containing 200 µg ml^{-1} DNase-free RNase and 2.5 µg ml^{-1} of propidium iodide and left at room temperature for one hour. They were then washed in PBS containing 0.01% Triton X-100 for 20 minutes followed by two washes in PBS for 20 minutes. To preserve fluorescence the follicles were equilibrated in 50% Vectashield™ (Vector). They could be stored overnight at 4 °C at this stage or transferred into 100% Vectashield™ (~ 50 µl) on a concave microscope slide, cover-slipped and sealed with nail polish for microscopic analysis.

6.2.3. Confocal microscopy

Follicles were examined using the Leica TCSNT Confocal system (Leica Microsystems). Using a 63x water corrected PL APO lens, a single scan was taken through the centre of each follicle as determined by central positioning of the propidium iodide stained germinal vesicle in the oocyte. Simultaneous scans at 488 nm (the green channel which shows any TUNEL labelled DNA) and 568 nm (the red channel which shows propridium iodide stained nuclear material) were taken to produce an amalgamated true colour RGB image. Each channel could also be viewed separately. Four accumulations were taken for each image saved which averaged the fluorescent

signal and removed electronic noise to produce a sharper image. Images were saved for later analysis.

6.2.4. Analysis of confocal images

A section through each follicle of interest was identified where the germinal vesicle within the oocyte could be clearly seen. A mosaic of images were scanned and stored at a 63x magnification that covered the entire section. Looking at each image in turn the complete number of cells present were counted. Only those that were sharply in focus were included. The number of TUNEL labelled cells that were in focus was also counted. In this way the proportion of apoptotic to healthy cells could be calculated (Figure 6.1).

6.2.5. Statistical analysis

The chi-squared test was used to compare follicles between different treatments.

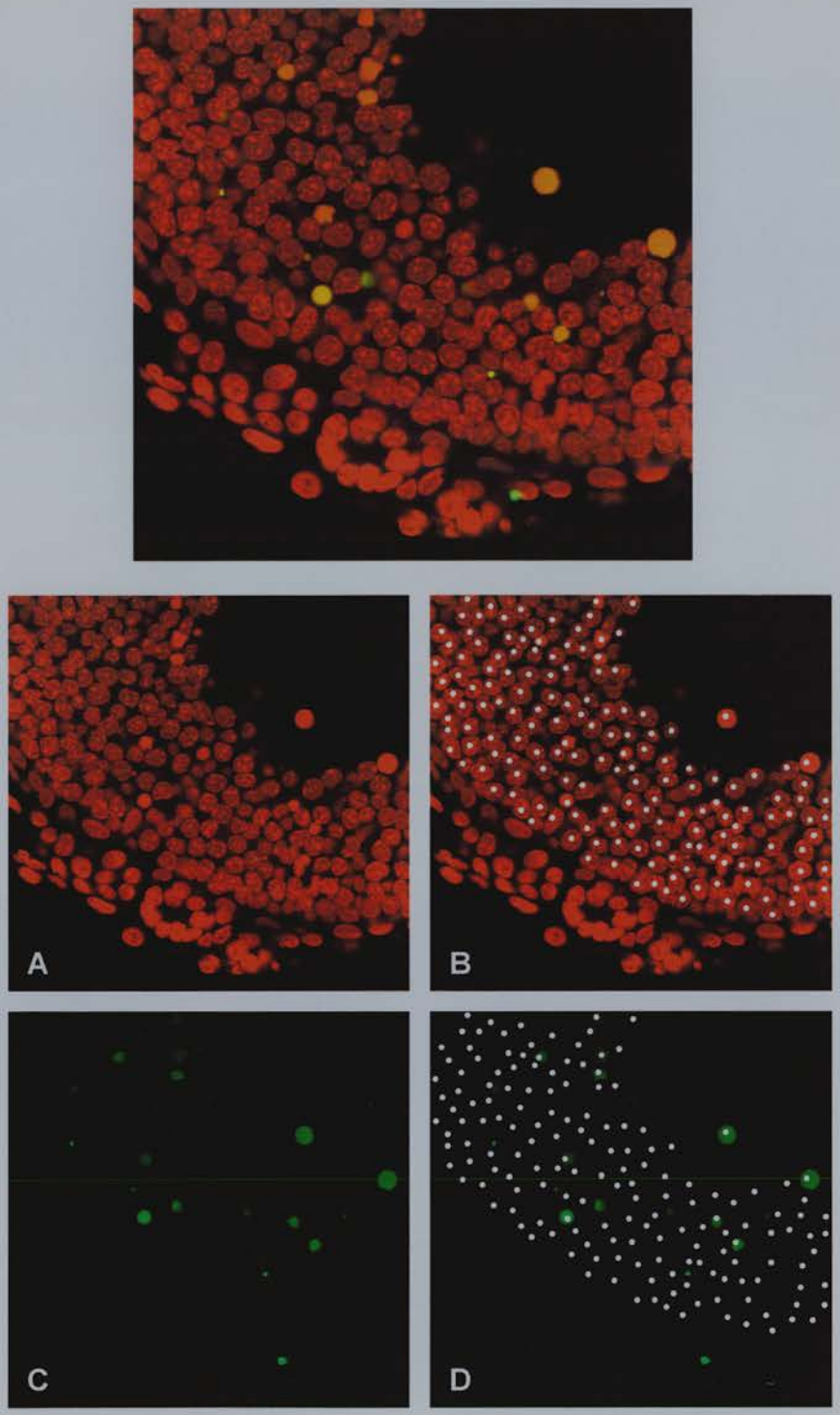


Figure 6.1 Apoptotic cell counting protocol. The confocal microscope scanned image (top photomicrograph) can be split into red and green channels (A and C respectively). Using Image Tool software (UTHSCA) the total number of cells can be counted and tagged in the red channel (B). By switching to the green channel the number of apoptotic TUNEL labelled cells within that population can be easily calculated. Only the green stained cells that have a tag are counted.

6.3 RESULTS

6.3.1. Single follicle cultures using TUNEL as an assay for atresia

Table 6.1 shows comparisons between follicles assayed using TUNEL labelling and those assayed using whole follicle DNA extraction and subsequent 'ladder' analaysis following agarose gel electrophoresis (see Chapter 5 for materials and methods). The data has been normalised in each case so that the follicles in the 1 IU ml^{-1} group are assigned a value of 1. The range and mean percentage of apoptotic cells counted using the TUNEL protocol is shown in Table 6.2. As before, the TUNEL assay shows that there is an increase in the degree of apoptosis as FSH concentration declines. There is a marked increase in the degree of apoptosis recorded when using the TUNEL protocol (e.g. a 21.1 fold increase in apoptosis recorded using the TUNEL method versus 4.1 when using the DNA extraction protocol when follicles are cultured in the absence of FSH for 24 hours). This suggests that TUNEL is a much more sensitive method for determining atresia. A key difference is that the TUNEL labelled cell count only includes granulosa cells. The DNA extraction method involves the digestion of the entire follicle, including the theca layer and the oocyte.

	Short cultures		Long cultures	
	DNA extraction	**TUNEL labelling**	**DNA extraction**	**TUNEL labelling**
1 IU	-	-	-	-
0.5 IU	1.3	-	1.0	3.4
0.25 IU	2.0	0.8	1.2	3.8
0.1 IU	2.5	5.1	1.4	3.9
0 IU	4.1	21.1	1.7	6.6

Table 6.1 Comparisons between TUNEL labelling and DNA extraction protocols for determining atresia. The data are normalised so that the 1 IU ml^{-1} FSH groups are assigned a value of 1. The 0.5 IU ml^{-1} FSH two-day culture follicles were lost during processing.

	Short Cultures		Long Cultures	
	% Apoptotic	Range (%)	% Apoptotic	Range (%)
1 IU	0.3 (5)	0 – 0.6	0.4 (4)	0.2 – 0.6
0.5 IU	-	-	1.2 (2)	0.8 – 1.6
0.25 IU	0.2 (4)	0 – 0.7	1.4 (4)	0.1 – 3.9
0.1 IU	1.6 (5)	0 – 2.6	1.4 (4)	0.2 – 2.3
0 IU	6.5 (5)	0.6 – 21	2.3 (3)	1.2 – 6.6

Table 6.2 The percentage of granulosa cell apoptosis calculated using the TUNEL protocol. The number of follicles included in the analysis is shown in brackets. The range of apoptosis between individual follicles is also shown.

6.3.2. The effect of FSH withdrawal on co-cultures

Figure 6.2 shows examples of co-cultured follicles cultured for five days in 1 IU ml^{-1} FSH (A), cultured for three days in 1 IU ml^{-1} FSH then for a further two days in the media prepared without FSH (B), and single control follicles cultured in 1 IU ml^{-1} FSH for five days (C). Cells showing green fluorescence have a high incidence of DNA laddering (apoptosis). It is of interest that the heaviest region of apoptosis appears to be in the mural granulosa cell region in the dominant follicle cultured for two days in the absence of FSH (B). The single control follicle in 1 IU ml^{-1} FSH also contains apoptotic cells. Complete granulosa cell counts from a cross-section through the middle of each follicle (as determined by central positioning of a clearly visible germinal vesicle) and the number of those cells staining positive for apoptosis were recorded. The percentage of apoptotic cells in each case is shown in Figure 6.3.

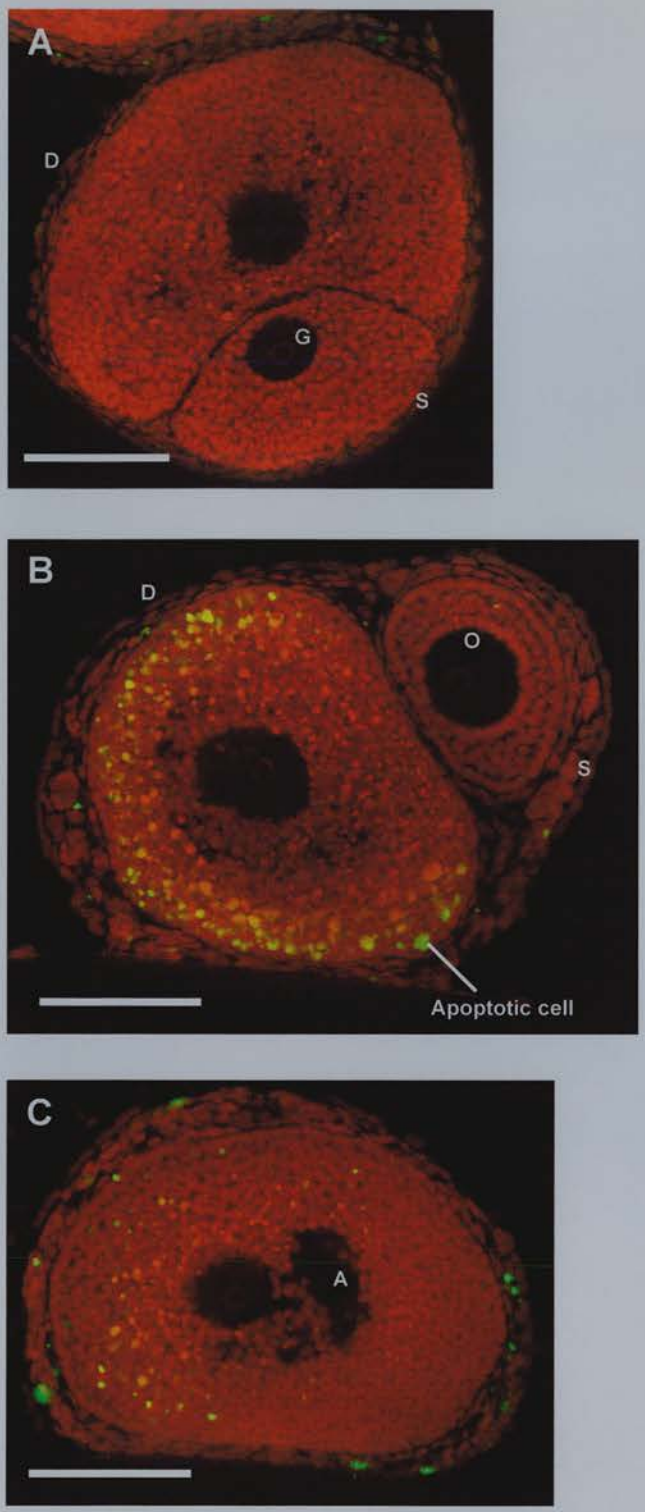


Figure 6.2 Confocal photomicrographs of follicles co-cultured for A: five days with 1 IU ml^{-1} FSH; B: three days with 1 IU ml^{-1} FSH then two days with 0 FSH, and C: single follicles cultured with 1 IU ml^{-1} FSH for five days. G = germinal vesicle; O = oocyte; D = dominant follicle; S = subordinate follicle, and A = antral cavity. Scale bar represent 125 μm.

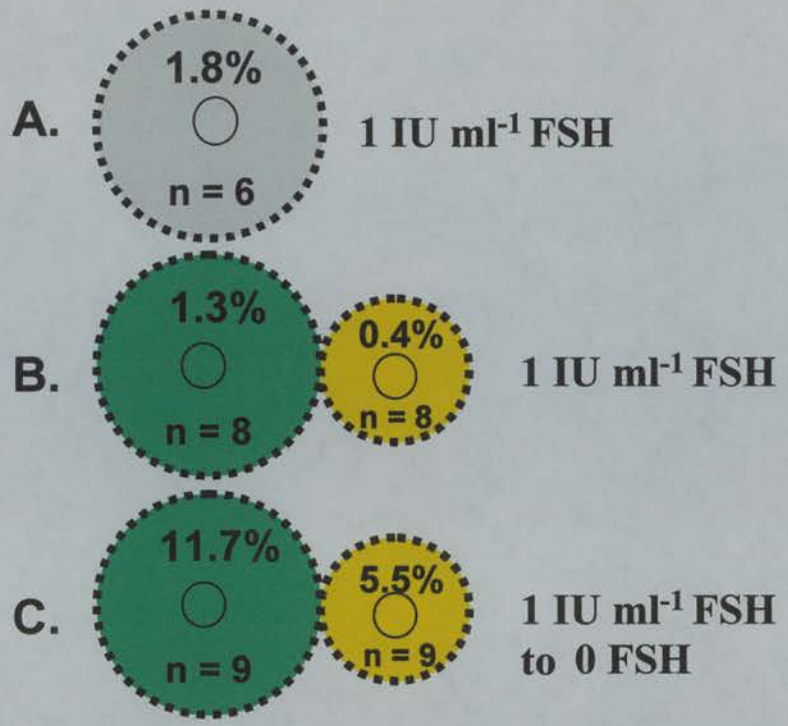


Figure 6.3 Percentage of apoptotic granulosa cells in cultured follicles. A. Single control follicles; B. co-cultured follicles in 'high' FSH throughout, B. co-cultured follicles in 'high' FSH for three days, then no FSH for two days.

The incidence of apoptosis in dominant follicles from both treatment groups was compared using the chi-squared test. This returned a highly significant difference ($p<0.001$) between the groups with a 10.4-fold increase in apoptosis in the dominant follicles from the 2-day zero FSH group in comparison with the 1 IU ml^{-1} FSH throughout group. Similarly, comparison of the subordinate follicles also returned a significant difference ($p<0.001$) with a 13.8-fold increase in apoptosis in the subordinate follicles from the FSH-deprived group compared with the 1 IU ml^{-1} FSH throughout group.

Follicles at different stages of development (such as the dominant and subordinate follicle within each co-cultured pair) have different characteristics and may behave differently in response to the same stimuli. Therefore, it cannot be assumed that it is possible to make comparisons of follicle health following FSH withdrawal between

follicles at different developmental stages (i.e. comparing the dominant and subordinate follicle after FSH withdrawal), unless it can be clearly demonstrated that they have the same 'baseline' in normal conditions (i.e. comparing dominant and subordinate follicles maintained in high FSH). A chi-squared test was therefore carried out first to compare the dominant and subordinate follicles maintained in high FSH levels throughout culture (control group). This comparison returned a significant difference ($p<0.005$) making the further comparison between dominant and subordinate follicle in the experimental (0 FSH) group meaningless.

A comparison of the proportional increase in the incidence of apoptosis when moving from 1 IU ml^{-1} FSH to 0 IU ml^{-1} FSH between dominant and subordinate follicles was made (10.4- and 13.8-fold respectively), also using the chi-squared test. In the context of these experiments, this was the most important test, as this illustrates the effect of co-culture on the subordinate follicle. Although a follicle group may exhibit a high percentage of apoptosis, this may represent the developmental stage of those follicles as much as their response to an environmental stimulus. Consequently it is the **proportional** increase in the incidence of apoptosis in follicles with FSH withdrawal as compared to 'normal' follicles maintained in high FSH levels, at the same developmental stage, that gives an indication of the effect of environmental change on follicle health. This statistical analysis returned a clearly significant result ($p<0.01$).

Comparison of the percentage of apoptosis in co-cultured follicles with single follicles from the initial experiments showed similarities between the subordinate follicles and the single follicles from the two-day cultures. The subordinate follicles cultured for five days in 1 IU ml^{-1} FSH exhibited 0.4% apoptosis, with the single follicles cultured for two days (without the influence of the dominance mechanism) exhibiting 0.3% apoptosis. The chi-squared test showed no significant difference between these groups. Subordinate follicles cultured without FSH for the last two days of culture exhibit 5.5% apoptosis, with single follicles cultured for two-days without FSH showing a 6.5% incidence of apoptosis. Again, the chi-squared test found no significant difference between these groups. No such correlation is seen when comparing dominant follicles from either group with singly cultured follicles. The

dominant follicles cultured with 1 IU ml^{-1} FSH throughout exhibited 1.3% apoptosis compared to 0.4% in single follicles also cultured for five days in the same FSH concentration, a significant difference, $p<0.001$. Dominant follicles subjected to FSH withdrawal displayed 11.7% apoptosis compared with 2.3% in the singly cultured equivalent follicles ($p<0.001$). Of note is the fact that the control, single follicles cultured in 1 IU ml^{-1} FSH at the same time as the co-cultured follicles, exhibited 1.8% apoptosis, an increase of 1.4% above follicles cultured in the same conditions during the initial TUNEL versus DNA extraction experiments. Comparing these control follicles with the dominant follicles cultured with 1 IU ml^{-1} FSH throughout, returns a narrowly significant difference, $p=0.05$.

6.4 DISCUSSION

The development of the whole follicle TUNEL labelling protocol with confocal microscope visualisation has provided a powerful tool for rapid follicle analysis. Currently, there are no reports in the literature documenting the use of this protocol for ovarian follicles, although a similar technique has been reported for the analysis of blastocysts (Brison and Schultz, 1997). From a technical standpoint, the use of this method for analysing follicles cultured *in vitro*, perhaps in combination with an existing protocol for *in situ* TUNEL labelling of *in vivo* sections (e.g. Billig *et al.*, 1994), offers a relatively simple indicator of follicle health.

The comparison between the TUNEL labelling method and the DNA extraction protocol clearly indicated that the former is a more sensitive assay for the detection of atresia. The higher incidence of apoptosis recorded when using this method could be attributed to the fact that only granulosa cells are included in the analysis. The DNA extraction assay reflects apoptosis in the entire follicle. Given that apoptosis in follicles at the developmental stages found in these cultures is found primarily in the granulosa cells, it is possible that the amount of apoptosis being detected is being 'diluted' by the healthy DNA from theca cells and the oocyte. Technically, DNA is more likely to be lost during processing of the DNA extractions and subsequent DIG labelling. There are several DNA precipitation and re-suspension steps throughout the protocol and varying quantities of the DNA will be lost at each of these. Additionally, the 'success' rate using the DNA extraction method was variable with 'blank' runs being a common occurrence.

The TUNEL assay reveals that within the same treatment group, follicles display a considerable range of apoptosis (e.g. when cultured for two days in 0.1 IU ml^{-1} FSH the percentage of granulosa cell apoptosis ranges from 0 – 2.6%). This is an observation that is lost when using the DNA extraction protocol, the follicle all being assayed together. This range of response to environment may reflect naturally occurring biochemical heterogeneity amongst the follicle population (Grant *et al.*, 1989). It is also possible that the follicles have experienced different degrees of mechanical trauma during the culture set-up which is being reflected in the variance of apoptotic profile.

The co-culture results build on previous work (detailed in Chapter 5) that investigated the response of follicles at different stages of development to FSH withdrawal. Large, singly cultured follicles were more able to withstand a decrease in FSH (as assessed by follicle apoptosis) presumably by being able to utilise available LH. Smaller follicles are unable to respond to environmental LH as they are unlikely to have functional LH receptors on their granulosa cells. I hypothesised that these findings could be extended to co-cultured follicles. The dominant follicle would behave like the singly cultured large follicles when subjected to FSH withdrawal. The dominance mechanism under investigation would retard the development of the subordinate follicle (despite the previous three days culture in 1 IU ml^{-1} FSH). The subordinate follicle would be expected to respond to the FSH removal in a manner analogous to the small, singly cultured follicles, with an increased incidence of apoptosis.

Statistical analysis of the results clearly shows that both dominant and subordinate follicles exhibit significantly more apoptosis when FSH is withdrawn. There is an increase from 0.4% to 5.5% in apoptotic granulosa cells in the subordinate follicles and from 1.3% to 11.7% in the dominant follicles. The singly cultured control follicles had 1.8% apoptotic granulosa cells. Although the dominant follicles in the absence of FSH have the highest proportion of apoptotic cells (11.7%), when viewed as a proportional **increase** in apoptosis, there is less of a rise than that shown by the subordinate follicle ($p<0.01$). Despite being a significant difference, it is perhaps less pronounced than might be expected if the dominant follicles were developmentally competent to rely on LH for trophic support. These results would suggest that the dominant follicles are not sufficiently mature enough to be completely FSH-independent at the time of FSH withdrawal. Alterations in the experimental design to extend the length of time follicles are cultured in 'high' FSH before withdrawal (perhaps by 24-48 hours) may be sufficient to allow the dominant follicle to become FSH-independent, revealing a more marked difference between subordinate and dominant follicle health.

The hypothesis that co-cultured subordinate follicles (pre-/early antral) might behave like single follicles cultured for a short time (also pre-/early antral), and conversely that co-cultured dominant follicles might be similar to single follicles

cultured for a longer time (both antral) is only partly substantiated. While there is a good correlation between the subordinate follicles and the small single follicles, the dominant follicles and the larger single follicles behave significantly differently. This may be a reflection on the nature of co-cultured follicles. It has been shown that dominant follicles from freely-contacting co-cultured pairs fail to achieve comparable sizes to those of single control follicles (Spears *et al.*, 1996). Presumably this means that the dominant follicles are less developmentally advanced than the single follicles despite starting the culture period at equivalent sizes and being cultured for comparable lengths of time. At the time of FSH withdrawal, it is possible that these dominant follicles are being 'caught' at an especially vulnerable time – the majority of the granulosa cells are capable of undergoing apoptosis and are highly FSH-dependent, but the population of LH-receptor-bearing granulosa cells is insufficient to sustain the follicle on LH alone. One way to test this theory would be to repeat the experiments but co-culture follicles across large-pored polycarbonate membranes (see Chapter 4 for materials and methods). This would have the advantage of maintaining follicle morphology and not compromising development but maintaining the influence of the dominance mechanism, but would be technically difficult due to the movement of follicles in these cultures.

The subordinate follicles cultured in 1 IU ml^{-1} FSH throughout exhibited less apoptosis than the single control follicles cultured at the same time in identical conditions (0.4% and 1.8% respectively). This may demonstrate a protective effect of the dominant follicle on its smaller partner, although I believe that this observation is more likely to be illustrative of differences in developmental stage and response to environment. While it has been reported that pre-/early antral follicle cells are capable of undergoing apoptosis *in vitro* (Robertson and Telfer, 1994), this may happen less readily than in more mature follicles. Apoptosis is a normal process of cell elimination in most healthy tissues (Hsueh *et al*, 1994). Large ovarian follicles have more cells than less developed follicles, many of which may play different roles in the follicular syncitium. Consequently there is more of a requirement for strict follicular organisation, including the elimination of unhealthy; inappropriately located, or unwanted cells. This may account for a higher baseline of apoptosis in more developmentally advanced follicles.

Of interest is the difference in the incidence of apoptosis in the two groups of single follicles cultured for five days in 1 IU ml^{-1} FSH (from the TUNEL versus DNA extraction experiments (0.4%) and the controls for the co-culture experiments, 1.8%). This would suggest that there may be poor reproducibility between repeat experiments using the TUNEL protocol (as compared to the DNA extraction method which requires a minimum of three different cultures to obtain a single result, thus averaging culture-to-culture variance). These differences may be attributable to the small sample size of the former group or variability in the effectiveness of the TUNEL labelling kits. If further experiments continue to show culture-to-culture variability caution should be used when comparing experiments conducted at different times, however, there is no problem when comparing treatments within the same culture.

The findings of these experiments offer support to the hypothesis that contact-mediated communication between follicles may play a role in follicle selection by holding back development of smaller follicles, rendering them more vulnerable to systemic changes in FSH concentration.

In the future these experiments should be consolidated with further studies looking at different concentrations of FSH and perhaps with a longer culture period before FSH levels are dropped. A complete absence of FSH in the culture medium after the initial culture period was used in these experiments as it was thought that the effects of this extreme would be easiest to detect. Confocal microscope analysis of TUNEL labelled whole follicles offers a straight-forward and sensitive method for assessing follicle health. Consequently even subtle changes in follicle health can be easily recorded. Systemically in the mouse we are unsure what circulating levels of FSH are at various times in the oestrous cycle. It may be that follicles are never subjected to such low levels. Reducing the FSH concentration to 0.1 IU or 0.25 IU ml^{-1} may still be sufficient to observe a significant affect on follicle health and would make the contact-mediated selection hypothesis more robust.

Chapter 7

Candidate genes involved in follicle-follicle interactions, *in vivo*

1.1. INTRODUCTION

The terms inductive signalling and lateral specification were introduced in Chapter 1 and some discussion given to the appropriateness of applying them to aspects of follicle dominance. A diagrammatic summary of these communication mechanisms is given in Figure 7.1.

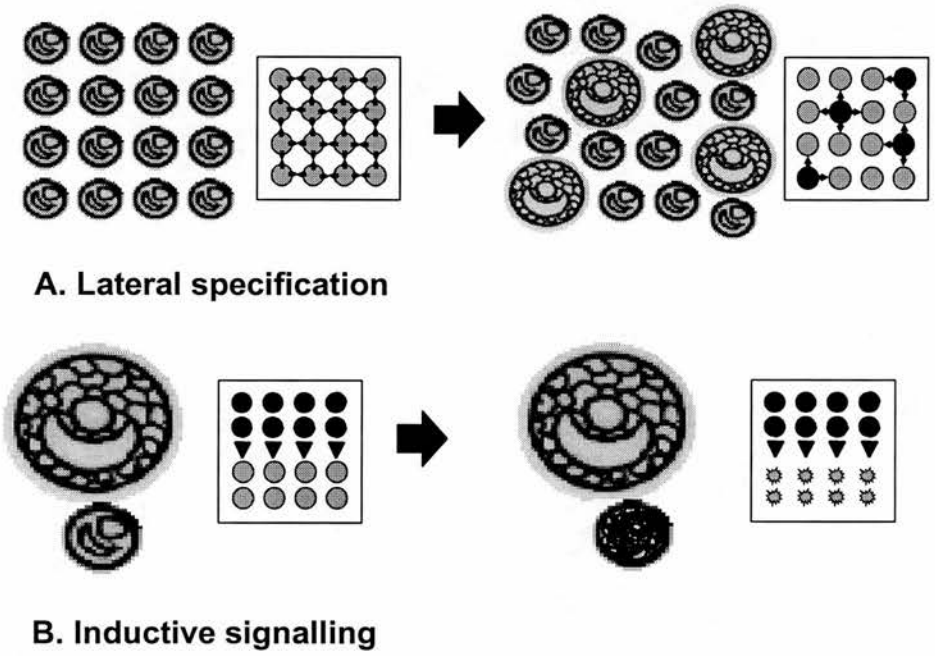


Figure 7.1 Lateral specification and inductive signalling: their speculative role in follicle selection In a process analagous to lateral specification (the process by which a group of cells forms two different populations in a patterned process), juxtacrine communication between an initially equivalent population of follicles results in some follicles emerging as dominant (A). Later, these dominant follicles maintain their eminence by holding back the growth of subordinates until the systemic FSH decline pushes them down the atretic pathway and/or or by directly inducing atresia. This later stage is analagous to inductive signalling between developing cells. (B).

A considerable body of evidence in the literature reports various factors that are secreted by an already dominant follicle to inhibit the development of neighbouring subordinates. These could be classified as examples of inductive signalling, as follicles at different developmental stages could be viewed as non-equivalent cell populations: one group of differentiated cells (the dominant follicle) communicates with another group of less differentiated cells (the subordinate follicle) to influence further development. In these cases the identified factor, e.g.

GCIF (Hynes *et al.*, 1996a, b), is itself the signalling molecule. Towards the end of Chapter 1, I hypothesised that a direct, contact-dependent mechanism acting between like-sized follicles is a vital process involved in follicle selection, and further that this may be a process analogous to lateral specification (since in both cases there is short range cell-cell communication between equivalent cells/follicles). Subsequent chapters have investigated the feasibility of a contact-mediated mechanism *in vivo* (Chapter 3), and shown how this might act in concert with systemic factors to cause atresia of subordinate follicles (Chapters 5 And 6). What is still lacking is an understanding of the signalling molecules involved in this process.

Signalling molecules in the Notch family of transmembrane receptor proteins are attractive candidates for this role. This family was one of the first group of regulatory genes to be isolated in *Drosophila*, and were initially implicated in the regulation of lateral specification. Signaling via the Notch receptor can control the ability of non-differentiated cells to respond to differentiation and proliferation cues, and is able to block the action of other differentiation signals. The Notch receptor contains a large extracellular domain that contains 36 EGF-like repeats and three cysteine-rich Notch/Lin-12 repeats (reviewed by Artavanis-Tsakonas *et al.*, 1995). These receptors have an array of possible ligands, including membrane-anchored extracellular ligands (each with EGF-like repeats) such as Delta and Serrate (Fehon *et al.*, 1990; Jönsson and Knust, 1996). *Notch* was first discovered due to its role in neurogenic cell fate in the developing *Drosophila*, an example of which was detailed in Chapter 1 with the formation of evenly spaced sensory bristles. It has since been shown to play a key role in both inductive signalling and lateral specification in the developing *Drosophila*: null mutations result in embryonic lethality. *Notch*, *Delta* and *Serrate* homologues have been found in *C. elegans* and in non-mammalian and mammalian vertebrates, including mice and humans (reviewed by Artavanis-Tsakonas *et al.*, 1995). In the mouse these are: *Notch 1* and *Notch 2* (Lardelli and Lendahl, 1993); *Notch 3* (Lardelli *et al.*, 1994); *Notch 4* (Uyttendaele *et al.*, 1996); *Delta-like 1* (Bettenhausen *et al.*, 1995); *Delta-like 3* (Dunwoodie *et al.*, 1997). In the rat, the *Serrate* homologues *Jagged 1*(Lindsell *et al*, 1995) and *Jagged 2* (Shawber *et al.*, 1996) have been identified.

Lateral signalling is usually defined as direct cell-cell communication within an initially equivalent population of cells which leads to those cells adopting

different developmental fates. In contrast, the ovarian follicle is a functional syncitium comprised of hundreds of cells from at least three distinct populations. If there are follicle-follicle communication mechanisms that are analogous to the process of lateral signalling there must be a requirement for the boundary of the follicle to be clearly delineated. In this way the follicle has a distinct 'edge' that makes contact with the 'edge' of neighbouring follicles. Signalling molecules expressed along these 'edges' would then have a clearly defined target. This boundary could be presented by the basal lamina or theca cells (see Chapter 4. for discussion) which would act in concert and express an appropriate gene(s).

Given that the Notch family of genes are known to be involved in juxtacrine communication between cell types in many developing systems, they seem plausible candidates as factors regulating follicle selection. Indeed, Notch has been shown to play vital roles in the *Drosophila* ovary (Xu *et al.* 1992), including evenly spacing the developing egg chambers. Notch 4 has been previously reported to be present in the mouse ovary (Uyttendaele, *et al.,* 1996), however, there are no previously published reports of expression of *Notch 1-3* in the mammalian ovary, or any of the other associated genes. Drawing parallels with the development of the *Drosophila* sensory bristle (see Chapter 1, Section 1.8.1) one possibility might be that Notch is activated in one follicle by the expression of *Delta* in the neighbouring follicle leading to enhanced development in the 'Notch-activated' follicle and retarded development in the 'Delta-activated' one.

More recent work has highlighted a further complexity in the 'Notch story'. This is due to the fact that, in some situations Notch can be widely expressed but only activated if co-expressed with other proteins. Looking again at *Drosophila* development, specifically boundary determination of the wing imaginal disc, it has been shown that *Delta* and *Notch* are broadly expressed during early wing development (Irvine and Wieschaus, 1994). *Fringe* and *Serrate* are restricted to dorsal cells. Fringe allows dorsal cells to respond to Delta which results in the activation of Notch (Panin *et al.*, 1997). Once activated, Notch initiates the transcription of downstream genes, one of which is *Serrate* (Figure 7.2). Serrate protein acts as a signal from the imaginal disc dorsal cells that causes activation of Notch in the ventral cells. Fringe blocks the ability of Serrate to signal to other dorsal cells (Panin *et al.*, 1997). *Serrate* and *Delta* expression becomes restricted to the

dorsal-ventral boundary with the proteins maintaining each others expression through a positive feedback loop (Figure 7.2). It is important that this expression is limited to the boundary or Notch would be activated throughout the wing resulting in abnormal development. This modulation of function may be achieved by altering glycosylation of cell-surface and/or secreted molecules (Yuan *et al.*, 1997).

The ability to mark a clear boundary within a large cell population makes mammalian homologues of the *fringe* gene particularly attractive candidates for having an involvement in follicle boundary determination and subsequent follicle-follicle 'lateral specification'.

Murine homologues of *D-fng* have been cloned in the mouse and the human (Cohen *et al.*, 1997; Johnston *et al.*, 1997). The murine homologues are: *Lunatic fringe* (*L-fng*); *Manic fringe* (*M-fng*), and *Radical fringe* (*R-fng*). The N-termini of the expressed proteins differ in length and sequence from the *Drosophila* homologue, but the C-termini 270 amino acids are highly conserved (Irvine and Wieschaus, 1994). The *fringe* genes have been shown to be widely expressed in both developing and adult tissues (Cohen *et al.*, 1997; Johnston *et al.*, 1997) at developmentally important boundaries. The expression of *L-fng* seems primarily restricted to undifferentiated cells, in contrast to both *M-fng* and *R-fng* which are usually expressed in cells that are undergoing or have completed terminal differentiation. This often results in the generation of an *L-fng* versus *M-fng*/*R-fng* boundary. An example of this is in the tongue epithelium (Cohen *et al.*, 1997). The basally located stem cells express *L-fng* initially but then switch to *M-fng* and *R-fng* expression as they move and differentiate to supra-basal cells. The resultant *fringe* expression boundary coincides with a *Notch*-ligand expression boundary, with the *Serrate* homologue *Jag2* mirroring *L-fng* expression in the basal stem cells and *Jag1* mirroring *M-fng*/*R-fng* in the supra-basal cells. *Dll1* and *Dll3* are likely to function together (Dunwoodie *et al.*, 1997) and are found coincident with L-fng at boundaries in the developing neural tube (Cohen *et al.*, 1997).

I have set out to use molecular biology techniques to investigate if the *Notch* family and related genes are being expressed in the ovary, and if so at which developmental stages. When a gene has been found to be expressed I have tried to establish if its pattern is one that might be meaningful in the context of follicle selection. Given the diverse range of influence that the expression and activation of

the *Notch* gene family has in the mammal, it is unproductive to speculate in depth about possible expression patterns. In broad terms, however, it would seem plausible that the expression of genes know to be delimited to boundaries, such as the *fringe*; *Jagged*, and *Delta* families, may be found at the periphery of follicles. The *fringe* genes could also be considered markers of development, with *M-fng* and *R-fng* being found in terminally differentiated cells and *L-fng* in less developed cells. Within the context of the follicle, this could mean that mural granulosa cells might express *L-fng* with the terminally differentiated granulosa cells lining the antral cavity expressing *M-fng*/*R-fng*: granulosa cells between these populations might then be expected to show a gradient of expression of these three genes.

If the *Notch* family and related genes do have a role to play in the selection of follicles it would be reasonable to assume that they would be found expressed in areas of the ovary where cohorts of follicles are found close together. This could be within clusters of pre-/early antral follicles like those illustrated throughout Chapter 3, or in clusters of follicles at early stages of development such as tightly packed primordial/ primary follicles.

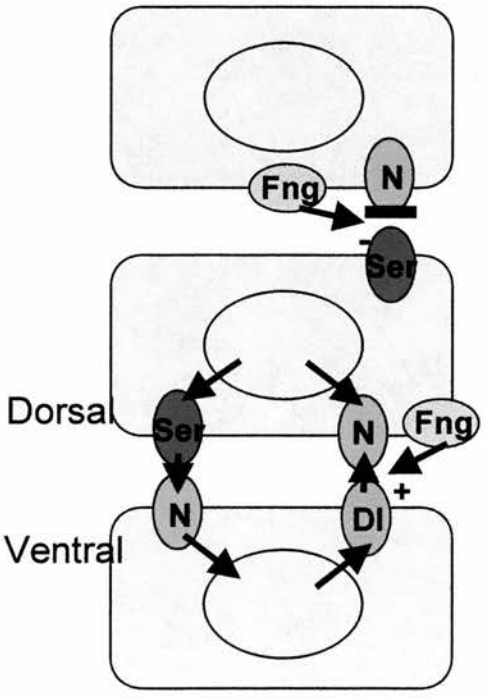


Figure 7.2 The *fringe* gene regulates Notch expression at developmentally important boundaries. Schematic representation of signalling interactions at the dorsal-ventral compartment border of the *Drosophila* wing imaginal disc. *Delta* (Dl) and *Notch* (N) are broadly expressed during early wing development, while *fringe* (Fng) and *Serrate* (Ser) are restricted to dorsal cells. Expression of *fringe* allows dorsal cells to respond to Dl, resulting in Notch activation. This leads to transcription of downstream genes, including Ser. Ser signals back from dorsal to ventral cells, activating Notch, and leading to the transcription of downstream genes including Dl. Fng blocks the ability of Ser to signal to other dorsal cells. After Panin *et al.* 1997.

7.2. MATERIALS AND METHODS

7.2.1. Reverse transcription polymerase chain reaction (RT-PCR)

RNA gel electrophoresis, Northern blotting and hybridisation with P^{32} labeled probes failed to detect Notch 1, Notch 2 or Notch 3 in ovarian tissues. Consequently a more sensitive approach was taken in an attempt to detect these, and other associated mRNAs, in mouse ovaries at different stages of development.

7.2.1.1. Extraction of mRNA from tissue

Messenger RNA (mRNA) was extracted from 12-13 day old ovaries, 3-4 week old ovaries, mature ovaries (greater than 8 weeks) and young brain (E19-P2). All tissue was taken from F_1 mice. Following aseptic dissection of the appropriate tissue (as detailed in Chapter 2, 2.1.1) the material was rapidly homogenized using a glass pestle and mortar which had been baked overnight to eliminate the risk of possible RNase contamination. mRNA was then extracted using a commercially available kit (mRNA Tissue Extraction kit, Pharmacia Biotech). Briefly, this involved the binding of the mRNA poly-A tail to microbeads, multiple washing steps to remove protein, DNA and other contaminants, and finally elution of the purified mRNA into 200 µl of buffer. The quantity of mRNA extracted was calculated following spectrophotometry. Typically, 20-80 ng of mRNA was recovered. To ensure that there was no DNA contamination, a PCR run was set up using primers that are known to amplify sections of genomic DNA. If no amplification occurred (with a convincing positive control) it was assumed that the mRNA sample was pure.

7.2.1.2. cDNA synthesis

Complementary DNA (cDNA) was constructed from the mRNA template using a commercially available kit (First-Strand cDNA synthesis, Pharmacia Biotech). A *Not* I oligo $d(T)_{18}$ primer was used in the reverse-transcriptase reaction. 20-150 ng of mRNA was denatured at 65 ^{0}C for 10 minutes then quenched on ice before addition to the kit reagents. The reaction mix was incubated at 37 ^{o}C for one hour, then stored at –20 ^{o}C. cDNA was constructed in a final volume of 33 µl.

7.2.1.3. RT-PCR

cDNA was heated to 95 °C for 5 minutes then chilled immediately on ice. This step denatures any enzyme still active from *in vitro* transcription and separates the cDNA:RNA duplex. 2-5 µl (~10-25 ng) of cDNA is then added to the following mixture:

10X Reaction buffer (Gibco)	5 µl
dNTPs (20 mM)	0.5 µl
Primer A (300 pmol μl^{-1})	3.4 µl
Primer B (300 pmol μl^{-1})	3.4 µl
Taq polymerase (Platinum™, Gibco)	0.25 µl
$MgCl_2$	To a final concentration of 1-1.5 mM
ddH_2O	up to 50 µl

The primers used were designed to span an intron to prevent the spurious amplification of any genomic DNA contaminant present in the cDNA sample, and manufactured commercially (MWG-Biotech). These were:

Notch 1 A[1]	5'-GTG AGG GTG ATG TCA ATG-3'
Notch 1 B[1]	5'-TGA AGT TGA GGG AGC AGT-3'
Notch 2 A[1]	5'-TAC AAC TGT ATC TGC CG-3'
Notch 2 B[1]	5'-GTC TTT GAA GTG GTC TGC-3'
Notch 3 A[2]	5'-ACA CTG GGA GTT CTC TGT-3'
Notch 3 B[2]	5'-GTC TGC TGG CAT GGG ATA-3'
Delta like 1 A[3]	5'-TGG ACT ATA ACC TCG TTC G-3'
Delta like 1 B[3]	5'-GAA AGA CTG GCT CAT A-3'
Delta like 3 A	5'-CAC TCA ACA ACC TGA G-3'
Delta like 3 B	5'-AGA AGC AGG TGG ATC T-3'
Jagged 1 A	5'-TCC AGC TGA CAG AGG TTT CC-3'
Jagged 1 B	5'-GAC CAG AAT GGC AAC AAA ACC TGC-3'
Jagged 2 A	5'-CCT GCC CAG ATG GCT A-3'
Jagged 2 B	5'-TAA CGC AGT GCC CGT G-3'

Lunatic fringe A	5'-GCG CCT GCT GCT GGC G-3'
Lunatic fringe B	5'-CAC ATT GCC TGT GAG C-3'
Manic fringe A	5'-AGC CGT CAC ACC CAC A-3'
Manic fringe B	5'-CAG CCC AAG ACC CCA T-3'
Radical fringe A	5'-ACA TCT ACC TGG GGC G-3'
Radical fringe B	5'-AGT TAC TGG AAA GCT C-3'
β–actin A[1]	5'-TAC CTC ATG AAG ATC CTG ACC GAG-3'
β-actin B[1]	5'-CTC CTG CTT GCT GAT CCA CAT CTG-3'

[1] Taken from Lardelli and Lendahl, 1993

[2] Taken from Lardelli *et al.*, 1994

[3] Taken from Bettenhausen *et al.*, 1995

The 50 µl reaction mix was contained in thin-walled PCR tubes (Alpha), overlaid with three drops of mineral oil to prevent evaporation and placed into the standard heat block of a thermocycler (Techne or Hybaid). The PCR cycle consisted of 35 cycles of denaturation at 95 °C for 1 minute, annealing at 55 °C for 1 minute, and extension at 72 °C for 1 minute.

For each gene of interest the following RT-PCR reactions were performed.

Tube 1: cDNA from 13 day-old ovaries, specific primers
Tube 2: cDNA from 13 day-old ovaries, β-actin primers
Tube 3: cDNA from 3 week-old ovaries, specific primers
Tube 4: cDNA from 3 week-old ovaries, β-actin primers
Tube 5: cDNA from mature ovaries, specific primers
Tube 6: cDNA from mature ovaries, β-actin primers
Tube 7: ddH_2O, specific primers (internal control)
Tube 8: genomic DNA, specific primers (primers control)
Tube 9: cDNA from young brain, specific primers (positive control)
Tube 10: cDNA from young brain, β-actin primers (positive control)

β-actin primers were included for each tissue sample as this is an almost ubiquitously expressed gene. Following agarose gel electrophoresis, crisp bands of the correct size for β-actin show that the cDNA is intact. Tube 7 is a 'blank' control. If any amplification occurs then there has been a possible contamination of the other tubes and the run is abandoned. Tube 8 acts as a control for the primers. If they have been designed correctly they should cross an intron and so should be unable to amplify DNA of the correct size from the genomic DNA.

7.2.1.4. Agarose gel electrophoresis

RT-PCR products were mixed with loading buffer (Orange G in glycerol, Sigma) in a ratio of 4:1 and loaded onto a 1-1.5% agarose gel made with Tris-borate/EDTA electrophoresis buffer (TBE), and containing 0.5 mg ml^{-1} ethidium bromide. 100 bp DNA markers (Gibco) were also loaded. The gels were run at 55 volts until the PCR products became satisfactorily separated. DNA bands could then be examined under ultraviolet illumination and compared to the DNA weight markers. Polaroid photographs were taken of the illuminated gels as a permanent record.

7.2.2. *In situ* hybridisation

The recipes for all solutions used during these procedures are contained in Appendix C.

7.2.2.1. Histology

6 μm sections of ovarian tissue were prepared according to protocols previously outlined (Chapter 2.3.5).

7.2.2.2. Making riboprobes

7.2.2.2.1. Transformation of cells with plasmids containing cDNA sequence of interest

A 1 μl volume of plasmid and insert (corresponding to ~ 10 ng DNA) was added to 100 μl of $CaCl_2$ competent DH5α cells on ice and left for 30 minutes. The mixture was then heat shocked at 42 °C for exactly two minutes before being returned to ice.

Two volumes of Luria-Bertani broth (LB) were added before vigorous shaking for 30 minutes at 37 °C. The broth was then plated out on agarose plates containing LB and L-Ampicillin and left overnight.

7.2.2.2.2. Amplifying the cDNA containing plasmid

A single colony was taken from the L-Ampicillin plates using a sterile wooden tooth pick and dropped into 5 ml of LB in a sterile tube, also containing L-Ampicillin at a concentration of 50 μg ml^{-1}, and shaken vigorously at 37 °C for 6 hours. After this time 1 ml of bacteria containing broth was removed and spun for one minute at 5000 r.c.f.. A pellet of bacterial cells was formed at the bottom of the eppendorf. The supernatant was removed and the pellet washed twice in sterile LB broth before being resuspended in 100 μl of broth and transferred to 50 ml of sterile LB broth containing 50 μg ml^{-1} of L-Ampicillin in a conical flask. The broth was shaken overnight at 37 °C. The following day the amplified plasmid was purified from the bacterial cells using a commercially available kit (Midi Prep Purification Kit, QIAgen).

7.2.2.2.3. Making a cDNA template and synthesising DIG labelled riboprobes

The purified plasmid was linearised by digestion with appropriate restriction enzymes. These were:

	Anti-sense	**Sense**
Notch 1	Hind III (T7)	Xba I (T3)
Notch 2	Bam HI (T3)	Eco RI (T7)
Notch 3	Bam HI (T3)	Hind III (T7)
Delta like 1	Sal I (T7)	Eco RI (SP6)
Delta like 3	Not I (T7)	Kpn (T3)
Lunatic fringe (Egan laboratories)	Eco RI (T7)	Bgl II (SP6)
Manic fringe (Egan laboratories)	Xba I (T7)	Hind III (T3)
Radical fringe (Egan laboratories)	Bam HI (T7)	Not I (T3)

Lunatic fringe (Vogt laboratories)	Hind III (T7)	Xba (T3)
Manic fringe (Vogt laboratories)	Not I (T3)	Eco RI (T7)
Radical fringe (Vogt laboratories)	Not I (T3)	Hind III (T7)

25 µl (~25 µg) was digested with 100 units of enzyme in a total volume of 250 µl (made up with 10 x appropriate enzyme buffer and ddH_2O). A double digest was also performed to release the inserted cDNA fragment. After digestion for 2.5 hours at 37 °C the linearised DNA was extracted by the addition of an equal volume (250 µl) of a phenol: chloroform: isobutyl acid (PCIA) mixture, removal of the DNA containing aqueous layer (repeated twice to maximize the yield) and precipitation by the addition of one tenth volume of 3M Na Acetate pH 5.5, two volumes of absolute ethanol and cooling to –70 °C for at least 30 minutes. The mixture was then spun at 14 000 r.c.f. for 30 minutes to form a DNA pellet which was washed with chilled 70% ethanol, spun at 14 000 r.c.f. for a further 10 minutes and allowed to air dry before resuspension in sterile ddH_2O. A small volume of the linearised DNA and the 'released' inserted fragment were run by agarose gel electrophoresis, stained with ethidium bromide and visualised using UV light as a check that enzymatic digestion had been complete and that the fragment was of the right size i.e. that the correct plasmid had been amplified and purified. A commercially available *in vitro* transcription kit (DIG *in vitro* transcription kit, Roche) was used to manufacture riboprobes that contained a DIG label. The polymerase enzymes used are denoted in brackets next to the appropriate restriction enzymes in the list above. The riboprobes were cleaned by ethanol precipitation as before with the exception that 4M LiCl was used in place of 3M Na Acetate. A dot blot was performed to ascertain the concentration of the riboprobes.

7.2.2.3. Pre-hybridisation treatment

Wax was removed from the histological sections by immersion in xylene for 10 minutes, twice, then they were hydrated through an ethanol series (100%; 100%;

95%; 90%; 70%; 50% and 30%). Following a five minute wash in 2 x SSPE the sections were permeabilised in 20 μg ml^{-1} Proteinase K (Roche) in 1 x P buffer for 7.5 minutes at 37 °C, washed for 30 minutes in 2 X SSPE then fixed in 4% paraformaldehyde made up in PBS for 15 minutes. A further five minute 2 x SSPE wash preceded 15 minutes acidification in 0.2M HCl, followed by another five minute 2 x SSPE wash. The mRNA was acetylated in 0.5% acetic anhydride in 0.1M TEA, with continuous stirring, then washed for at least five minutes in 2 x SSPE.

7.2.2.4. Hybridisation

For each slide, 2 μl of riboprobe (see section 7.2.3.2 for preparation) was added to 60 μl of hybridisation mix. This mixture was then heated to 80 °C for five minutes then quenched immediately on ice before addition to the slides which were then coverslipped. The slides were incubated overnight at 50 °C in a humid atmosphere (50% formamide in 1 x salts).

7.2.2.5. Post-hybridisation

The slides were soaked in 2 x SSC at 50 °C for at least 10 minutes to let the coverslips detach, before a 45 minute incubation at 65 °C in 2 x SSC made with 50% formamide. Sections were then washed in 4 x SSPE at 50 °C for five minutes followed by RNase A digestion (20 μg ml^{-1} in 4 x SSPE) for 30 minutes at 37 °C. This step removes any unbound mRNA and riboprobe but is unable to affect bound mRNA/riboprobe duplexes. The slides were incubated in 2 x SSC in 50% formamide at 65 °C for 45 minutes before being allowed to cool to room temperature in 2 x SSC initially at 50 °C. Slides were washed in PBST for 10 minutes then equilibrated in 1% blocking buffer in PBST for 30 minutes before being incubated in antibody solution which had been pre-absorbed overnight (1 x blocking reagent; 2% heat inactivated sheep serum; 1 x PBS; 0.1% Tween 20 and 0.02% anti-digoxygenin antibody, in ddH_2O). Slides were left overnight in a dark place at 4 °C. The following day the slides were thoroughly washed (three 20 minute washes in PBST), equilibrated in alkaline phosphatase buffer for five minutes then transferred to colour detection buffer (0.4% NBT/BCIP in alkaline phosphatase buffer with a few crystals of levamisole to block endogenous alkaline phosphatase activity) and left overnight

at 25 °C. After colour development the slides were counter-stained in Nuclear Fast Red (Vector) for three seconds, rinsed in ddH_2O, dehydrated through an ethanol series (70%; 90%; 95%; 99%; 100%, twice), equilibrated in xylene (twice for 30 minutes) and coverslipped with DPX.

7.3 RESULTS

7.3.1. The presence of developmental neuro-genes: RT-PCR

Table 7.1 summarises the results of the RT-PCR reactions as shown by agarose gel electrophoresis and staining with ethidium bromide. Figures 7.3-7.11 show photographs made of these gels. Southern blots followed by probing with DIG-labelled oligonucleotides were also performed as a verification that the correct sequence had been amplified. These results are not shown as they were only intended as an in-house check before proceeding to the *in situ* hybridisation experiments. They in each case confirmed the results of the RT-PCRs. It was not possible to obtain a definitive answer as to whether Delta-like 3 is present in the mouse ovary at any of the developmental stages examined, due to technical difficulties. The primers used produced multiple bands of similar sizes to the product of interest. Attempts to clarify the RT-PCR results by excision of the band at the appropriate size, DNA extraction from the agarose and subsequent nested PCR also failed to produce a clear band. Southern blotting and probing with DIG labelled DNA sequences were unable to produce a convincing result.

7.3.2. The expression of developmental neuro-genes: *in situ* hybridisation

Optimisation of the *in situ* hybridisation protocol for ovaries took considerable time. Consequently it has, to date, only been possible to gather results for two of the Notch gene family, Notch 2 and Notch 3. These results are shown in Figure 7.12 and 7.13 respectively. The anti-sense probes for Notch 2 resulted in the 3-week-old ovary sections developing a dark colour in all cell compartments, an observation not found in the sense-probed sections (Figure 7.12). Within the sections, the granulosa cells appeared to stain more darkly than the neighbouring theca layer (marked as 'g' and 't' respectively in all sections). Both granulosa and theca layers were more darkly coloured than the oocyte. A small number of the granulosa cells adjacent to developing antral patches stained very darkly (marked as 'w' on Figure 7.12, B and C) compared to other neighbouring granulosa cells (marked 'y') in the anti-sense sections. Some 'antral' granulosa cells in

	13 day ovary	3 week ovary	Mature ovary	Young brain		Figure
Notch 1	H	H	H	√		7.3
Notch 2	F	H	X	√		7.4
Notch 3	√	√	X	√		7.5
Delta like 1	X	√	√	X		7.11
Delta like 3	-	-	-	-		-
Jagged 1	F	√	X	√		7.9
Jagged 2	√	√	√	√		7.10
Lunatic fringe	X	√	√	√		7.6
Manic fringe	√	√	√	√		7.7
Radical fringe	√	√	F	√		7.8

Table 7.1 RT-PCR results for the presence of neuro-genes in the mouse ovary √ = present, X = absent, F = faint, H = only detectable when a higher starting concentration of cDNA used.

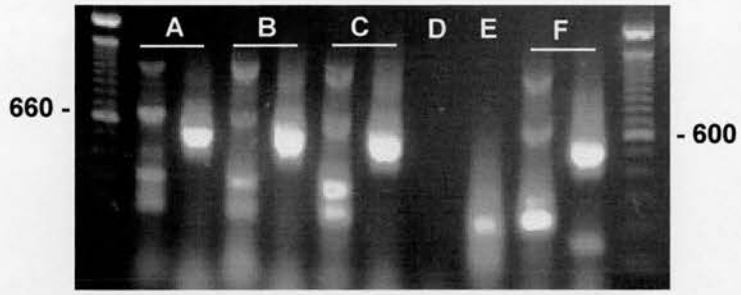


Figure 7.3 Notch 1 RT-PCR results. The amplified product is 660 bp. Lanes A are: 13-day-old ovary; B: 3-week-old ovary; C: mature ovary; D: ddH_2O; E: genomic DNA, and F: young brain. The right lane shows β-actin in each case.

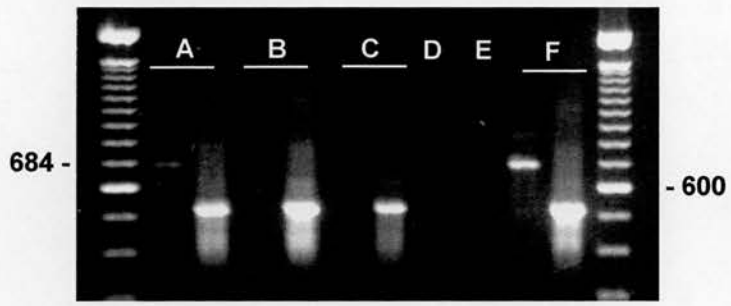


Figure 7.4 Notch 2 RT-PCR results. The amplified product is 684 bp. Lanes A are: 13-day-old ovary; B: 3-week-old ovary; C: mature ovary; D: ddH_2O; E: genomic DNA, and F: young brain. The right lane shows β-actin in each case.

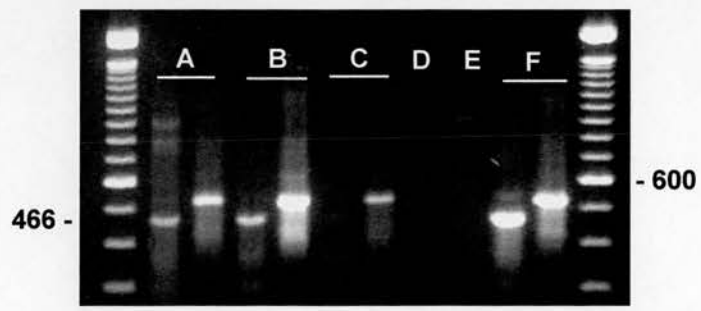


Figure 7.5 Notch 3 RT-PCR results. The amplified product 466 bp. Lanes A are: 13-day-old ovary; B: 3-week-old ovary; C: mature ovary; D: ddH_2O; E: genomic DNA, and F: young brain. The right lane shows β-actin in each case.

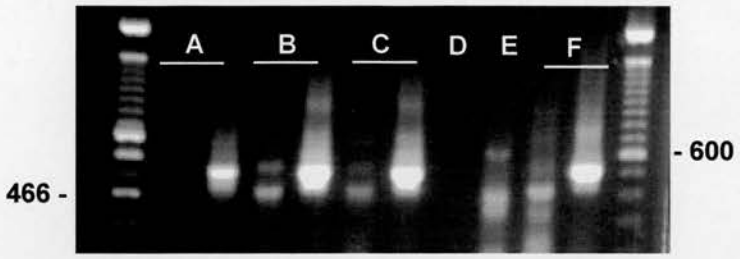


Figure 7.6 Lunatic fringe RT-PCR results. The amplified product is 466 bp. Lanes A are: 13-day-old ovary; B: 3-week-old ovary; C: mature ovary; D: ddH_2O; E: genomic DNA, and F: young brain. The right lane shows β-actin in each case.

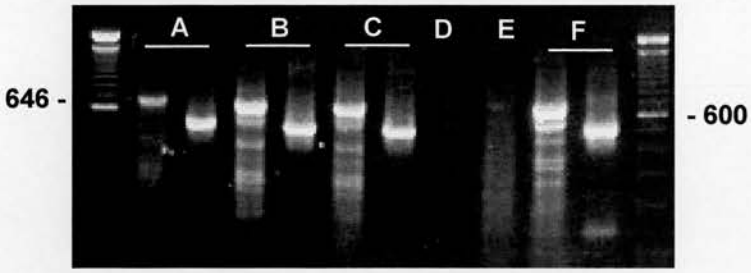


Figure 7.7 Manic fringe RT-PCR results. The amplified product is 646 bp. Lanes A are: 13-day-old ovary; B: 3-week-old ovary; C: mature ovary; D: ddH_2O; E: genomic DNA, and F: young brain. The right lane shows β-actin in each case.

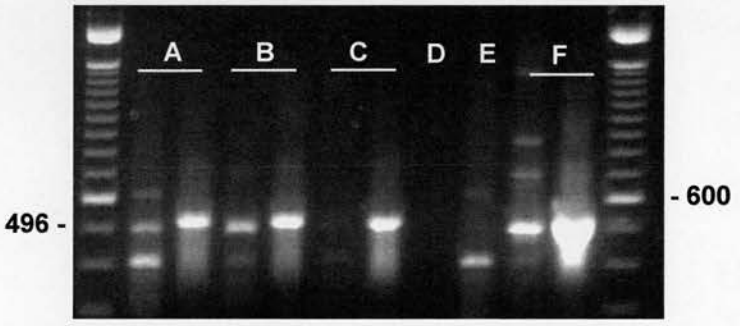


Figure 7.8 Radical fringe RT-PCR results. The amplified product is 496 bp. Lanes A are: 13-day-old ovary; B: 3-week-old ovary; C: mature ovary; D: ddH_2O; E: genomic DNA, and F: young brain. The right lane shows β-actin in each case.

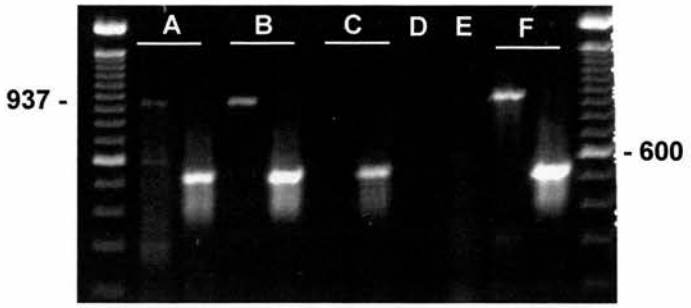


Figure 7.9 Jagged 1 RT-PCR results. The amplified product is 937 bp. Lanes A are: 13-day-old ovary; B: 3-week-old ovary; C: mature ovary; D: ddH_2O; E: genomic DNA, and F: young brain. The right lane shows β-actin in each case.

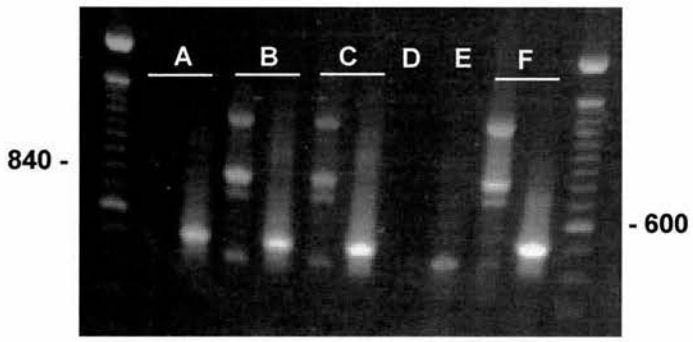


Figure 7.10 Jagged 2 RT-PCR results. The amplified product is 840 bp. Lanes A are: 13-day-old ovary; B: 3-week-old ovary; C: mature ovary; D: ddH_2O; E: genomic DNA, and F: young brain. The right lane shows β-actin in each case.

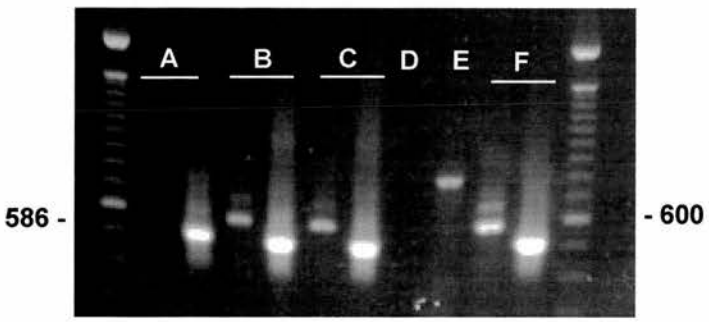


Figure 7.11 Delta like 1 RT-PCR results. The amplified product is 586 bp. Lanes A are: 13-day-old ovary; B: 3-week-old ovary; C: mature ovary; D: ddH_2O; E: genomic DNA, and F: young brain. The right lane shows β-actin in each case.

the sense control also appeared to stain more darkly than neighbouring cells (marked 'x' and 'z' respectively). Unlike the Notch 2 *in situ* experiments, there was no apparent 'background' or general staining in either the anti-sense or sense probed sections for Notch 3 (Figure 7.13). At high magnification there appears to be staining in the theca cell layer (marked 'v' in Figure 7.13 A, B and C, controls marked 'r'). Figure 7.13C was a result obtained from an early *in situ* experiment at the time the protocol was being refined. Consequently there was no sense control slide for this run.

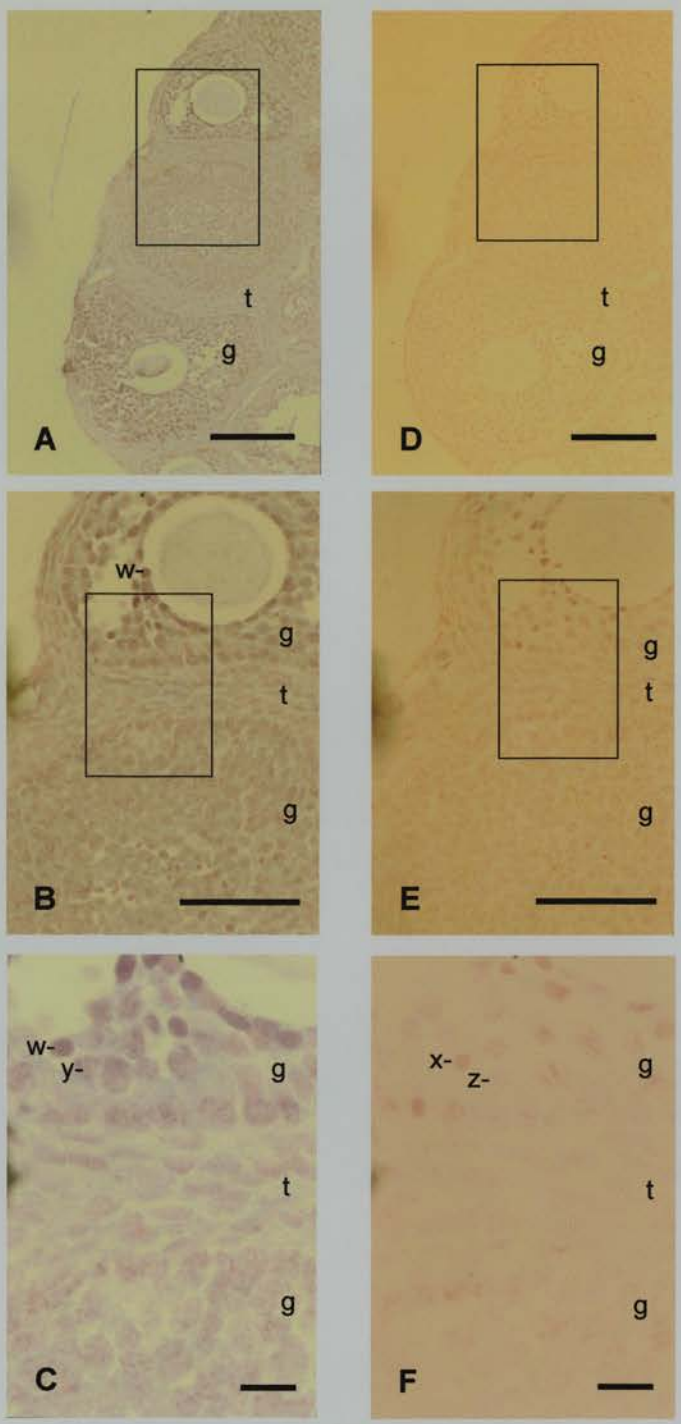


Figure 7.12 *In situ* hybridisation results for Notch 2 in three-week-old mouse ovary sections. A-C have been hybridised with anti-sense probes, D-F are corresponding sections hybridised with sense probes. Black boxes illustrate the subsequent section. G=granulosa cell, t=theca cell, w=condensed stained cell, x=condensed control, y='normal' stained cell, z= 'normal' control. Scale bars represent 50 μm, except A and D which represent 125 μm.

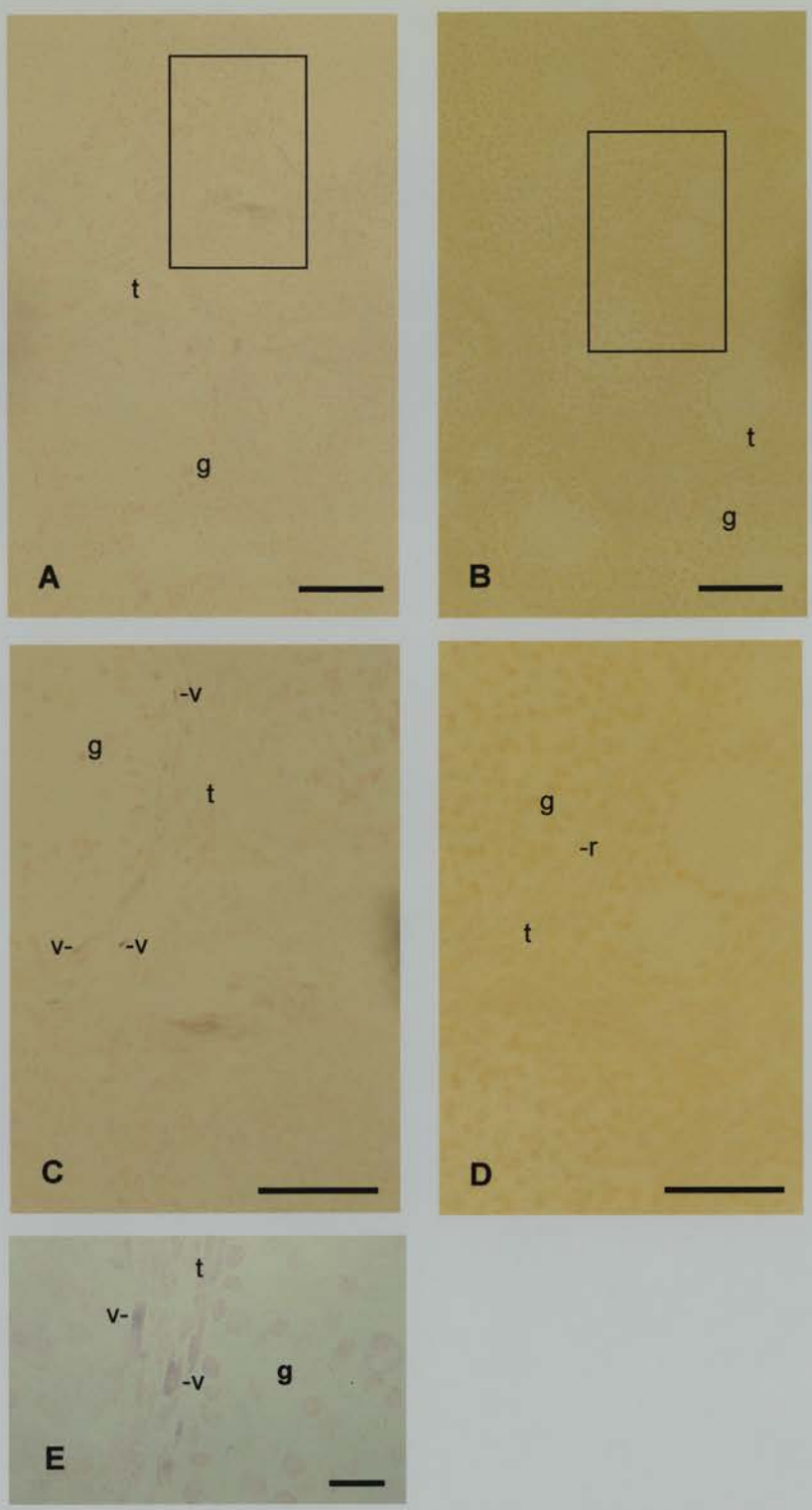


Figure 7.13 *In situ* hybridisation results for Notch 3 in three-week-old mouse ovary sections. A, C and E have been hybridised with anti-sense probes, B and D are sections from the same ovary as A and C, hybridised with sense probes. g=granulosa cells, t=theca cells, v=positive staining, r=un-stained control. Black boxes illustrate the subsequent section. Scale bars represent 125 μm in sections A and B, 50 μm in sections C and E, and 20 μm in E .

7.4 DISCUSSION

This pilot investigation into the expression of the *Notch* family and associated genes in the ovary has clearly demonstrated that all of the genes examined are being expressed during at least one developmental stage (with the presence/absence of the *Dll3* product still to be clarified). Furthermore some of the results from the RT-PCR investigation concur with previous investigations in other systems. *L-fng* and *Dll1* are found to be co-expressed in the developing neural tube (Cohen *et al.*, 1997): in the 13-day-old ovary both these gene products are absent, however they are both found in the 3-week-old and mature ovaries. All of the genes under investigation were found in the 3-week-old ovary. This is perhaps not surprising (assuming that the genes have a role in ovarian function) as this is the age when the mouse is very close to completing puberty and undergoing the first oestrous cycle. Consequently the morphology of the ovary reflects this developmental transition with many small follicles still present (as in the 13-day-old ovary) but also containing much larger antral follicles (reflecting the adult state). This transition stage may mean that genes that are only expressed early-on in the animals development may still be active but genes that are only active during adulthood have begun to be expressed. It is feasible that this age of ovary reflects a unique period of gene expression. While it is likely that gene expression levels will be consistent across mRNA extractions at different times from the 13-day-old and 3-week-old animals there may be much greater variability between mature ovary mRNA extractions. The extent of gene expression will depend on the timing of the extraction relative to the oestrous cycle, as the cycling ovary is a highly dynamic organ and will have rapidly changing proportions of pre-antral and antral follicles and corpora lutea.

It is unclear what role these genes are having in the ovary, with the possibility that any given gene might perform multiple functions (as mentioned previously, Notch 1 has been shown to play a variety of roles in other developing tissue). This thesis investigation is concerned primarily with follicle selection and dominance via contact-mediated mechanisms. Looking at the *in situ* hybridisation results within that context it is encouraging that the expression of *Notch 3* seems limited to the theca layer (at least in

the 3-week-old mouse). Figure 7.13 C and E show theca cells that have stained more darkly than neighbouring cells (marked 'v'). The presence of Notch 3 in the theca layer makes it feasible that this gene has a role in direct follicle-follicle communication. However, this is highly speculative as Notch 3 may equally be involved in different processes such as theca cell organisation.

The nature of Notch 2 expression is harder to interpret. The observation that the entire ovary section seems to stain in the anti-sense sections, but not the sense controls, would suggest that Notch 2 is widely expressed in all cell compartments. The granulosa cell layers (marked as 'g') have stained more strongly than either the oocyte or the theca cells in the lower magnification sections (Figure 7.12.A and B), although differences in theca and granulosa staining are less evident in the high magnification section (Figure 7.12 C).

Looking at the anti-sense sections, the strongest staining cells would appear to be found in some of the granulosa cells found around the developing antrum (marked 'w'), in so far as they exhibit the darkest staining of all cells in the ovary. Comparing these cells to equivalents found in the sense control section (marked 'x') it can be seen that some granulosa cells in the same region are also staining more darkly with the counter-stain. It can also be seen that these cells are condensed. Caution should be used therefore, before attributing too much significance to the condensed cells in the anti-sense probed section. It is entirely possible that these cells are undergoing apoptosis, picking up more background stain and appearing more 'positive' as a result, rather than actually expressing more Notch 2 protein. Conversely, it is possible that the reason they are staining so darkly is due to the Notch 2 protein being more concentrated on the reduced cell membrane surface. This would give a darker signal as there would be more probe bound in that area. A clearer result is presented with the comparison of non-condensed granulosa cells (marked 'y' in the anti-sense sections and 'z' in the sense control). The anti-sense probed cells clearly exhibit a higher degree of staining than the controls, providing the most compelling evidence that Notch 2 is being expressed in these cells.

If Notch 2 is being broadly expressed throughout ovarian tissue it would have to exhibit a specific pattern of activation to be directly involved in follicle selection. This could be bought about by the patterned expression of other associated genes, such as the *fringe* family. If follicle selection (or other processes of intra-ovarian organisation) do involve an inter-play between such genes, this would be analagous to gene interactions in the developing *Drosophila* which bring about limitation of the wing imaginal disc. From an evolutionary standpoint it is highly feasible that gene product interactions of this nature have been conserved between species, with homologous signalling cascades having different consequences from species to species and from tissue to tissue. I believe the fact that the products of the *Notch* family and associated genes are present in the ovary makes it highly likely that the resultant proteins will interact in ways already documented in other systems. To what end these interactions influence ovarian function still remains a matter of conjecture.

In conclusion, I believe that this work has offered a promising start to a much larger investigation into the gene expression behind juxtacrine communication in the ovary. I had begun this work with the intention of seeing if *Notch/Delta* regulated contact-mediated follicle selection, however, during the course of the investigation it became clear that other gene products (such as Jagged and Fringe) could also be involved. In view of this, it became evident that to examine the possible role of this group of genes in follicle selection would be a major project in itself. Having shown that these genes are being expressed in the ovary, a full investigation of their roles (whatever these are) is surely warranted. A comprehensive program of work is underway to perform *in situ* hybridisation reactions for each of the genes, looking at ovary sections at different developmental stages. If necessary this investigation will be extended to developing *in situ* RT-PCR protocols for genes that are being expressed at a low copy number. This study will be extended to incorporate follicles cultured *in vitro*, singly and in pairs. Hopefully these investigations will be able to offer an insight into possible gene function through distinct geographical localisation. It would then be possible to begin testing hypothesised function by altering *in vitro* culture conditions, or manipulating the

systemic environment *in vivo* (e.g. would superovulated animals express more/less of genes hypothesised to be involved in selection?).

Chapter 8

General discussion and concluding remarks

8.1 GENERAL DISCUSSION

The aims of this thesis were:

I. To establish if direct contact between follicles plays a part in the selection of the ovulatory follicle(s), and if this appeared to be the case,

II. To investigate the precise nature of direct follicle-follicle communication.

I believe that this investigation has gone some way towards addressing the first aim of this thesis, offering further insight into the role of follicle-follicle communication in selection. Whilst the precise nature of direct follicle-follicle contact is likely to involve a complex interplay between various factors, this investigation has clarified the requirement for the 'dominance mechanism' to be observed *in vitro*. Figure 8.1 summarises the experimental chapters and puts them into the overall context of follicle selection.

Starting with a histological examination of ovaries taken from several different mammalian species, it was revealed that it is the norm for pre-antral and early antral follicles to be found in close contact with one another *in vivo*. This makes 'contacting' follicle-follicle interactions *in vivo* feasible, consolidating the hypothesis of Spears *et al.* (1996) that intra-ovarian communication of this nature might have a role in follicle dominance and selection. Furthermore, these contacting follicles *in vivo* frequently had a very thin, shared theca layer separating them, an observation reflected *in vitro* when pre-antral follicles are co-cultured in contact. This adds further evidence that the use of whole follicle culture systems offers a close physiological paradigm.

With the knowledge that co-culturing follicles in close contact was reflecting a situation found *in vivo* I used polycarbonate membrane constructions to regulate the nature of communication between adjacent follicles. These experiments revealed that pre-/early antral follicles produce a locally diffusible factor that is capable of effecting the development of near, similarly-sized follicles. In contrast to the original publication from this laboratory (Spears *et al.*, 1996) the dominance effect appeared to be growth enhancing. Addressing the second aim of this thesis, these experiments have highlighted

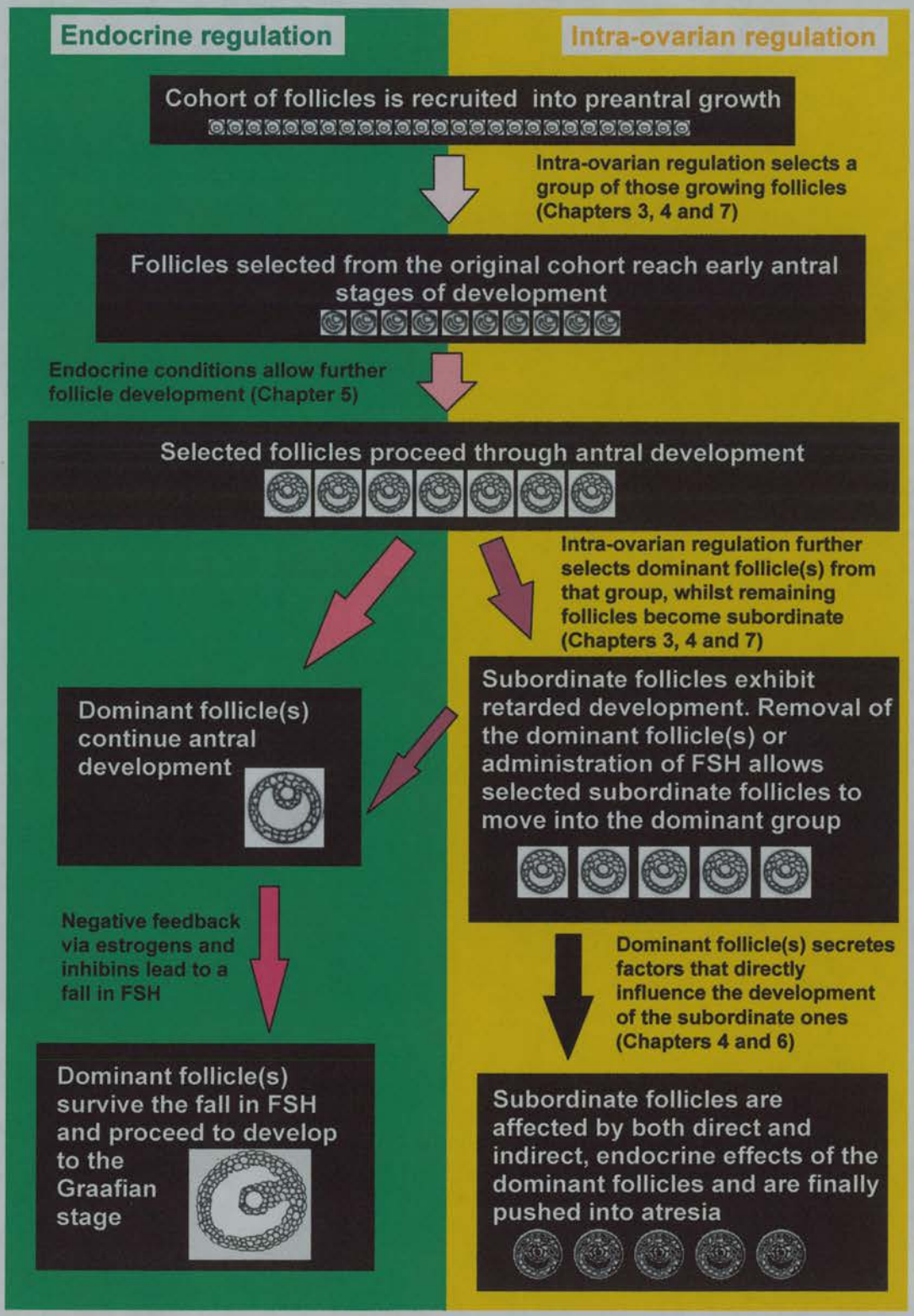


Diagram 8.1 Hypotheses for the selection of ovulatory follicles from a growing cohort, with references to areas investigated during the course of this thesis. Taken from Baker and Spears 1999.

a novel inter-follicular factor that may be involved in the selection of follicles from a growing cohort, and as such could represent an important intra-ovarian regulatory process. Currently there are no other reports in the literature regarding follicles at this developmental stage influencing the development of other like-sized follicles. The elucidation of a locally acting diffusible factor(s) acting between developmentally similar follicles opens up the potential for extensive further investigations. Recent work by others in this laboratory has shown that this factor(s) is/are stable in culture medium. This should make it possible to further investigate its/their effect on the development of follicles at various developmental stages and ultimately to characterise the factor(s). This could be a very time consuming pursuit with the likelihood that these follicles are secreting more than one factor making it difficult to identify a specific factor as having a particular function. It may be that an investigation of this nature would not represent a productive investment of time and resources.

Although it is possible to observe the emergence of dominance within co-cultured follicles without direct physical contact it can be seen *in vivo* that follicles do make close contact with one another. This makes the involvement of cell surface signalling molecules in follicle-follicle communication feasible. Investigation of genes known to be involved in lateral specification in other developmental systems has shown that some of these genes are being expressed in the ovary. While their expression patterns still largely remain to be established and their precise role is unclear, this work offers a start to an exciting investigation into the role of neuro-genes in the ovary and as such may ultimately be an extension of the second aim of this thesis.

Addressing the first aim of this thesis, the role of follicle-follicle contact in the selection of ovulatory follicles, is still speculative. However, I believe this investigation has provided compelling evidence suggesting that this may be the case. Confirming reports in the literature relating to other species, I have shown that murine antral follicles are less vulnerable to a decline in FSH than less developed pre-/early antral follicles *in vitro*, when a source of LH is available. This provided the backdrop to investigating the inter-relationship between follicle-follicle contact, environmental (systemic) gonadotrophin concentration and follicle health. These experiments clearly showed that

being co-cultured in contact with an antral follicle made pre-/early antral follicles susceptible to decreased FSH, with a marked negative effect on follicle health. These results offer strong evidence that follicle-follicle contact could have an important role to play in follicle selection, under-pinning the gonadotrophin hypothesis for follicle selection.

8.2. CONCLUDING REMARKS

To date, most of the research into follicular dominance has concentrated on its endocrine regulation. While endocrine control of follicle dominance can explain much of the later processes that occur (such as ensuring that subordinate follicles ultimately undergo atresia), intra-ovarian interactions are also involved in its regulation. Follicle-follicle interactions have various possible roles both at early stages when follicles are being selected from amongst a cohort or cluster of follicles and/or later, when dominant follicles are 'holding back' challengers. Furthermore, intra-follicular processes also mediate the response of a follicle to endocrine changes. I believe this thesis has been successful in highlighting the possible importance of follicle-follicle interactions in ovary regulation. However, as such it could be viewed as a starting point for a much larger investigation into this little understood component of ovarian function.

References

Adashi, E.Y. and Rohan, R.M.
Intraovarian regulation: Peptidergic signalling systems
J. Steroid. Biochem. Mol. Biol. 1992 3: 243-248

Alberts, A., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D.
Molecular Biology of the Cell 3rd Edition
Garland Publishing, Inc., New York 1994

Armstrong, D.G. and Webb, R.
Ovarian follicular dominance: the role of intraovarian growth factors and novel proteins
Rev. Reprod. 1997 2: 139-146

Armstrong, D.T. and Dorrington, J.H.
Estrogen biosynthesis in the ovaries and testes
In Regulatory mechanisms affecting Gonadal Hormone action eds. Thomas, J.A. and Singhal, R.L.
University Park Press, Baltimore 1979 2: 217-258

Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M.E.
Notch Signalling
Science 1995 268: 225-268

Bacharova, R.
In 'Developmental Biology' ed Browder, L.W.
Plenum, New York 1985 453-524

Bagavandos, P., Midgley, A.R. and Wicha, M.
Developmental changes in the Ovarian Follicular Basal Lamina detected by Immunofluorescence and electron microscopy
J. Hist. Cyt. 1983 31: 633-640

Baird, D.T.
A model for follicular selection and ovulation: Lessons from superovulation
J. Steroid. Biochem. 1987 27: 15-23

Baker, S.J. and Spears, N.
The Role of Intra-ovarian Interactions in the Regulation of Follicle Dominance
Human Reproduction Update 1999 5: 153-165

Baker, T.G.
Oogenesis and ovulation. In Reproduction in mammals 2nd ed. eds Austin, C.R. and Short, R.V.
Cambridge: Cambridge University Press 1982 17-45

Beaumont, H.M. and Mandl, A.M.
A quantitative and cytological study of oogonia and oocytes in the foetal and neonatal rat ovary
Proc. R. Soc. London, Series B 1962 155: 557-579

Bergh, C., Carlsson, B., Olsson, J.H., Selleskog, U. and Hellensjö, T.
Regulation of androgen production in cultured human thecal cells by insulin-like growth factor and insulin
Fertil. Steril. 1993 59: 323-331

Bettenhausen, B., Hrabě de Angelis, M., Simon, D., Guénet, J-L., Gossler, A.
Transient and restricted expression during mouse embryogenesis of Dll1, a murine gene closely related to Drosophila Delta
Dev. 1995 121: 2407-2418

Billig, H., Furuta, I. And Hsueh, A.J.W.
Gonadotropin-Releasing Hormone Directly Induces Apoptotic Cell Death in the Rat Ovary: Biochemical and in situ Detection of Deoxyribonucleic Acid Fragmentation in Granulosa cells
Endocrinology 1994 134: 245-252

Birchmeier, C. and Birchmeier, W.
Molecular aspects of mesenchymal-epithelial interactions
Ann. Rev. Cell. Biol. 1993 9: 511-540

Boland, N.I. and Gosden, R.G.
Clonal analysis of chimaeric mouse ovaries using DNA in situ hybridization
J. Reprod. Fert. 1994 100: 203-210

Boone, D.L. and Tsang, B.K.
Identification and localisation of Deoxyribonuclease I in the rat ovary
Biol. Reprod. 1997 57: 813-821

Boone, D.L., Yan, W. and Tsang, B.K.
Identification of a deoxyribonuclease I-like endonuclease in rat granulosa and luteal cell nuclei
Biol. Reprod. 1995 53: 1057-1065

Brison, D.R. and Schultz, R.M.
Apoptosis during Mouse Blastocyst Formation: Evidence for a Role for Survival Factors Including Transforming Growth Factor α
Biol. Reprod. 1997 56: 1088-1096

Brown, J.B.
Pituitary control of ovarian function - concepts derived from gonadotrophin therapy
Aust. NZ. J. Obstet. Gyn 1978 18: 47

Byskov, A.G.
The anatomy and ultrastructure of the rete system in the fetal mouse ovary
Biol. Reprod. 1978 19: 720-735

Cahill, L.P., Driancourt, M.A., Chomely, W.A. and Finlay, J.K.
Role of intrafollicular regulators and FSH in growth and development of large antral follicles in sheep
J. Reprod. Fert. 1985 75: 599-607

Campbell, B. K., Picton, H.M., Mann, G.E., McNeilly, A.S. and Baird, D.T.
Effect of steroid- and inhibin-free ovine follicular fluid on ovarian follicles and ovarian hormone secretion
J. Reprod. Fert. 1991 93: 81-96

Campbell, B.K. and McNeilly, A.S.
Follicular dominance and oocyte maturation
Zygote 1996 4:3 27-334

Cara, J.F. and Rosenfield, R.L.
Insulin-like growth factor I and insulin potentiate luteinizing hormone-induced androgen biosynthesis by rat ovarian theca-interstitial cells
Endocrinology 1988 123: 733-739

Carnevale, E.M., McKinno, A.O., Squires, E.L. and Voxx, J.L
Ultrasonographic characteristics of the preovulatory follicle preceding and during ovulation in mares
Equine Veterinary Science 1988 8: 428-431

Carson, R.S., Robertson, D.M. and Findlay, J.K.
Ovine follicular fluid inhibits thymidine incorporation by 3T3 fibroblasts <u>*in vitro*</u>
J. Reprod. Fert. 1988 82: 447-455

Chakravorty, A., Mahesh, V.B. and Mills, T.M.
Control of peptides regulating mitosis of granulosa cells in immature rat ovary by oestrogen and gonadotrophin
J. Reprod. Fert. 1993 97: 91-100

Check, J.H., Dietterich, C. and Houck, M.A.
Ipsilateral versus Contralateral ovary selection of dominant follicle in succeeding cycle
Obs. Gyn. 1991 77: 247-249

Chun, S.-Y., Eisenhauer, K.M., Minami, S., Billig, H., Perlas, E. and Hsueh, A.J.W.
Hormonal regulation of Apoptosis in early antral folliciles: Follicle-stimulating Hormone as a major survival factor
Endocrinology 1996 137: 1447-1456

Cohen, B., Bashirullah, A., Dagnino, L., Campbell, C., Fisher, W.W., Leow, C.C., Whiting, E., Ryan, D., Zinyk, D., Boulianne, G., Hui, C-C., Gallie, B., Phillips, R.A., Lipshitz, H.D. and Egan, S.E.
Fringe boundaries coincide with Notch-dependent patterning centres in mammals and alter Notch-dependent development in Drosophila
Nature Genetics 1997 16: 283-288

di Zerega, G.S., Goebelsmann, U. and Nakamura, R.M
Identification of protein(s) secreted by the pre-ovulatory ovary which suppresses the follicle response to gonadotrophins
J. Clin. Endo. Metab. 1982 54: 1091-1096

Dierschke, D.J., Hutz, R.J. and Wolf, R.C.
Induced follicular atresia in rhesus monkeys: strength-duration relationships of the estrogen stimulus.
Endocrinology 1985 117: 1397-1403.

Driancourt, M.A.
Lack of between follicle interactions in the sheep ovary
Rep. Nutrition. Dev. 1994 34: 249-260

Dong, J., Albertini, D.F., Nishimori, K., Kumar, T.R., Lu. N. and Matzuk, M.M.
Growth differentiation factor-9 is required during early ovarian folliculogenesis.
Nature 1996 383: 531-535

Donovan, P.J., Stott, D., Cairns, L.A., Heasman, J. and Wylie, C.C.
Migratory and postmigratory mouse primordial germ cells behave differently in culture
Cell 1986 44: 831-838

Dukelow, W.R.
Ovulatory cycle characteristics of macoca fasicularis
J. Med. Primatol. 1977 6: 33-42

Dunwoodie, S.L., Henrique, D., Harrison, S.M. and Beddington, S.P.
Mouse Dll3: a novel divergent Delta gene which may complement the function of other Delta homologues during early pattern formation in the mouse
Dev. 1997 124: 3065-3076

Eckery, D.L., Moeller, C.L., Nett, T.M. and Sawyer, H.R.
Localisation and quantification of binding sites for FSH, LH, GH and IGF-1 in sheep ovarian follicles
Biol. Reprod. 1997 57: 507-513

Eckternkamp, S.E., Howard, H.J., Roberts, A.J., Grizzle, J. and Wise, T.
Relationships amongst concentrations of steroids, insulin-like growth factor-1, and insulin-like growth factor binding proteins in ovarian follicular fluid of beef cattle
Biol. Reprod. 1994 51: 971-981

Edwards, R.G., Fowler, R.E., Gore-Langton, R.E., Gosden, R.G., Jones, E.C., Redhead, C. and Steptoe, P.C.
Normal and abnormal follicular growth in mouse, rat and human ovaries
J. Reprod. Fert. 1977 51: 237-263

Erickson, G.F. and Danforth, D.R.
Ovarian control of follicle development
Am. J. Obstet. Gynecol. 1995 172: 736-747

Erickson, G.F., Magoffin, D.A., Dyer, C.A. and Hofeditz, C.
The ovarian androgen producing cells: a review of structure function relationships
Endocr. Rev. 1985 6: 371-399

Fehon, R.G., Kooh, P.J., Rebay, I., Regan, C.L., Xu, T., Muskavitch, M.A. and Artavanis-Tsakonas, S.
Molecular interactions between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in Drosophila
Cell 1990 61: 523-534

Forabosco, A., Sforza, C., De Pol, A., Vizzotto, L., Marzona, L. and Ferrario VF.
Morphometric study of the human neonatal ovary.
Anatomical Record 1991 231: 201-208.

Fortune, J.E.
Ovarian follicular growth and development in mammals
Biol. Reprod. 1994 50: 1320-1327

Freeman, M.E.
In 'The Phyiology of Reproduction' eds Knobil, E. and Neil, J
Raven Press, New York 1988 115-139

Fukuda, M., Fukuda, K., Andersen, C.Y. and Byskov, A.G.
Contralateral selection of dominant follicle favours pre-embryo development
Hum. Reprod. 1996 11: 1958-1962

Giannakis, C., Forbes, I.J. and Zalewski, P.D.
Ca2+/Mg2+ dependent nuclease: tissue distribution, relationship to inter-nucleosomal DNA fragmentation and inhibition by Zn2+
Biochem. Biophys. Res. Commun. 1991 181: 915-920

Gibbons, J.R., Wiltbank, M.C. and Ginther, O.J.
Functional Interrelationships between follicles greater than 4mm and the Follicle Stimulating surge in heiffers
Biol. Reprod. 1997 57: 1066-1073

Gilbert, A.B.
Female genital organs
In Form and Function in birds., eds King, A.S. and McLelland, J.
Academic Press, London 1979 1, 237-360

Gillman, J.
The development of the gonads in man, with a consideration of fetal endocrines and histogenesis of ovarian tumours
Contrib. Embryol. 1948 32: 81-131

Ginther, O.J., Knopf, L. and Kastelic, J.P.
Temporal associations among ovarian events in cattle during oestrous cycles with two and three follicular waves
J. Reprod. Fertil. 1989 87: 223-230

Gondos, B.
In Gonadtrophins and Ovarian Development eds Butt, W.R., Crooke, A.C. and Ryle, M.
Livingstone, Edinburgh 1970 239-247

Gong, J.G., McBride, D., Bramley, T.A. and Webb, R.
Effect of recombinant bovine somatotropin, insulin-like growth factor-1 and insulin on the proliferation of bovine granulosa cells in vitro.
J. Endocrinol. 1993 139: 67-75

Gore, M.A., Nayudu, P.L. and Vlaisavljevic, V.
Attaining dominance in vivo: distinguishing dominant from challenger follicles in humans.
Hum. Reprod. 12: 2741-2747 1997

Gosden, R.G. and Telfer, E.E.
Scaling of follicular sizes in mammalian ovaries.
Journal of Zoology, 1987 211: 157-168.

Gosden, R.G., Boland, N.I., Spears, N., Murray, A.A., Chapman, M., Wade, J.C., Zhody, N.I. and Brown, N.
The biology and technology of follicular oocyte development <u>*in vitro*</u>
Reprod. Med. Rev. 1993 2: 129-152

Gougeon, A. and Lefèvre, B.
Histological evidence of alternating ovulation in women
J. Reprod. Fert. 1984 70: 7-13

Gougeon, A.
Regulation of ovarian follicular development in primates: Facts and hypotheses
Endocrinology Rev. 1996 17: 121-155

Grant, S.A., Hunter, M.G. and Foxcroft, G.R.
Morphological and biochemical characteristics during ovarian follicular development in the pig
J. Reprod. Fert. 1989 86: 171-183

Greenwald, G.S. and Terranova, P.F.
Follicular selection and its control
Raven Press Ltd, New York 1988 387-445

Gwynne, J.T. and Strauss, J.F.
The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands
Endocr. Rev. 1982 3: 299-329

Hakuno, N., Koji, T., Yano, T., Kobayashi, N., Tsutsumi, O., Taketani, Y. and Nakane, P.K.
Fas/APO-1/CD95 system as a mediator of granulosa cell apoptosis in ovarian follicle atresia
Endocrinology 1996 137: 1938-1948

Hanrahan, J.P. and Quirke, J.F.
Contribution of variation in ovulation rate and embryo survival to within breed variation in litter size. In Genetics of reproduction in sheep Eds Land, R.B. and Robinson, D.W.
Butterworths, London 1985 193-201

Hartenstein, V. and Posakony, J.W.
A dual function of the Notch gene in Drosophila sensillum development
Dev. Biol. 1990 142: 13-30

Hay, M.R., Cran, D.G., Moor, R.M.
Structural changes occurring during atresia in sheep ovarian follicles
Cell. Tiss. Res. 1976 169: 515-529

Heitzler, P. and Simpson, P.
The choice of cell fate in the epidermis of Drosophila
Cell 1991 64: 1083-1092

Heller, D.T., Cahil, D.M., Schultz, R.M.
Biochemical studies of mammalian oogenesis: Metabolic cooperativity between granulosa cells and growing mouse oocytes
Dev. Biol. 1981 84: 455-464

Hickey, G.J., Chen, S., Besman, M.J., Shively, J.E., Hall, P.F., Gaddy-Kurten, D. and Richards, J.S.
Hormonal regulation, tissue distribution, and content of aromatase cytochrome P450 messenger ribonucleic acid and enzyme in rat ovarian follicles and corpora lutea: relationship to estradiol biosynthesis
Endocrinology 1988 122: 1426

Hillier, S.G., Whitelaw, P.F., Smyth, C.D.
Sex Steroid Metabolism in the Ovary
The Biochemist 1994 Apr/May 22-26

Hillier, S.G., Yong, E.L., Illingworth, P.I., Baird, D.T., Schwall, R.H. and Mason, A.J.
Effect of recombinant inhibin on androgen synthesis in cultured human thecal cells
Mol. Cell. Endocrinol. 1991 75, R1-R6: 79, 177

Hirshfield, A.N.
Size-frequency analysis of atresia in cycling rats
Biol. Reprod. 1988 38: 1181-1188

Hirshfield, A.N.
Granulosa cell proliferation in very small follicles of cycling rats studied by long-term continuous tritiated-thymidine infusion
Biol. Reprod. 1989 41: 309-316

Hirshfield, A.N.
Development of follicles in the mammalian ovary
Internat. Rev. Cytol. 1991a 124: 43-101

Hirshfield, A.N.
Theca cells may be present at the outset of follicular growth
Biol. Reprod. 1991b 44: 1157-1162

Hirshfield, A.N.
Patterns of Ovarian Cell Proliferation in Rats during the Embryonic Period and the First Three Weeks Postpartum
Biol. Reprod. 1995 53: 1208-1221

Hirshfield, A.N. and Midgley Jr, R.
Morphometric analysis of follicular development in the rat
Biol. Reprod. 1978 19:597-606

Hodgen, G.D.
The dominant ovarian follicle
Fertil. Steril. 1982 38: 281-300

Horster, M., Huber, S., Tschöp, J., Dittrich, G. and Braun, G.
Epithelial nephrogenesis
Pflüg. Arch. Eur. J. Physiol. 1997 434: 647-660

Hsueh, A.J.W., Billig, H. and Tsafriri, A.
Ovarian Follicle Atresia: A Hormonally Contolled Apoptotic Process
Endocrinology Rev. 1994 15: 707-725

Huet, C., Monget, P., Pisselet, C. and Monniaux, D.
Changes in Extracellular Matrix components and Steroidogenic Enzymes during growth and atresia of antral ovarian follicles in the sheep
Biol. Reprod. 1997 56: 1025-1034

Hughes, F.M. and Gorospe, W.C.
Biochemical identification of apoptosis (programmed cell death) in granulosa cells: evidence for a potential mechanism underlying follicular atresia.
Endocrinology 129: 2415-2422.

Hurle, J.M., Ros, M.A., Climent, V. and Garcia-Martinez, V.
Morphology and significance of programmed cell death in the developing limb bud of the vertebrate embryo
Microscopy Research and Technique 1996 34: 236-246

Hurwitz, A. and Adashi, E.Y.
Ovarian follicular atresia is an apoptotic process.
In The Ovary eds. Adashi, E.Y. and Leung, C.K.
Raven Press Ltd, New York 1993 473-485

Hynes, A.C., Kane, M.T. and Sreenan, J.M.
Partial purification from bovine follicular fluid of a factor of low molecular mass with inhibitory effects on the proliferation of bovine granulosa cells in vitro and on rat follicular development in vivo
J. Reprod. Fert. 1996 108: 185-191

Hynes, A.C., Sreenan, J.M. and Kane, M.T.
Modulation of the effects of FSH, androstenedione, EGF and IGF-1 on bovine granulosa cells by GCIF a growth inhibitory factor of low molecular mass from bovine follicular fluid
J. Reprod. Fert. 1996 108: 193-197

Hynes, A.C., Kane, M.T. and Sreenan, J.M.
Immunization of rats and sheep against granulosa cell-inhibitory factors from bovine follicular fluid increases the number of large follicles in rats and the ovulation rate in sheep
J. Reprod. Fert. 1999 108: 116: 35-41

Ireland, J.J. and Roche, J.F.
Development of nonovulatory antral follicles in heiffers: changes in steroids in follicular fluid and receptors for gonadotrophins
Endocrinology 1983 112: 150-156

Irvine, K.D. and Wieschaus, E.
Fringe, a boundary specific molecule, mediates interactions between dorsal and ventral cells during Drosophila wing development
Cell 1994 79: 595-606

Johnston, S.H., Rauskolb, C., Wilson, R., Prabhakaran, B., Irvine, K.D. and Vogt, T.F.
A family of mammalian Fringe genes implicated in boundary determination and the Notch pathway
Dev. 1997 124: 2245-2254

Jönsson, F. and Knust, E.
Distinct functions of the Drosophila genes Serrate and Delta revealed by ectopic expression during wing development
Dev. Genes. Evol 1996 206: 91-101

Kerr, J.F., Wylie, A.H. and Currie, A.R.
Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics
Br. J. Cancer 1972 26: 239-257

Kigawa, T., Ogawa, T., Miyamura, K. and Iino, Y.
Inhibitory activity of a low molecular weight substance from porcine small follicles on oestradiol and progesterone secretion by rat granulosa cells in vitro and rat ovaries in vivo
Endocrinologica Japonica 1986 83: 597-607

Knobil, E. and Neill, J.D.
The Physiology of reproduction
Raven Press, New York 1988 387-445

Ko, J.C.H., Kastelic, J.P., Del Campo, M.R. and Ginther, O.J.
Effects of a dominant follicle on ovarian follicular dynamics during the oestrous cycle in heifers
J. Reprod. Fert. 1991 91: 522-519

Koering, M.J., Danforth, D.R. and Hodgen, G.D.
Early follicle growth in the juvenile Macaca monkey ovary: the effects of estrogen priming and follicle-stimulating hormone.
Biol. Reprod. 1994 50: 686-694.

Lardelli, M., Dahlstrand, J. and Lendahl, U.
The novel Notch homologue Notch 3 lacks specific epidermal growth factor repeats and is expressed in proliferating neuroepithelium
Mech. Dev. 1994 46: 123-136

Lardelli, M. and Lendahl, U.
Motch A and Motch B- two mouse Notch homologues coexpressed in a wide varity of tissues.
Exp. Cell Research 1993 204: 364-372.

Le Nestour, E., Marraomi, J., Lahlou, N., Roger, M., de Ziegler, D. and Bouchard, P.
Role of oestradiol in the rise in follicle stimulating hormone levels during the luteal follicular transition
J. Clin. Endocrin. Met. 1993 77: 439-442

Linsell, C.E., Shawber, C.J., Boulter, J. and Weinmaster, G.
Jagged: A mammalian ligand that activates Notch
Cell 1995 80: 910-917

Lintern-Moore, S and Moore, G.P.
The initiation of follicle and oocyte growth in the mouse ovary
Biol. Reprod. 1979 20: 773-778

Luck, M.R.
The gonadal extracellular matrix
Oxford Reviews of Reproductive Biology 1994 16: 34-85

McGee, E., Spears, N., Minami, S., Hsu, S-Y., Chun, S-Y., Billig, H. and Hsueh, A.J.W.
Preantral Ovarian Follicles in Serum-Free Cultures: Suppression of Apoptosis after Activation of the cGMP pathway and stimulation of Growth and Differentiation by FSH
Endocrinology 1997 138: 2417-2424

McIntush, E.W. and Smith, M.F.
Matrix metalloproteinases and tissue inhibitors of metalloproteinases in ovarian function
Reviews of Reproduction 1998 3: 23-30.

Marinho, A.O., Sallam, H.N., Goessens, L., Collins, W.P. and Campbell, S.
Ovulation side and occurrence of mittelschmerz in spontaneous and induced ovarian cycles
B.M.J. 1982 284: 632

Matton, P., Adelakoun, V., Couture, Y. and Dufour, J.J.
Growth and replacement of the bovine ovarian follicles during the oestrous cycle
J. Anim. Sci. 1981 52: 813-820

Mauléon, P.
In Control of Ovulation eds Crighton, D.B., Foxcroft, G.R., Haynes, N.B. and Lamming, G.E.
Butterworth, London 1978 141-158

Merchant, H. and Zamboni, L.
Presence of connections between follicles in juvenile mouse ovaries
Am. J. Anat. 1972 134: 127-132

Mihm, M., Good, T.E.M., Ireland, J.L.H., Ireland, J.J., Knight, P.G. and Roche, J.F.
Decline in serum follicle-stimulating hormone concentrations alters key intrafollicular growth factors involved in selection of the dominant follicle in heifers.
Biol. Reprod. 1997 57: 1328-1337

Mizunuma, H., Liu, X., Andoh, K., Abe, Y., Kobayashi, J., Yamada, K., Yokota, H., Ibuki, Y. and Hasegawa, Y.
Actvin from secondary follicles causes small preantral follicles to remain dormant at the resting stage
Endocrinology 1999 140: 37-42

Morse, A.H. and van Wagenen, G.
Observations upon ovulation in primates
Am. J. Obs. Gyn. 1936: 823-832

Murray, A.A., Molinek, M.D., Baker, S.J., Hillier, S.G. and Spears, N.
Ascorbic acid supports survival of murine ovarian follicle in vitro
In preparation

Naruse, I. And Keino, H.
Apoptosis in the developing CNS
Progress in Neurobiology 1995 47: 135-155

Nayudu, P.L. and Osborn, S.M.
Factors influencing the rate of preantral and antral growth of mouse ovarian follicle in vitro
J. Reprod. Fertil. 1992 95: 349-362

Nebert, D.W., Nelson, D.R., Coon, M.J., Estabrook, R.W., Feyereisen, R., Fujii-Kuriyama, Y., Gonzales, F.J., Guengerich, F.P., Gunsalus, I.C., Johnson, E.F., Loper, J.C., Sato, R., Waterman, M.R. and Waxman, D.J.
The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature
DNA cell Biology 1991 10: 1-14

Nicosia, S.V. and Nicosia, R.F.
Neoplasms of the ovarian mesothelium
In Pathology of Human Neoplasms ed. Azar, H.A.
Raven Press, New York 1988 435-486

O'Shea, J.D.
Smooth muscle-like cells in the theca externa of ovarian follicles in sheep
J. Reprod. Fert. 1971 24: 283-285

Ohno, S. and Smith, J.B.
Role of fetal follicular cells in meiosis of mammalian oocytes
Cytogenetics 1964 3: 324-333

Palumbo, A. and Yeh, J.
In situ Localization of Apoptosis in the Rat Ovary during Follicular Atresia
Biol. Reprod. 1994 51: 888-895

Panin, V.M., Papayannopoulos, V., Wilson, R. and Irvine, K.D.
Fringe modulates Notch-ligand interactions
Nature 1997 387: 908-912

Pearson, O.P.
Reproduction of a South American rodent, the mountain viscacha
Amer. J. Anat. 1949 84: 143-198

Peng, X-R., Hsueh, A.J.W., LaPolt, P.S., Bjersing, L. and Ny, T.
Localization of Luteinizing Hormone Receptor Messenger Ribonucleic Acid Expression in Ovarian Cell Types during Follicle Development and Ovulation
Endocrinology 1991 129: 3200-3207

Peters, H., Byskov, A.G. and Faber, M.
Intraovarian regulation of follicular growth in the immature mouse
In Peters, H., ed. The development and maturation of the ovary and its functions
Excerpta Medica, Amsterdam 1973a 20-23

Peters, H., Byskov, A.G., Lintern-Moore, S. and Faber, M.
Proceedings: Follicle growth initiation in the immature mouse ovary: Extraovarian or intraovarian control?
J. Reprod. Fert. 1973b 35: 619-620

Peters, H.
In The Vertebrate Ovary ed Jones, R.E.
Plenum, New York 1978 121-144

Potashnik, G., Insler, V. and Meizner, I.
Frequency, sequence, and side of ovulation in women menstruating normally
B.M.J. 1987 294: 219

Prickett, M.E.
Pathology of the equine ovary
Proceedings of the 12th annual convention of American Association of Equine Practioners 1966 145-154

Quirk, S.M., Cowan, R.G., Joshi, S.G. and Henrikson, K.P.
Fas antigen-mediated apoptosis in human granulosa/luteal cells
Biol. Reprod. 1995 52: 279-287

Redmer, D.A. and Reynolds, L.P.
Angiogenesis in the ovary
Rev. Reprod. 1996 1: 182-192

Richards, J.S., Jahnsen, T., Hedin, L., Lifka, J., Ratoosh, S.L., Durcia, J.M. and Goldring, N.B.
Ovarian follicular development: from physiology to molecular biology
Recent Prog. Horm. Res. 1987 43: 231-270

Robertson, M.A. and Telfer, E.E.
Programmed cell death in murine follicles grown in vitro
J. Reprod. Fertil. Abstract series 1994 13: 28

Rodgers, H.F., Lavranos, T.C., Vella, C.A. and Rodgers, R.J.
Basal lamina and other extracellular matrix produced by bovine granulosa cells in anchorage-independent culture
Tissue Res. 1995 282: 463-471

Rodgers, R.J., Vella, C.A., Rodgers, K., Scott, K. and Lavranos, T.C.
Production of Extracellular Matrix, Fibronectin and Steroidogenic Enzymes, and Growth of Bovine Granulosa Cells in Anchorage-independent Culture
Reprod. Fert. Dev. 1996 8: 249-257

Rühl, A.

Regelmässigkeit im wechsel der Ovarial funktion

Arch. Gynäk. 1925 124: 1-26

Ryan, A.K., Blumberg, B., Rodriguez-Esteban, C., Yonei-Tamura, S., Tamura, K., Tsukui, T., de la Pena, J., Sabbagh, W., Greenwald, J., Choe, S., Norris, D.P., Robertson, E.J., Evans, R.M., Rosenfeld, M.G. and Belmonte, J.C.I.
Pitx2 determines left-right asymmetry of internal organs in vertebrates.
Nature 1998 394: 545-551.

Saxen, L. and Lehtonen, E.
Transfilter induction of kidney tubules as a function of the extent and duration of intercellular contacts
J. Embryol. Exp. Morphol 1978 47: 97-109

Schams, D., Amselgruber, W., Kamhuber, M. and Einspanier, R.
The Importance of Basic fibroblastic growth factor for follicle selection
Biol. Reprod. 1996 54 (Supplement 1) Abstract 6

Schawber, C., Boulter, J., Linsell, C.E. and Weinmaster, G.
Jagged 2: A serrate-like gene expressed during rat embryogenesis
Dev. Biol. 1996 180: 370-376

Slack, J.M.W.
The nature of the mesoderm-inducing signal in Xenopus: a transfilter induction study
Dev. 1991 113: 661-669

Snow, M.H.L. and Monk, M.
Emergence and migration of mouse primordial germ cells.
In Current Problems in Germ cell Differentiation eds. McClaren, A. and Wylie, C.C. Cambridge University Press, Cambridge 1983 115-135

Song, L., Porter, D.G. and Coomber, B.L.
Production of Gelatinases and Tissue Inhibitors of Matrix Metalloproteinases by Equine Ovarian Stromal cells In Vitro
Biol. Reprod. 1999 60: 1-7

Spears, N., deBruin, J.P. and Gosden, R.G.
The establishment of follicular dominance in co-cultured mouse ovarian follicles
J. Reprod. Fert. 1996 106: 1-6

Spicer, L.J., Eckternkamp, S.E., Canning, S.F. and Hammond, J.M
Relationship between concentrations of immunoreactive insulin-like growth factor-1 in follicular fluid and various biochemical markers of differentiation in bovine antral follicles
Biol. Reprod. 1988 39: 573-580

Sunderland, S.J., Crowe, M.A., Boland, M.P., Roche, J.F. and Ireland, J.J.
Selection, dominance and atresia of follicles during the oestrous cycle of heiffers
J. Reprod. Fert. 1994 101: 547-555

Tamura, T., Kitawaki, J., Yamamoto, T., Osawa, Y., Kominami, S., Takemori, S., Okada, H.
Immunohistochemical localization of 17α-hydroxylase/C17-20 lyase and aromatase cytochrome P450 in the human ovary during the menstrual cycle
J. Endocrinol. 1992 135: 589-595

Telfer, E.E. and Robertson, M.A.
Programmed cell death in murine preantral follicles: from birth to death
Biol. Reprod. Supplement 1 1993 48: 332

Tilly, J.L., Billig, H. and Hsueh, A.J.W.
Gonadotropins suppress apoptotic cell death in cultured ovarian follicles
Proceedings from the 4oth Annual meeting of the Society of Gynecological Investigation, Toronto, Canada 1993 p.147

Tilly, J.L. and Furuta, I.
A role for IGF-1 in FSH mediated suppression of apoptosis in cultured rat ovarian follicles
Biol. Reprod. 1993 supplement 1 48:99

Tilly, J.L., Kowalski, K.I., Johnson, A.L. and Hseuh, A.J.
Involvement of apoptosis in ovarian follicular atresia and post-ovulatory regression
Endocrinology 1991 129: 2799-2801

Tilly, J.L.
Apoptosis and ovarian function
Rev. Reprod 1996 1: 162-172

Tilly, J.L.
When cells die: A comprehensive evaluation of apoptosis and programmed cell death. eds.
Lockshin, R.A., Zakeri, Z. and Tilly, J.L.
Wiley-Liss, New York 1998 431-452

Ueno, S., Kuroda, T., Maclaughlin, D.T., Ragin, R.C., Manganaro, T.F. and Donahoe, P.K.
Mullerian inhibiting substance in the adult rat ovary during various stages of the estrous cycle
Endocrinology 1989 125: 1060-1066

Uyttendaele, H., Marazzi, G., Wu, G., Yan, Q., Sassoon, D., and Kitajewski, J.
Notch 4 / int-3, a mammary proto- oncogene, is an endothelial cell-specific mammalian Notch gene.
Dev. 1996 122: 2251-2259

van Wezel, I.L. and Rodgers, R.J.
Morphological characterization of bovie primordial follicles and their environment in vivo
Biol. Reprod. 1996 55: 1003-1011

Vigne, J.-L., Halburnt, L.L. and Skinner, M.K.
Characterization of bovine ovarian surface epithelium and stromal cells: Identification of secreted proteins
Biol. Reprod. 1994 51: 1213-1221

Wallach, E.E., Virutamasen, P. and Wright, K.H.
Menstual cycle characteristics and side of ovulation in the rhesus monkey
Fertil. Steril. 1973 24: 715-721

Webb, R. and England, B.G.
Identification of the ovulatory follicle in the ewe: associated changes in the follicular size, thecal and granulosa cell luteinizing hormone receptors, antral fluid steroids, and circulating hormones during the preovulatory period.
Endocrinology 1982 110: 873-881

Werlin, L.B., Weckstein, L., Weathersbee, P.S., Parenicka, K., White, D. and Stone, S.C.
Ultrasound: a technique useful in determining the side of ovulation
Fertil. Steril. 1986 46: 814-817

Whitelaw, P.F., Smyth, C.D., Howles, C.M. and Hillier, S.G.
Cell-specific expression of aromatase and LH receptor mRNAs in the rat ovary
J. Mol. Endocrinol. 1992 9: 309-313

Wright, C.S., Becker, D.L. and Hardy, K.
Expression of Multiple Connexin Proteins in Mouse Ovarian Follicle Gap Junctions
J. Reprod. Fertil. 1997 Abstract Series 20: 34

Wyllie, A.H.
What is apoptosis?
Histopathology 1986 10: 995-998

Xu, T., Caron, L.A., Fehon, R.G. and Artavanis-Tsakonas, S.
The involvement of the Notch locus in Drosophila oogenesis
Development 1992 115: 913-922

Yuan, Y.P., Schultz, J., Mlodzik, M. and Bork, P.
Secreted Fringe-like signaling molecules may be glycosyltransferases
Cell 1997 88: 9-11

Yong, E.L., Baird, D.T., Yates, R., Reichert, L.E., Jr. and Hillier, S.G.
Hormonal regulation of the growth and steroidogenic function of human granulosa cells
J. Clin. Endocrinol. Metab. 1992 74: 842-849

Zamboni, L. and Merchant, H.
Interfollicular connections in the ovaries of immature mice. *The Development and Maturation of the Ovary and its Function.*
Exerpta Medica, Amsterdam 1973 53-54.

Zeleznik, A.N. and Hillier, S.G.
The role of gonadotrophins in the selection of the pre-ovulatory follicle
Clin. Obstet. Gyn. 1984 27: 927-940

Zeleznik, A.J., Schuler, H.M. and Reichert, L.E. Jr.
Gonadotropin-binding sites in the rhesus monkey ovary: Role of the vasculature in the selective distribution of human chorionic gonadotropin to the preovulatory follicle.
Endocrinology 1981 109: 356-362

Zhao, Y. and Luck, M.R.

Gene expression and protein distribution of collagen, fibronectin and laminin in bovine follicles and corpora lutea

J. Reprod. Fertil. 1995 104: 115-123

Appendix A. Suppliers Addresses

All chemicals used during this thesis investigation from **Sigma-Aldrich**, **Merck Ltd** or **Fisher Scientific UK**.

Acumedic Ltd London. U.K.

Apple MacIntosh Scotsys Computer Systems, Edinburgh, U.K.

Astecair Weston-Super-Mare, U.K.

B. Braun Melsungen AG. Aesculap, Sheffield, U.K.

Becton Dickinson and Co. New Jersey, USA.

BDH Supplies see Merck Ltd

Bibby Sterilin Ltd Aberbargoed, U.K.

Bio-Rad Hemel Hempstead, U.K.

Boehringer Mannheim see Roche

Corning Costar Cambridge, MA. USA

Dow Corning see Merck Ltd

Fisher Scientific UK Loughborough, U.K.

Forma Scientific Marietta, OH, USA

Gibco BRL Renfrew, U.K.

Iwaki see Bibby Sterilin Ltd

Leica UK Ltd Milton Keynes, U.K.

Linkam Tadworth, U.K.

Merck, Lutterworth, U.K.

Millipore Watford, U.K.

Pharmacia Biotech St. Albans, U.K.

Phoenix Pharmaceuticals Ltd

QIAgen Ltd Crawley, U.K.

Reichert-Jung see Leica UK Ltd

Roche Lewes, U.K.

Roebling Berlin, Germany

Sigma-Aldrich Company Ltd Poole, Dorset. U.K.

Sherwood-Davis and Geck Gosport, U.K.

TAAB Laboratories Ltd Aldermaston, U.K.

Vector Burlingame, CA, USA

Zeiss (Carl Zeiss Ltd.), Hertfordshire, U.K.

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Appendix B

a) Coating slides with gelatine for histology.

1g powdered gelatine
0.1g chromic potassium sulphate
200 ml H_2O

Mix and microwave ~30 seconds until the gelatine has dissolved. Slides can either be wiped with the solution or completely immersed before being left to dry.

b) TESPA coating slides for *in situ* hybridisation.

1) Wash slides in sulphur chromic acid over night.
2) Wash each slide individually in tap water.
3) Leave overnight in running tap water.
4) Rinse in ethanol (can be stored like this).
5) Bake overnight at 180°C.
6) Mix 8ml of TESPA (Sigma) with 392ml acetone (BDH).
7) Dip slides and allow to dry (~ 1 minute).
8) Dip slides in acetone and allow to dry. Repeat.
9) Rinse in ddH_20 (10 minutes) and air dry.

Appendix C Solutions required for wax *in situ* hybridisation

20 x P buffer

1M Tris HCL, pH 7.5
0.1M EDTA

20 x SSPE

3.6M NaCl
0.2M NaH_2PO_4
0.02M EDTA
(pH 7.4)

Hybridisation mix

5ml formamide
1ml 20 x SSPE
500µl 100 x Denhardts
2ml 50% dextran sulphate
200µl 10mg/ml tRNA
500µl 10% SDS
800µl depC H_2O

20 x SSC

3M NaCl
0.3M Na_3Citrate

10 x Salts

3M NaCl
0.1M Tris pH6.8
50mM EDTA
0.1M Na/PO_4

PBST

1 x PBS with 0.1% Tween 20

Alkaline Phosphatase buffer

3.75ml 4M NaCl
7.5ml 1M $MgCl_2$
7.5ml 2M Tris, pH9.5
150µl Tween 20
ddH_2O upto 150ml
15 drops levamisole

The role of mammalian neuro-genes in follicle development

Stuart J. Baker, Helen I. Cameron and Norah Spears

Throughout a female's reproductive life, a continual trickle of primordial follicles leave the ovarian resting pool to resume growth. Development continues unheeded until the follicles reach the early antral stage, at which time they become highly FSH sensitive. If FSH is sufficiently elevated to maintain growth, the follicles are selected to develop further: in the absence of this gonadotropic effect they will become atretic and die. The number of follicles that make it past this checkpoint is greater than the desired species-specific ovulatory number, so a second phase of selection occurs, known as follicular dominance. Recently, co-culture experiments from this laboratory have highlighted a contact-mediated dominance mechanism *in vitro,* leading to the idea that dominant follicles may be actively and directly involved in inducing atresia in neighbouring subordinates. This poses an interesting question: what is the nature of this follicle-follicle communication?

With this phenomenon in mind, we have begun to look for the involvement of mammalian homologues of genes regulating development via cell-cell contact in *Drosophila*. We have used RT-PCR and oligonucleotide probing of Southern blots and shown that a number of genes belonging or related to the *Notch* family are expressed in the ovary at different developmental stages. These genes are known to be vital in the establishment of boundaries and the divergence of cell/tissue fate. We hypothesise, therefore, that a dominant follicle might express certain members of this gene family resulting in the neighbouring follicle adopting a subordinate fate. Expression analysis by *in situ* hybridisation is currently under way. Although the role of these genes in follicle dominance is highly speculative, this expression analysis may give us a clearer insight to their roles within the ovary.

Follicle Stimulating Hormone inhibits apoptosis in pre- and early- antral murine follicles *in vitro*.

Stuart J Baker and Norah Spears, Department of Physiology, Edinburgh University Medical school, Teviot Place, Edinburgh. EH8 9AG

A whole follicle culture system was used to investigate the role of Follicle Stimulating Hormone (FSH) as a survival factor for pre- and early- antral murine follicles. Following microdissection from the ovaries of three week old mice, pre-antral follicles (~190 μm in diameter) were cultured after Boland *et al* (1993) in standard medium with serum and varying concentrations of FSH. Concentrations used ranged from 1 IU/ml, the standard level for optimal growth and oestradiol output, to 0.1 IU. Follicles were also cultured in the absence of FSH.
After 48 hours in culture, follicles were collected on dry ice (~ 8-10 per sample), and genomic DNA extracted and purified prior to 3' end-labelling with digoxygenin (DIG). Once labelled, samples were run by electrophoresis on a 2% agarose gel and Southern blotted over night. Visualisation of the resultant blot was conducted in accordance with the 'DIG users Handbook' protocol (Boehringer Mannheim).
DNA 'ladders', a well documented biochemical marker of apoptosis, and therefore atresia, were clearly evident in the samples collected from follicles cultured in low levels or the absence of FSH. Follicles cultured in FSH concentrations greater than 0.25 IU FSH exhibited little evidence of genomic DNA laddering. These results give support to the role of FSH as an important survival factor and back up previous findings in the rat (Chun *et al* 1996). We believe that this is the first time DIG has been used to visualise DNA ladders from such small sample sizes.

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The role of intra-ovarian interactions in the regulation of follicle dominance

S.J.Baker and N.Spears[1]

Department of Biomedical Sciences (Physiology), Edinburgh University Medical School, Teviot Place, Edinburgh EH8 9AG, UK

The processes that precisely control the selection of ovulatory follicles from a growing cohort are poorly understood. This reduction in follicle number occurs through several phases of selection, consequently we limit the use of the term 'selection' to the first major reduction of growing follicles, at the pre- to early antral stage. The final process of selection, achieving the appropriate ovulatory number, is referred to as 'dominance'. We discuss possible mechanisms that could bring about these reductions and highlight intra-ovarian involvement, particularly via follicle–follicle interactions. Analogies are drawn between local ovarian events and processes commonly reported in the determination of cell fate in developmental biology. Two facets of intra-follicular interactions are proposed: initially that follicle–follicle interactions mediate early selection processes at the preantral stage, and later that during antral development dominant follicles directly affect the fate of the subordinate cohort members.

Key words: cell fate/follicular dominance/follicle–follicle interactions/follicle selection/ovary

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Introduction

Follicle dominance

By or shortly after birth, the mammalian ovary contains a female's complete supply of oocytes or potential eggs. These oocytes (which are arrested in prophase of the first meiotic division) are housed within supporting cells to form primordial follicles. Throughout reproductive life, a small proportion of primordial follicles continually escape their arrested state and resume growth and development, in response to unknown cues (Baker, 1982). From this point onwards development continues until the oocyte reaches maturity and is ovulated or, more commonly, until the follicle becomes atretic. More than 99% of follicles entering the growing phase are destined to undergo an atretic fate, thus ensuring that only an appropriate species specific number will successfully ovulate (Gougeon, 1996). Correct regulation of this process is vital as this is the main way by which most mammals regulate their litter size (e.g. sheep: Hanrahan and Quirke, 1985).

Following recruitment from the primordial resting pool, a gonadotrophin independent process (Peters *et al.*, 1973), most follicles will develop at least until the acutely follicle stimulating hormone (FSH) dependent early antral stage (Figure 1). At that point, if FSH concentrations are low the follicles will undergo atresia (Hirshfield, 1991a). Conversely, if FSH concentrations are suitably elevated at that time (in response to the decline in oestrogen production from the regressing corpus luteum of the previous cycle: Le Nestour *et al.*, 1993), a certain number of follicles in the cohort will continue development to the later antral stages. This is probably the first process of selection which follicles undergo after leaving the resting pool and it is temporally regulated, i.e. if a follicle reaching the FSH-dependent stage finds itself in the 'window' when FSH is elevated it can proceed to the next developmental stage. Despite significantly reducing the number of contenders, the number of follicles in the cohort that continue to develop is greater than the desired ovulatory number, so a further process of reduction occurs. This is the second phase of selection, involving the emergence of dominant follicles among the growing cohort. Confusion frequently arises when making comparisons between multi- and mono-ovular species although the processes involved are broadly similar. The key difference is the extent of the final stage of selection:

[1]To whom correspondence should be addressed. E-mail: Norah.Spears@ed.ac.uk

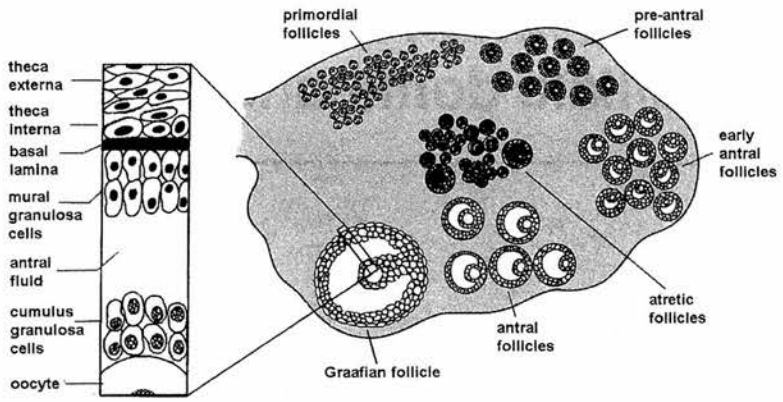

Figure 1. The different developmental stages of ovarian follicles. More than 99% of primordial follicles that resume growth and development will become atretic.

the emergence of the dominant follicle(s). In cattle the final selection process results in just one follicle attaining dominance in comparison with six to eight in mice. Henceforth we shall refer to this second selection process as follicular dominance. Dominant follicles continue to the final stages of development while the remaining subordinate follicles in the cohort ultimately undergo atresia and regress (Figure 2).

The emergence of dominant and subordinate follicles is the result of complex interplay between a range of factors, and our understanding of many of these is still poor. For the purpose of this review we shall define follicular dominance as having two principle components: indirect endocrine actions and direct intra-ovarian regulation. The latter can modulate endocrine regulation of dominance within a follicle (intra-follicular) or can initiate or exacerbate differences between follicles (inter-follicular). This review focuses mainly on effects of intra-ovarian follicle–follicle interactions, concentrating on both primate and murine species where data are available.

Endocrine regulation via the hypothalamic–pituitary system

This is the aspect of follicular dominance into which most research has been conducted to date. The larger follicles in a cohort indirectly cause the cessation of growth and development in subordinate members of the same cohort by releasing increasing concentrations of oestradiol and inhibin into the systemic circulation (Zeleznik and Hillier, 1984; Gibbons *et al.*, 1997). These act on the hypothalamic–pituitary system to decrease FSH to concentrations that will not support the continued growth and development of the highly FSH-dependent, less-developed subordinate follicles (Brown, 1978). The slightly more mature follicles that initiated the fall in FSH will withstand this decline in trophic support due to an up-regulation in functional LH receptors (Webb and England, 1982; Ireland and Roche, 1983) coupled to the aromatase systems in granulosa cells and a possible increase in FSH receptor concentrations (Ireland and Roche, 1983; Zeleznik and Hillier, 1984). The ovary is also the site of extremely high levels of angiogenesis (for recent review, see Redmer and Reynolds, 1996), and the dominant follicles acquire more vascular theca, perhaps due to an increase in basic fibroblast growth factor (bFGF), positively correlated with oestradiol concentrations (Schams *et al.*, 1996). This allows dominant follicles to obtain an increased uptake of serum gonadotrophins (Zeleznik *et al.*, 1981). The dominant follicles have, therefore, several mechanisms for sequestering more of the available gonadotrophins and surviving the decline in circulating FSH concentrations (Figure 2). In contrast, subordinate follicles are highly susceptible to a decline in circulating gonadotrophins: granulosa cells undergo apoptosis and follicular atresia results (Hughes and Gorospe, 1991; Hsueh *et al.*, 1994; Tilly, 1998).

Artificially increasing systemic FSH concentrations can result in greater-than-normal numbers of follicles reaching maturity and hence subsequent superovulation (Baird, 1987), a technique with enormous clinical, veterinary and agricultural applications. It seems likely that the FSH decline is the major endocrine selection mechanism by which the ovulatory quota is determined, with dominant follicles proceeding to ovulation and subordinate follicles being forced down the atretic pathway. However, we believe that this mechanism alone fails adequately to explain how the appropriate number of follicles first emerges as dominant.

Endocrine action between ovaries

Ovulation had been considered to occur from alternating ovaries in mono-ovular species since original observations by Rühl (1925), an opinion bolstered by appearances in seminal reproductive texts (e.g. Knobil and Neil, 1988). If this is the case, it could be due to a locally suppressive effect of the corpus luteum from the previous cycle, or alternatively it is possible that some as yet undefined communication mechanism is acting between the bilateral ovaries to regulate this

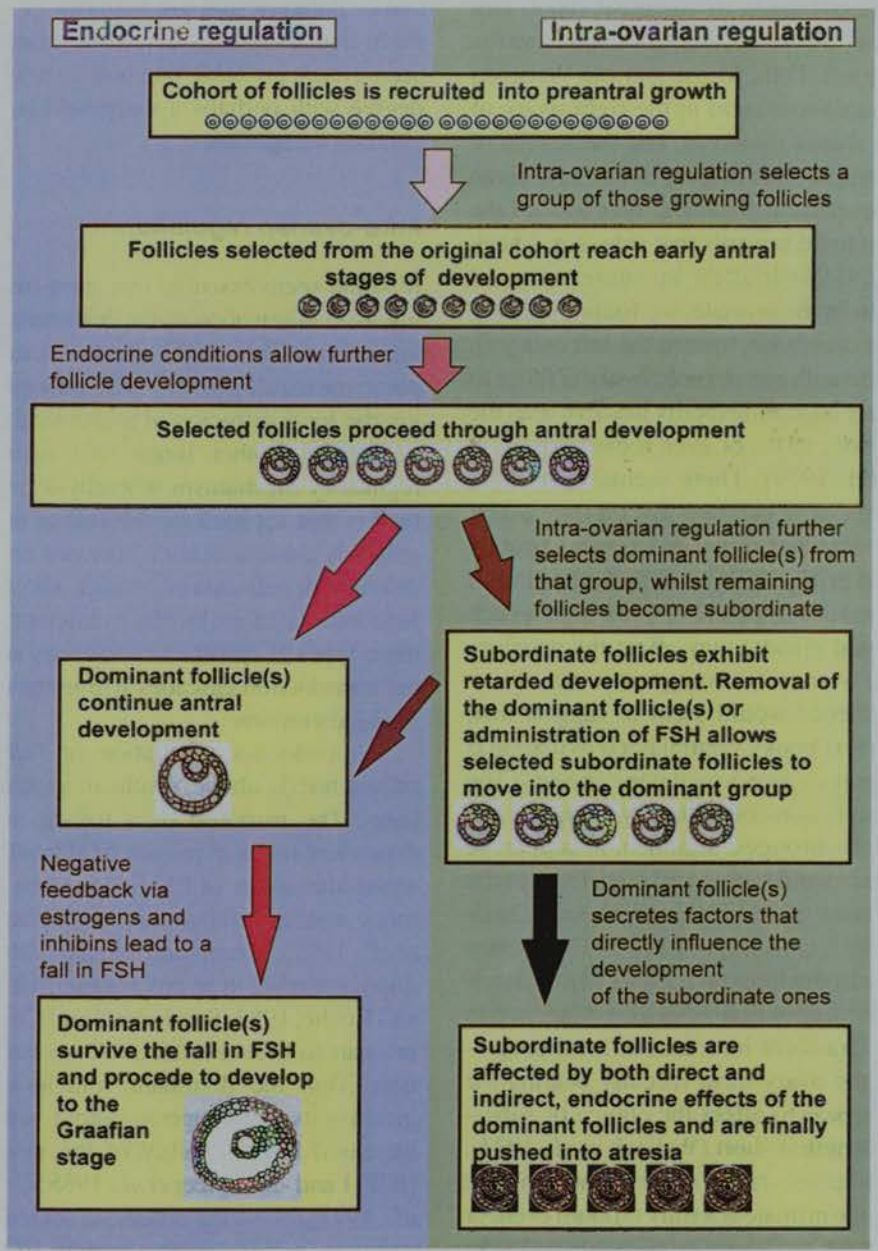

Figure 2. Both endocrine and intra-ovarian regulation are involved in the selection of ovulatory follicles. FSH = follicle stimulating hormone.

'turn-about' process. Similarly in multi-ovular species this ovary-to-ovary 'talk' could divide up the total number of ovulatory follicles ensuring that each uterine horn receives equal numbers of fertilized embryos. Evidence in the literature regarding consecutive ovulation sites is conflicting. Support for the contralateral theory comes from a histological study of ovaries obtained from 25 women by Gougeon and Lefèvre (1984) in which corpora lutea were identified and assigned an age on the basis of morphology. A chronological order of ovulations was then calculated which suggested that ovulation occurred in a turn-about manner. Other studies in the human (Marinho *et al.*, 1982) and non-human primate (Dukelow, 1977; Hodgen, 1982) also implicate contralateral ovulation. In contrast, Werlin *et al.* (1986) suggest that ipsilateral ovulations are the norm in women.

A third possibility is that selection of the ovary containing the dominant follicle is random. Considerable weight was lent to this hypothesis by the sonographic data of Check *et al.* (1991), due to its large sample size. Obtained from a study of 572 cycles in 92 women, they demonstrated a 52.4% incidence of ipsilateral ovulation and a 47.6% incidence of contralateral ovulation which is a non-significant difference. This study supported an earlier finding by Wallach *et al.* (1973) in the rhesus monkey. Doubt has also been cast on the

incidence of contralateral ovulation by the observation that ovulation in the primate appears to be more common from the right ovary, than from the left. Thus, Morse and van Wagenen (1936) report a bias towards ovulation in the right ovary of 60% in a study of eight rhesus monkeys, and the results of Potashnik *et al.* (1987) support this finding in humans. Even studies that do not demonstrate a statistical bias toward the right ovary report a slight trend towards that side (e.g. Check *et al.*, 1991; Fukuda *et al.*, 1996). In all the literature examined detailing side of ovulation in the primate, we found no trend, statistically significant or otherwise, toward the left ovary. In some species, the trend towards one or other ovary is taken to an extreme, with one ovary becoming totally inactive (e.g. the mountain viscacha; Pearson, 1949) or even regressing (as in the domestic hen, Gilbert, 1979). There seems to be little discussion as to why there should be a bias towards one ovary, although anatomical asymmetries possibly brought about by genes such as *Pitx2* (Ryan *et al.*, 1998), such as the origin and drainage of vasculature and development of other organs such as the kidneys (and adrenal glands) may affect the development and function of the ovaries, favouring one side.

The main body of evidence would seem to suggest that, despite what is written in text books, contralateral ovulation is not the physiological norm in mono-ovulatory species. The detailed histological examination conducted by Gougeon and Lefèvre (1984) presents the strongest argument in support of this proposal, although this would appear to conflict with the larger clinical investigations of Potashnik *et al.* and Check *et al.* (Potashnik *et al.*, 1987; Check *et al.*, 1991). Difficulties in accurately ageing the corpora lutea and consequently determining the sequence of ovulations may offer an explanation to these different findings. Transient increases in local progesterone concentration of the ovary most recently bearing the ovulatory follicle only appear to affect the choice of subsequent ovary when cycle length is short (Wallach *et al.*, 1973). It still remains unclear whether choice of ovary housing the next dominant follicle in the primate is a truly random event or if there is a bias towards the ipsilateral or right-handed side, the last two proposals being mutually compatible. Even less clear is an understanding of how dominance is established between ovaries if ovulation does not occur in a turn-about manner driven by the intra-ovarian environment. Interestingly from a clinical viewpoint, whether ovulation is from the contra- or ipsilateral ovary may have implications for subsequent oocyte retrieval, fertilization, cleavage and embryo transfer during assisted fertility treatments: Fukuda *et al.* demonstrated that the success rate of all these procedures was significantly higher if ovulation was on the contralateral side (Fukuda *et al.*, 1996). Whatever the mechanism(s) at work, extrapolating these findings to non-primate species, particularly to multi-ovulatory species, may be harder. The ability of embryos to migrate along the uterine horns in some species means that bilateral ovulation is not an absolute requirement for evenly distributed embryonic implantation. However, it seems unlikely that the majority of the oocytes released in multi-ovular species originate from one ovary. Instead it seems more probable that both ovaries contribute to a similar degree with perhaps a marginal bias towards one side, depending on species.

Intra-ovarian regulation

It would seem essential that intra-ovarian communication is involved in selection of the dominant follicle(s) from a growing cohort. This could occur via three possible pathways: paracrine regulation, the 'talk' between different cells, involving the local diffusion of a chemical messenger produced in one cell to another 'target' cell; autocrine regulation, a self-regulatory mechanism whereby a certain cell type produces factors that act back on the cell of origin; and juxtacrine regulation, communication between cells as the result of direct cell–cell or cell-matrix contact, allowing cell- or matrix surface-associated molecules to interact with one another. All of these types of communication may subsequently lead to signal transduction cascades within the cell, giving rise to functional alterations.

The endocrine regulation of follicular dominance, discussed briefly above, results in a lowering of FSH concentrations. The response of a follicle to that drop in FSH is dependent on its dominant or subordinate status, with differential alterations in FSH-dependent growth factor and hormone concentrations directing ultimate follicle fate (Mihm *et al.*, 1997). Dominant follicles continue to grow and produce oestradiol in an environment of decreased FSH (Ireland and Roche, 1983; Sunderland and Crowe, 1994) whereas subordinate follicles exhibit markedly reduced oestradiol production. That the dominant follicle continues to grow and increase its steroidogenic output is thought to be due to the increased bioavailability of the insulin-like growth factors (IGF-1 and -2) (Spicer *et al.*, 1988; Gong *et al.*, 1993; Mihm *et al.*, 1997) following enhanced secretion of this peptide and a decrease in IGF binding protein (IGFBP) production (Echternkamp *et al.*, 1994). During this period of selection, the subordinate follicles exhibit increased IGFBP production reducing the concentration of available IGF-1 (Mihm *et al.*, 1997). As a result of these differential responses to the reduced concentrations of FSH, the dominant follicles continue to grow and develop whilst the subordinate follicles undergo follicular atresia and die. The role of such intra-follicular factors on follicle dominance has been the subject of several comprehensive reviews, (e.g. Adashi and Rohan, 1992; Erickson and Danforth, 1995; Campbell and McNeilly, 1996; Armstrong and Webb, 1997).

There is, however, an additional method of intra-ovarian regulation of follicular dominance, namely through interactions between follicles. Such interactions could enhance the effect of endocrine regulation of follicular dominance, with the dominant follicle also producing factors that will directly

affect the development of subordinate ones. For example, interfollicular interactions may have a role in maintaining dominance once it has been established, by 'holding back' challengers. This could explain the phenomenon of follicular waves exhibited by some species. If the dominant follicle found itself in a hostile environment upon reaching the ovulatory stage and consequently regressed, the inhibitory influence would be removed and the follicles that had been held in check could resume development and contend for the dominant position (Matton *et al.*, 1981; Ko *et al.*, 1991). Alternatively, follicle–follicle interactions could initiate differences between follicles upon which endocrine action can subsequently act. These possibilities are explored more fully later.

General morphology of the follicle

Follicle structure

The majority of follicles found within the ovary are in the primordial stage (mouse: Peters *et al.*, 1973; human: Forabosco *et al.*, 1991). These follicles consist of an oocyte arrested in prophase I of the first meiotic division, surrounded by flattened pregranulosa cells (Hirshfield, 1991a), and a basal lamina. In the young mouse these follicles are found in closely packed clusters, at synchronized stages of development and frequently connected to each other by interfollicular bridges (Zamboni and Merchant, 1973). The oocytes in these follicles are ~15 μm in diameter in the mouse and 30 μm in humans (Gosden and Telfer, 1987). Thecal cells are generally regarded as being indistinguishable until the follicle attains a multilaminar stage. A steady trickle of primordial follicles becomes activated and leaves the resting pool, forming primary follicles (Figure 1). Due to the avascular nature of the part of the ovary in which the primordial follicles are located, locally produced growth factors are more likely to regulate this process than systemic factors (Greenwald and Terranova, 1988; Hirshfield, 1991a; van Wezel and Rodgers, 1996). During preantral development, the oocyte enlarges and the zona pellucida is formed between the oocyte and the granulosa cells. The granulosa cells become cuboidal and are the site of rapid synthesis of matrix components, including the basal laminae. As the follicle continues its growth it acquires a fluid-filled antral cavity. Antral formation begins when the granulosa cell population reaches ~2000 cells for all species studied (Gosden *et al.*, 1993). It is during antral development that granulosa cells differentiate to form two major populations, the mural granulosa cells which are proximal to the basal lamina, and the cumulus granulosa cells which surround the oocyte (cells in the stalk may possess characteristics of both). The granulosa cells regulate oocyte development, for example, 85% of oocyte metabolites are of granulosa cell origin (Heller *et al.*, 1981). Conversely the mural granulosa cells are the recipients of instruction from the oocyte, e.g. growth differentiation factor-9 (GDF-9), an oocyte secreted factor that is involved in granulosa cell development (Dong *et al.*, 1996).

The mature follicle also has many associated thecal cells. The highly vascularized theca interna, the layer most closely associated with the convex surface of the basal lamina, is readily identifiable, the cells containing prominent lipid droplets and being more rounded than the theca externa (O'Shea, 1971). Likewise, there is a marked change in extracellular matrix composition at the interface between the interna and externa.

The ovarian extracellular matrix (ECM)

In the ovary, as in all other tissues, the ECM provides the architectural framework that supports and compartmentalizes the different cell types (Alberts *et al.*, 1994). Increasingly, research is highlighting the additional role of the ECM in regulating cell behaviour in all aspects of development and maintenance. This seems to be particularly true of the ovary, a highly dynamic organ which exhibits rapid tissue remodelling throughout reproductive life (Luck, 1994). An illustration of this is provided by the ECM found in the follicular theca. This contains laminin and collagen fibrils types I, III and IV (reviewed by Luck, 1994) and fibronectin in some species (e.g. sheep: Huet *et al.*, 1997; rat: Bagavandos *et al.*, 1983). This ECM used to be thought of as primarily supportive, but is now also considered to act as a binding and storage site for many factors that regulate the growth, development and function of follicular cells (Armstrong and Webb, 1997; McIntush and Smith, 1998).

Extracellular proteases

ECM is constantly remodelled by the action of extracellular proteases, mainly matrix metalloproteinases (MMP) (such as collagenase and gelatinase) and the plasminogen activator/plasmin family (Luck, 1994; McIntush and Smith, 1998). Around the follicles, degradation of the ECM results in release of sequestered ovarian factors. Inhibition of these proteases, such as through the action of TIMP (tissue inhibitors of metalloproteinases), maintains the ECM and hence favours retention of growth factors and cytokines. This site of storage and release is a fundamental control mechanism of follicle development.

Intra-ovarian interactions help determine follicle fate

We consider that selection of the correct number of follicles for ovulation cannot easily be achieved by endocrine mechanisms alone. Variations in systemic concentrations of follicular trophic factors do not seem subtle enough, or targeted in any way, making it hard to envisage how they could account for such precise and regimented control of ovulatory number. If this is the case, it seems essential that intra-ovarian factors

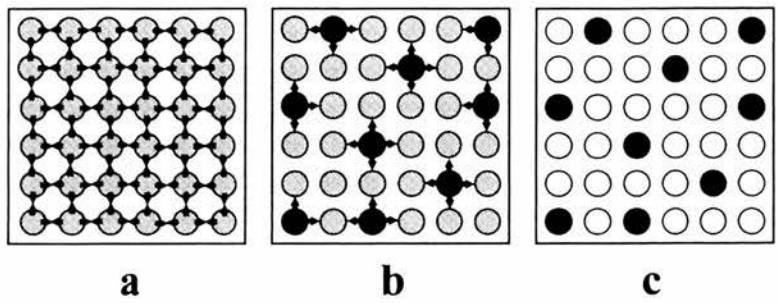

Figure 3. Lateral specification. (**a**) An initially equivalent population of cells all possessing the ability to differentiate and simultaneously send an inhibitory signal to the neighbouring cells. (**b**) A few cells have been able to differentiate to a greater degree than their neighbours. They produce a stronger inhibitory signal preventing the further differentiation of the surrounding cells which in turn lose the ability to inhibit the dominant cell. (**c**) Two distinct cell types have arisen from an initially equipotential population. A regular 'pattern' has been generated.

play a role in regulating the development of the correct number of follicles. These factors could be acting in two ways, predisposing certain follicles for successful growth culminating in ovulation and/or condemning the unsuccessful contenders to an atretic pathway (Figure 2). Once follicle dominance has been established, intra-ovarian factors could also 'hold back' any challengers.

Such follicle–follicle interactions could either establish differences between a group of initially equivalent follicles or, at a later stage, allow a follicle to influence the fate of another non-equivalent (subordinate) follicle group. These processes bear striking resemblance to methods of cell fate determination commonly described in developmental biology, namely those of inductive signalling and of lateral specification. We discuss below whether they may indeed be analogous, citing examples to draw parallels, and consider the use of these terms in ovarian physiology.

Inductive signalling and lateral specification

Inductive signalling is a method of communication between adjacent, non-equivalent cell populations, whereby one cell type influences the fate of another, and can thus generate new cell types. Lateral specification (also called lateral inhibition) is the short-range cell–cell 'talk' between initially equivalent cells, an example of juxtacrine communication. This cell–cell dialogue may give rise to signal transduction cascades within the cells causing functional alterations. Thus, from an initially equivalent and equipotent group of cells, interactions between these cells lead to the generation of two distinct cell fates.

Examples of inductive signalling can be found in many developing systems in a diverse range of organisms that includes plants, invertebrates and mammals. One example recently reviewed by Horster *et al.* describes the cell–cell interactions that bring about the formation of the mammalian metanephric kidney (Horster *et al.*, 1997). Two types of tissue with distinct embryological origins, the metanephric mesenchymal blastema and the ureteric bud, come into contact with one another at the site of the future kidney. The mesenchymal cells aggregate around the branching ureteric bud tip, allowing the two cell types to communicate with each other via inductive signalling. The signalling between the different cell populations is successful as the two cell types express ligands and receptors in a complementary pattern (Birchmeier and Birchmeier, 1993). Interactions with the ureteric bud tip cause the mesenchymal cells in contact with the ureteric bud to adopt epithelial morphology and function. These newly created epithelial cells subsequently differentiate into the variety of cell populations that comprise the nephron.

Lateral specification is involved in the patterning of differentiated cell types. An often-cited example of lateral specification can be found in the developing *Drosophila* bristles where evenly spaced sensory mother cells are created from an initial population of equivalent proneural ectodermal cells. These cells would all differentiate to become sensory mother cells unless prevented from doing so. As the ectodermal cells begin down the pathway to sensory differentiation they send an inhibitory signal to their neighbours. A 'battle' is then fought as each cell attempts to suppress the differentiation of the adjacent cells and gain a slight developmental edge, allowing it to escape the inhibitory influence. Consequently this eminence is enhanced as the cell becomes more differentiated and produces a stronger inhibitory signal, preventing the contacting cells both from becoming sensory mothers and from producing an inhibitory signal (Figure 3). These 'weak' cells consequently develop into epidermal cells (Hartenstein and Posakony, 1990; Heitzler and Simpson, 1991).

Some of the genes regulating local cell interactions necessary for inductive signalling and lateral specification are known, more so for the latter process. The first group of regulatory genes to be isolated, in *Drosophila*, were initially implicated in the regulation of lateral specification. These encode for the Notch family of transmembrane receptor proteins. Signalling via the Notch receptor can control the ability of non-differentiated cells to respond to differentiation and proliferation cues, and is able to block the action of other differentiation signals. The Notch receptor contains a large

extracellular domain that contains 36 epidermal growth factor (EGF)-like repeats and three cysteine-rich Notch/Lin-12 repeats (reviewed by Artavanis-Tsakonas *et al.*, 1995). These receptors have an array of possible ligands, including membrane-anchored extracellular ligands (each with EGF-like repeats) such as Delta and Serrate and those implicated in intracellular signalling, such as Deltex or Suppressor of Hairless (SuH). Some downstream genes (or gene cascades) have also been identified. *Notch* was first discovered due to its role in neurogenic cell fate in the developing *Drosophila*, an example of which is given above. It has since been shown to play a key role in both inductive signalling and lateral specification in the developing *Drosophila*: null mutations result in embryonic lethality. Homologues have been found in *C.elegans* and in non-mammalian and mammalian vertebrates, including mice and humans (reviewed by Artavanis-Tsakonas *et al.*, 1995). Their expression in the mammalian ovary is described below. *Notch* mutations in humans have been linked to cancer (Ellisen *et al.*, 1991; Robbins *et al.*, 1992), and one of its human ligands, *Jagged*, has been implicated in Alagile syndrome, an autosomal dominance disorder with a range of developmental abnormalities (Li *et al.*, 1997; Oda *et al.*, 1997).

'Inductive signalling' and follicular dominance

The traditional view of interfollicular regulation of follicular dominance is that an already dominant follicle secretes some factor(s) that inhibits the development of its subordinate neighbours. As this is one population of cells affecting the fate of another, non-equivalent population of cells, it is analogous to inductive signalling, and the factors produced by the dominant follicles can be thought of as 'inductive signalling' molecules.

Several putative 'dominance' factors have been reported. Di Zerega identified a protein with a molecular weight between 14 000 and 18 000 kDa secreted by the dominant follicle in humans which suppressed the follicular response to gonadotrophins (di Zerega, 1982). Cahill *et al.* demonstrated that ovine follicular fluid inhibited the development of follicles >2 mm in diameter in the ovary and reduced the mitotic index of the granulosa cells of follicles <2 mm (Cahill *et al.*, 1985). Later, substances with molecular weights of 18 000 and <10 000 kDa were identified in ovine follicular fluid that inhibited the mitotic activity of murine embryonic lung fibroblasts (Carson *et al.*, 1988). Campbell *et al.* reported a substance in the ovine pre-ovulatory follicle that is atresia-inducing and results in a loss of steroidogenic activity following injection into the cycling sheep (Campbell *et al.*, 1991). How a follicular fluid-derived factor would act on neighbouring follicles was called into question when Driancourt (Driancourt, 1994) failed to demonstrate interfollicular interactions in the Booroola sheep. Using aromatase activity as a measure of follicle development, no positive role of the largest atretic follicle or negative role of the dominant follicle was found on other follicles following his experiments using ovarian serum or conditioned media. However, he substantiated the previous literature reporting the presence of an inhibitory factor in the follicular fluid, which was shown to significantly reduce the activity of aromatase in large follicle pieces, as compared to cultures with serum or conditioned medium. Perhaps then his failure to observe either an inhibitory or stimulatory effect was an in-vitro artefact, the mechanisms by which the follicular fluid factors are transported from the antrum being absent or disabled *in vitro*. Another possibility is that such factors were indeed present but not at sufficient concentrations in culture to exert an effect.

Most recently, work on the bovine follicle resulted in the characterization of granulosa cell-inhibitory factor (GCIF) which was shown to inhibit the proliferation of small and medium follicles (Hynes *et al.*, 1996a,b). Steroid-free bovine follicular fluid was separated into high and low molecular weight fractions and purified. A factor with a molecular weight <5 kDa was shown to inhibit granulosa cell proliferation *in vitro*, inhibit the proliferation of granulosa cells taken from small and medium follicles but not large follicles, and, following systemic administration to cycling rats, inhibit the formation of large follicles and increase the number of small follicles. The authors report similarities between GCIF and factors found in porcine (Kigawa *et al.*, 1986) and rat follicular fluid (granulosa cell mitostatic protein, GCMP; Chakravorty *et al.*, 1993). Gore *et al.* note the disappearance of 'challenger' follicles from around dominant follicles in humans and speculate that oestrogen may be responsible for this phenomenon (Gore *et al.*, 1997). They cite the studies of Dierchke *et al.* (1985) and Koering *et al.* (1994) which demonstrated a detrimental role of oestrogen on follicles *in vivo*.

'Lateral specification' and early determination of follicular fate

While a process analogous to that of inductive signalling can help explain how follicle dominance is maintained once established, it does not address the issue of how dominance has arisen (with the correct, species-specific number of follicles continuing to develop). Based on morphological criteria, the emergence of dominant and subordinate follicles would seem to arise from a cohort of initially equipotential follicles. Although we cannot discount the possibility that differences are established at the time of gonadal formation, marking the follicles destined to ovulate, this must at the very least be a readily reversible designation, as the number of ovulating follicles can be manipulated with ease. Experiments where the dominant follicle is ablated (e.g. Matton *et al.*, 1981; Ko *et al.*, 1991) show that a new dominant follicle rapidly emerges from the cohort of antral follicles. Presumably, had the original dominant follicle been allowed to ovulate, the 'new' replacement dominant follicle would have become atretic. Similarly,

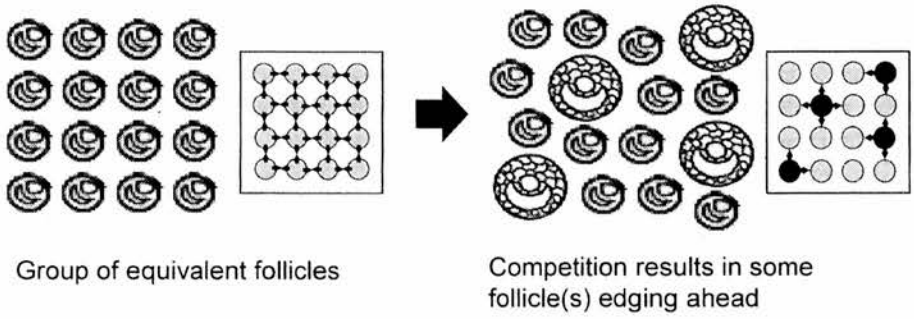

Figure 4. Follicles are selected for continued growth from a larger cluster of preantral follicles in a manner analogous to lateral specification.

superovulation (e.g. Baird, 1987) results in a far greater number of follicles than would be expected in a normal cycle, suggesting that at least some, if not the majority, of follicles have been deflected from an atretic fate. We propose that differences in follicle development may have arisen through a process early in follicle development equivalent to lateral specification.

At the onset of follicle development, a cohort of primordial follicles enters the growth phase. At this point they are most likely equipotential. As they start to grow, there are presumably fluctuations in the production of signalling molecules setting up transient differences between neighbouring follicles. These differences could become magnified as selected 'stronger' follicles inhibit development of their immediate neighbours (similar to the differentiation of cell types in the developing *Drosophila*, as detailed previously). In this manner, a pattern of selected and non-selected follicles, or later, of dominant and subordinate follicles, would emerge (Figure 4): the endocrine loop would then act on those differences.

Recent work from this laboratory has highlighted a contact-mediated mechanism whereby 'dominance' is established between co-cultured murine follicles *in vitro* (Spears *et al.*, 1996), although this may be more analogous to the process of selection *in vivo*, particularly in large mammals. Using a whole follicle culture system which allows the growth of follicles from the preantral to the Graafian stage, experiments were conducted to investigate the influence of follicle–follicle interactions on growth and development. It was found that when pairs of follicles were co-cultured in contact, one follicle invariably became dominant over its partner. When follicles were cultured in similar conditions but placed slightly apart, this phenomenon was not seen, implying a requirement for follicle–follicle contact. We believe that this observation may be an example of 'lateral specification' between neighbouring follicles, resulting in the initially equivalent follicles adopting different fates and only the successful follicle being selected for further development and maturation (Figure 5a,b).

For our in-vitro observations and proposed hypothesis to be relevant *in vivo*, there would be a requirement for follicles to be in direct contact with others at the same stage for at least part of their development. Histological examination of sections from a range of mammalian species (including the mouse, rat, rabbit, cat, pig, tiger and marmoset) reveals that preantral follicles are frequently found in close contact with each other, as shown in some of the examples in Figure 5c–e. Zamboni and Merchant report that bi- and tri-laminar follicles are found interconnected by granulosa cell projections in young mice (Zamboni and Merchant, 1973), presumably as a result of persisting intercellular bridges between primordial germ cells. Connected 'strings' of primordial/primary follicles can be seen in mice ~3 weeks of age (S.Baker and N.Spears, unpublished observation), and in young cats (J.Mullan, personal communication; Figure 5f). It would be interesting to see if these 'strings' of follicles resume growth as a unit, giving rise to closely contacting preantral follicles at equivalent stages of development. Whole ovary sections illustrate the degree of closeness that neighbouring preantral follicles assume, often with a very thin dividing thecal layer (Figure 5c–e), which would enhance the possibility of juxtacrine communication. Interestingly, this thin shared theca layer was also observed in our co-cultured follicles (Figure 5b). Detailed analyses of serial sections from 3-week-old mice revealed that most preantral/early antral follicles are found in clusters (of up to 20–50 follicles), frequently in contact with two or more like-sized follicles (Figure 6). Similar clusters of like-sized preantral follicles have also been described in the rat (Hirshfield and De Santi, 1995). It is thus clear that preantral follicles do develop in contact with other similar-sized follicles, making it at least feasible that 'lateral specification' (or an equivalent process) could influence follicular fate, resulting in only certain follicles from within each cluster proceeding on to further stages of follicular development.

As discussed previously, the *Notch* family of genes is known to be involved in juxtacrine communication between cell types in many developing systems. As such, they seem plausible candidates as factors regulating follicle selection. Indeed, *Notch* has been shown to play vital roles in the *Drosophila* ovary (Xu *et al.*, 1992) including evenly spacing the developing egg chambers. There are four mammalian *Notch* genes identified to date (*Notch* 1–4). Although *Notch* 4 has

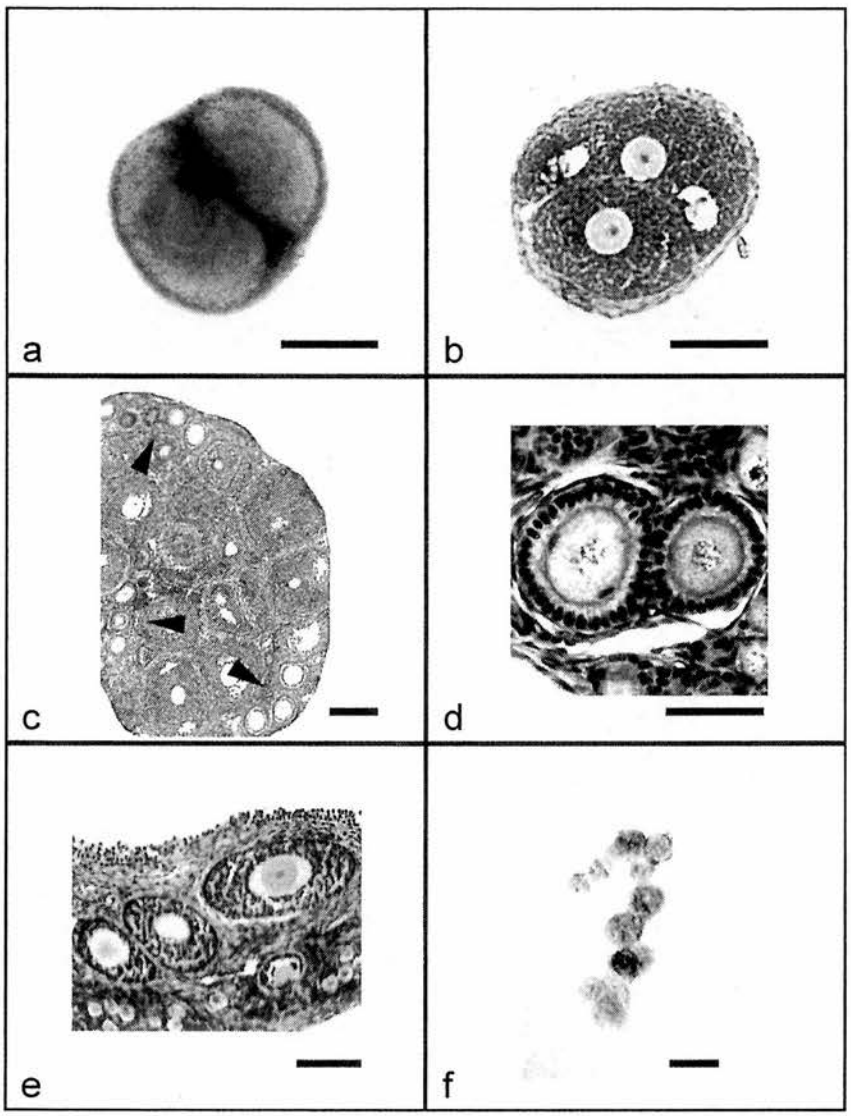

Figure 5. Preantral follicles are frequently found in close contact with one another, with similar characteristics to co-cultured follicles. (**a**) Photomicrograph of a pair of co-cultured murine follicles after 48 h in culture. (**b**) Photomicrograph of a histological section of co-cultured follicles showing a thin, shared theca layer (follicles were embedded in resin and sections stained with haematoxylin and eosin). (**c**) Photomicrograph of a section through a 3 week old mouse ovary showing that preantral follicles tend to grow in clusters (arrowheads). (**d**) Photomicrograph of contacting preantral follicles in a kitten ovary. (**e**) Photomicrograph of closely contacting preantral follicles in a marmoset ovary. (**c–e** were obtained from wax sections of ovaries and stained with haematoxylin and eosin.) (**f**) Photomicrograph of a connecting 'string' of primary follicles dissected from a young cat ovary. All scale bars represent 100 μm except (**d**) which represents 50 μm.

been reported to be expressed in the mouse ovary (Uyttendaele, *et al.*, 1996), there are no previously published reports of expression of *Notch 1–3* in the mammalian ovary. As a first step towards examining the role of *Notch* genes in the mammalian ovary, we have used reverse transcriptase–polymerase chain reaction to examine expression of Notch 1–3 in the mouse ovary (Figure 7). All three genes are expressed in the ovary and we are currently examining their expression patterns with a view to possible involvement in follicle fate (Baker, Cameron and Spears, manuscript in preparation).

Inductive signalling, lateral specification and interfollicular interactions

The major differences between lateral specification and inductive signalling in developing systems and the processes that are occurring in the ovary are those of scale and maturity. Lateral specification has been described to date only as occurring between individual cells and inductive signalling between populations of cells. Each follicle will have several hundred or even several thousand cells. For equivalent pro-

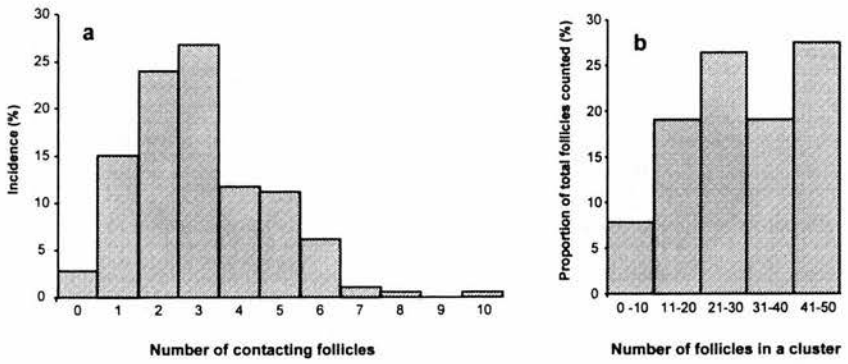

Figure 6. Serial sections of ovaries from three 3 week old mice were examined sequentially and all follicles around the stage of antrum formation were identified and marked. For each highlighted follicle, numbers of contacting similarly sized follicles were noted. It was found that: (**a**) >95% of late preantral follicles are in direct contact with other like-sized follicles; (**b**) calculations of total cluster size reveal that >90% of late pre-antral follicles are found in groups of 11–50 follicles.

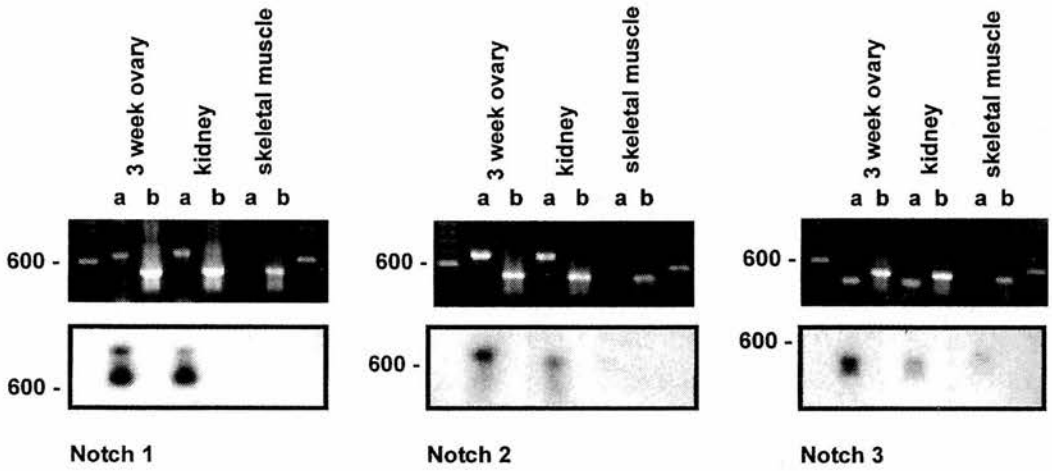

Figure 7. Reverse transcriptase–polymerase chain reaction (PCR) gels (upper row) and probed Southern blots (lower row) showing the expresseion of *Notch 1, 2* and *3* in 3-week-old murine ovaries, kidney and skeletal muscle. Band **a** has been produced using primer pairs specific for each gene's mRNA sequence, band **b** shows expression of β-actin message in each tissue. mRNA was extracted from the three tissue types and cDNA synthesized using Pharmacia Biotech kits (St Albans, UK), prior to amplification by PCR using specific primers for *Notch 1, 2* and *3* mRNA (as in Lardelli and Lendahl, 1993). PCR products were run by electrophoresis and visualized under UV light following staining with ethidium bromide. *Notch 1* primers yielded a 660 bp product; *Notch 2* a 684 bp product and *Notch 3* a 466 bp product. To check for specificity gels were then blotted overnight onto nylon membranes, transferred DNA fixed by baking and then hybridized with digoxigenin (DIG)-labelled oligonucleotide probes specific to the gene of interest (*Notch 1*: CTGGCCACACTGGACGC; *Notch 2*: CAAGGCTCGGGA; *Notch 3*: CCATGCAGCGCATACTC), according to the DIG users Handbook (Boehringer Mannheim, Lewes, UK). Final detection of hybridized probe was by reaction to CDP-Star (Boehringer Mannheim) and subsequent exposure to X-ray film.

cesses to be viable between adjacent follicles there would be a requirement for many thousands of cells to act in concert and present a unified front towards the neighbouring follicle. The oocyte has a profound effect on the somatic follicular cells (e.g. Dong *et al.*, 1996), so it is possible that the process could be oocyte-driven. Thus despite being comprised of thousands of cells, the follicle could behave in a manner analogous to that of an individual cell. Furthermore, the population of granulosa cells arises from a small number of progenitors in the primordial follicle (Boland and Gosden, 1994). Recent work by Hirshfield (Hirshfield, 1991b) has even suggested that the theca cell population may have arisen from a few follicle-associated interstitial cells. If populations have arisen from few progenitor cells, that syncytium of cells—all being of similar characteristics—could act in concert.

The other key dissimilarity between our proposed follicle–follicle communication mechanisms and the classical examples of inductive signalling and lateral specification is

the age of the animal. We are suggesting that this form of cell fate determination is present in the ovary throughout reproductive life, whereas examples of inductive signalling and lateral specification in the literature appear to be confined to developing embryos. However, the mature ovary can be regarded as being in a constant state of development. As previously mentioned, the ovary is the site of the most rapid angiogenesis and apoptosis in the female body, processes normally associated with development. Growth and development of follicles continues and indeed only recommences fully in adult life.

We are only beginning to understand aspects of the molecular regulation of follicle dominance, while regulation of inductive signalling and lateral specification during embryonic development is fairly well understood. If analogies between the systems are close, it would be productive to look for further parallels, at the molecular/genetic level: such an approach has proved invaluable in many other areas of biology.

Conclusion

To date, most of the research into follicular dominance has concentrated on its endocrine regulation. While endocrine control of follicle dominance can explain much of the later processes that occur (such as ensuring that subordinate follicles ultimately undergo atresia), intra-ovarian interactions are also involved in its regulation. Follicle–follicle interactions have various possible roles both at early stages when follicles are being selected from a cohort or cluster of follicles and/or later, when dominant follicles are 'holding back' challengers (Figure 2). Furthermore, intrafollicular processes also mediate the response of a follicle to endocrine changes. These intra-ovarian processes have received less attention than endocrine changes, perhaps because they are less amenable to investigation. Further understanding of intra-ovarian interactions will help us to determine how each species selects the correct number of follicles for continued development during an ovulatory cycle.

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References

Adashi, E.Y. and Rohan, R.M. (1992) Intraovarian regulation: Peptidergic signalling systems. *J. Steroid Biochem. Mol. Biol.*, **3**, 243–248.

Alberts, A., Bray, D., Lewis, J. *et al.* (1994) *Molecular Biology of the Cell*, 3rd edn. Garland Publishing, New York.

Armstrong, D.T. and Webb, R. (1997) Ovarian follicle dominance: the role of intraovarian growth factors and novel proteins. *Rev. Reprod.*, **2**, 139–146.

Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M.E. (1995) Notch signalling. *Science*, **268**, 225–232.

Baker, T.G. (1982) In Austin, C.R. and Short, R.V. (eds), *Oogenesis and Ovulation. Reproduction in Mammals*, 2nd edn. Cambridge University Press, Cambridge, pp. 17–45.

Bagavandos, P., Midgley, A.R. and Wicha, M. (1983) Development changes in the ovarian follicular basal lamina detected by immunofluorescence and electron microscopy. *J. Histochem. Cytochem.*, **31**, 633–640.

Baird, D.T. (1987) A model for follicular selection and ovulation: lessons from superovulation. *J. Steroid. Biochem.*, **27**, 15–23.

Birchmeier, C. and Birchmeier, W. (1993) Molecular aspects of mesenchymal–epithelial interactions. *Annu. Rev. Cell. Biol.*, **9**, 511–540.

Boland, N.I. and Gosden, R.G. (1994) Clonal analysis of chimeric mouse ovaries using DNA *in situ* hybridisation. *J. Reprod. Fertil.*, **100**, 203–210.

Brown, J.B. (1978) Pituitary control of ovarian function—concepts derived from gonadotrophin therapy. *Aust. NZ Obstet. Gynaecol.*, **18**, 47–54.

Cahill, L.P., Driancourt, M.A., Chamely, W.A. *et al.* (1985) Role of intrafollicular regulators and FSH in growth and development of large antral follicles in sheep. *J. Reprod. Fertil.*, **75**, 599–607.

Campbell, B.K. and McNeilly, A.S. (1996) Follicular dominance and oocyte maturation. *Zygote*, **4**, 327–334.

Campbell, B.K., Picton, H.M., Mann, G.E. *et al.* (1991) Effect of steroid- and inhibin-free ovine follicular fluid on ovarian follicles and ovarian hormone secretion. *J. Reprod. Fertil.*, **93**, 81–96.

Carson, R.S., Robertson, D.M. and Findlay, J.K. (1988) Ovine follicular fluid inhibits thymidine incorporation by 3T3 fibroblasts *in vitro*. *J. Reprod. Fertil.*, **82**, 447–455.

Chakravorty, A., Mahesh, U.B. and Mills, T.M. (1993) Control of peptides regulating mitosis of granulosa cells in immature rat ovary by oestrogen and gonadotrophin. *J. Reprod. Fertil.*, **97**, 91–100.

Check, J.H., Dietterich, C. and Houck, M.A. (1991) Ipsilateral versus contralateral ovary selection of dominant follicle in succeeding cycle. *Obstet. Gynecol.*, **77**, 247–249.

di Zerega, G.S., Goebelsmann, U. and Nakamura, R.M. (1982) Identification of protein(s) secreted by the preovulatory ovary which suppresses the follicle response to gonadotropins. *J. Clin. Endocrinol. Metab.*, **54**, 1091–1096.

Dierschke, D.J., Hutz, R.J. and Wolf, R.C. (1985) Induced follicular atresia in rhesus monkeys: strength–duration relationships of the estrogen stimulus. *Endocrinology*, **117**, 1397–1403.

Dong, J., Albertini, D.F., Nishimori, K. *et al.* (1996) Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature*, **383**, 531–535.

Driancourt, M.A. (1994) Lack of between follicle interactions in the sheep ovary. *Reprod. Nutr. Dev.*, **34**, 249–260.

Dukelow, W.R. (1977) Ovulatory cycle characteristics of Macaca fasicularis. *J. Med. Primatol.*, **6**, 33–42.

Echternkamp, S.E., Howard, H.J., Roberts, A.J. *et al.* (1994) Relationships amongst concentrations of steroids, insulin-like growth factor-1, and insulin-like growth factor binding proteins in ovarian follicular fluid of beef cattle. *Biol. Reprod.*, **51**, 971–981.

Ellisen, L.W., Bird, J., West, D.C. *et al.* (1991) TAN-1, the human homolog of the *Drosophila* notch gene is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell*, **66**, 649–661.

Erickson, G.F. and Danforth, D.R. (1995) Ovarian control of follicle development. *Am. J. Obstet. Gynecol.*, **172**, 736–747.

Forabosco, A., Sforza, C., De Pol, A. *et al.* (1991) Morphogenic study of the human neonatal ovary. *Anat. Rec.*, **231**, 201–208.

Fukuda, M., Fukuda, K., Andersen, C.Y. *et al.* (1996) Contralateral selection of dominant follicle favours pre-embryo development. *Hum. Reprod.*, **11**, 1958–1962.

Gibbons, J.R., Wiltbank, M.C. and Ginther, O.J. (1997) Functional interrelationships between follicles greater than 4mm and the FSH surge in heifers. *Biol. Reprod.*, **57**, 1066–1073.

Gilbert, A.B. (1979) Female genital organs. In King, A.S. and McLelland, J. (eds), *Form and Function in Birds*. Academic Press, London, vol. 1, pp. 237–360.

Gong, J.G., McBride, D., Bramley, T.A. *et al.* (1993) Effect of recombinant bovine somatotropin, insulin-like growth factor-1 and insulin on the proliferation of bovine granulosa cells *in vitro*. *J. Endocrinol.*, **139**, 67–75.

Gore, M.A., Nayudu, P.L. and Vlaisavljevic, V. (1997) Attaining dominance *in vivo*: distinguishing dominant from challenger follicles in humans. *Hum. Reprod.*, **12**, 2741–2747.

Gosden, R.G. and Telfer, E.E. (1987) Scaling of follicular sizes in mammalian ovaries. *J. Zool.*, **211**, 157–168.

Gosden, R.G., Boland, N.I., Spears, N. *et al.* (1993) The biology and technology of follicular oocyte development *in vitro*. *Reprod. Med. Rev.*, **2**, 129–152.

Gougeon, A. (1996) Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocr. Rev.*, 121–155.

Gougeon, A. and Lefèvre, B. (1984) Histological evidence of alternating ovulation in women. *J. Reprod. Fertil.*, **70**, 7–13.

Greenwald, G.S. and Terranova, P.F. (1988) Follicular selection and its control. In Knobil, E. and Neil, J. (eds), *The Physiology of Reproduction*. Raven Press, New York, pp. 387–445.

Hanrahan, J.P. and Quirke, J.F. (1985) Contribution of variation in ovulation rate and embryo survival to within breed variation in litter size. In Land, R.B. and Robinson, D.W. (eds), *Genetics of Reproduction in Sheep*. Butterworths, London, pp. 193–201.

Hartenstein, V. and Posakony, J.W. (1990) A dual function of the Notch gene in *Drosophila* sensillum development. *Dev. Biol.*, **142**, 13–30.

Heitzter, P. and Simpson, P. (1991) The choice of cell fate in the epidermis of *Drosophila*. *Cell*, **64**, 1083–1092.

Heller, D.T., Cahil, D.M. and Schultz, R.M. (1981) Biochemical studies of mammalian oogenesis: Metabolic cooperativity between granulosa cells and growing mouse oocytes. *Dev. Biol.*, **84**, 455–464.

Hirshfield, A.N. (1991a) Development of follicles in the mammalian ovary. *Int. Rev. Cytol.*, **124**, 43–99.

Hirshfield, A.N. (1991b) Thecal cells may be present at the outset of follicular growth. *Biol. Reprod.*, **44**, 1157–1162.

Hirshfield, A.N. and DeSanti, A. (1995) Patterns of ovarian cell proliferation in rats during the embryonic period and the first three weeks postpartum. *Biol. Reprod.*, **53**, 1208–1221.

Hodgen, G.D. (1982) The dominant ovarian follicle. *Fertil. Steril.*, **38**, 281–300.

Horster, M., Huber, S., Tschop, J. *et al.* (1997) Epithelial nephrogenesis. *Pflüg. Arch. Eur. J. Physiol.*, **434**, 647–660.

Hsueh, A.J.W., Billig, H. and Tsafriri, A. (1994) Ovarian follicle atresia: a hormonally controlled apoptotic process. *Endocr. Rev.*, **15**, 707–724.

Huet, C., Monget, P., Pisselet, C. *et al.* (1997) Changes in ECM components and steroidogenic enzymes during growth and atresia of antral ovarian follicles in the sheep. *Biol. Reprod.*, **56**, 1025–1034.

Hughes, F.M. and Gorospe, W.C. (1991) Biochemical identification of apoptosis (programmed cell death) in granulosa cells: evidence for a potential mechanism underlying follicular atresia. *Endocrinology*, **129**, 2415–2422.

Hynes, A.C., Kane, M.T. and Sreenan, J.M. (1996a) Partial purification from bovine follicular fluid of a factor of low molecular mass with inhibitory effects on the proliferation of bovine granulosa cells *in vitro* and on rat follicular development *in vivo*. *J. Reprod. Fertil.*, **108**, 185–191.

Hynes, A.C., Sreenan, J.M. and Kane, M.T. (1996b) Modulation of the effects of FSH, androstenedione, EGF and IGF-1 on bovine granulosa cells by GCIF, a growth inhibitory factor of low molecular mass from bovine follicular fluid. *J. Reprod. Fertil.*, **108**, 193–197.

Ireland, J.J. and Roche, J.F. (1983) Development of nonovulatory antral follicles in heifers: changes in steroids in follicular fluid and receptors for gonadotrophins. *Endocrinology*, **112**, 150–156.

Kigawa, T., Ogawa, T., Miyamura, K. *et al.* (1986) Inhibitory activity of a low molecular weight substance from porcine small follicles on oestradiol and progesterone secretion by rat granulosa cells *in vitro* and rat ovaries *in vivo*. *Endocrinol. Japonica*, **83**, 597–607.

Knobil, E. and Neill, J.D. (eds) (1988) *The Physiology of Reproduction*. Raven Press, New York, pp. 387–445.

Ko, J.C.H., Kastelic, J.P., Del Compo, M.R. *et al.* (1991) Effects of a dominant follicle on ovarian follicle dynamics during the oestrus cycle in heifers. *J. Reprod. Fertil.*, **91**, 511–519.

Koering, M.J., Danforth, D.R. and Hodgen, G.D. (1994) Early follicle growth in the juvenile Macaca monkey ovary: the effects of estrogen priming and follicle-stimulating hormone. *Biol. Reprod.*, **50**, 686–694.

Lardelli, M. and Lendahl, U. (1993) Motch A and Motch B — two mouse Notch homologues coexpressed in a wide variety of tissues. *Exp. Cell Res.*, **204**, 364–372.

Le Nestour, E., Marraomi, J., Lahlou, N. *et al.* (1993) Role of oestradiol in the rise in follicle stimulating hormone levels during the luteal follicular transition. *J. Clin. Endocrinol. Metab.*, **77**, 439–442.

Li, L., Krantz, I.D., Deng, Y. *et al.* (1997) Alagille syndrome is caused by mutations in human Jagged 1, which encodes a ligand for Notch 1. *Nature Genet.*, **16**, 243–251.

Luck, M.R. (1994) The gonadal extracellular matrix. *Oxf. Rev. Reprod. Biol.*, **16**, 33–85.

Marinho, A.O., Sallam, H.N., Goessens, L. *et al.* (1982) Ovulation side and occurrence of mittelschmerz in spontaneous and induced ovarian cycles. *Br. Med. J.*, **284**, 632

Matton, P., Adelakoun, V., Couture, Y. *et al.* (1981) Growth and replacement of the bovine ovarian follicles during the oestrous cycle. *J. Anim. Sci.*, **52**, 813–820.

McIntush, E.W. and Smith, M.F. (1998) MMP and TIMPs in ovarian function. *Rev. Reprod.*, **3**, 23–30.

Mihm, M., Good, T.E.M., Ireland, J.L.H. *et al.* (1997) Decline in serum FSH concentrations alter key intrafollicular growth factors involved in selection of the dominant follicle in heifers. *Biol. Reprod.*, **57**, 1328–1337.

Morse, A.H. and van Wagenen, G. (1936) Observations upon ovulation in primates. *Am. J. Obstet. Gynecol.*, **32**, 823–832.

Oda, T., Elkahloun, A.G., Pike, B.L. *et al.* (1997) Mutations in the human Jagged 1 gene are responsible for Alagille syndrome. *Nature Genet.*, **16**, 235–242.

O'Shea, J.D. (1971) Smooth muscle-like cells in the theca externa of ovarian follicles in the sheep. *J. Reprod. Fertil.*, **24**, 283–285.

Pearson, O.P. (1949) Reproduction of a South American rodent, the mountain viscacha. *Am. J. Anat.*, **84**, 143–198.

Peters, H., Byskov, A.G., Lintern-Moore, S. *et al.* (1973) Proceedings: follicle growth initiation in the immature mouse ovary: extraovarian or intraovarian control? *J. Reprod. Fertil.*, **35**, 619–620.

Potashnik, G., Insler, V. and Meizner, I. (1987) Frequency, sequence, and side of ovulation in women menstrating normally. *Br. Med. J.*, **294**, 219.

Redmer, D.A. and Reynolds, L.P. (1996) Angiogenesis in the ovary. *Rev. Reprod.*, **1**, 182–192.

Robbins, J., Blondel, B.J., Gallahan, D. *et al.* (1992) Mouse mammary tumor gene int-3, a member of the Notch gene family transforms mammary epithelial cells. *J. Virol.*, **66**, 2594–2599.

Rühl, A. (1925) Regelmassigkeit im wechsel der Ovarial funktion. *Arch. Gynak.*, **124**, 1–26.

Ryan, A.K., Blumberg, B., Rodriguez-Esteban, C. *et al.* (1998) Pitx2 determines left–right asymmetry of internal organs in vertebrates. *Nature*, **394**, 545–551.

Schams, D., Amselgruber, W., Kamhuber, M. *et al.* (1996) The importance of basic fibroblast growth factor for follicle selection. *Biol. Reprod.*, **54**, Abstract 6.

Spears, N., de Bruin, J.P. and Gosden, R.G. (1996) The establishment of follicular dominance in co-cultured mouse ovarian follicles. *J. Reprod. Fertil.*, **106**, 1–6.

Spicer, L.J., Eckternkamp, S.E., Canning, S.F. *et al.* (1988) Relationship between concentrations of immunoreactive insulin-like growth factor-1 in follicular fluid and various biochemical markers of differentiation in bovine antral follicles. *Biol. Reprod.*, **39**, 573–580.

Sunderland, S.J. and Crowe, M.A. (1994) Selection, dominance and atresia of follicles during the oestrus cycle of heifers. *J. Reprod. Fertil.*, **101**, 547–555.

Tilly, J.L. (1998). Cell death and species propagation: molecular and genetic aspects of apoptosis in the vertebrate female gonad. In Lockshin, R.A., Zakeri, Z. and Tilly, J.L. (eds). *When cells die: A comprehensive evaluation of apoptosis and programmed cell death*.Wiley–Liss, New York, pp. 431–452.

Uyttendaele, H., Marazzi, G., Wu, G. *et al.* (1996) Notch 4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. *Development*, **122**, 2251–2259.

Van Wezel, I.L. and Rodgers, R.J. (1996) Morphological characterization of bovine primordial follicles and their environment *in vivo*. *Biol. Reprod.*, **55**, 1003–1011.

Wallach, E.E., Virutamasen, P. and Wright, K.H. (1973) Menstrual cycle characteristics and side of ovulation in the rhesus monkey. *Fertil. Steril.*, **24**, 715–721.

Webb, R. and England, B.G. (1982) Identification of the ovulatory follicle in the ewe: associated changes in the follicular size, thecal and granulosa cell luteinizing hormone receptors, antral fluid steroids, and circulating hormones during the preovulatory period. *Endocrinology*, **110**, 873–881.

Werlin, L.B., Weckstein, L., Weathersbee, P.S. *et al.* (1986) Ultrasound: a technique useful in determining the side of ovulation. *Fertil. Steril.*, **46**, 814–817.

Xu, T., Caron, L.A., Fehon, R.G. *et al.* (1992) The involvment of the Notch locus in *Drosophila* oogenesis. *Development*, **115**, 913–922.

Zamboni, L. and Merchant, H. (1973) Interfollicular connections in the ovaries of immature mice. *The Development and Maturation of the Ovary and its Function*. Exerpta Medica, Amsterdam, pp. 53–54.

Zeleznik, A.J. and Hillier, S.G. (1984) The role of gonadotropins in the selection of the preovulatory follicle. *Clin. Obstet. Gynecol.*, **27**, 927–940.

Zeleznik, A.J., Schiler, H.M. and Reichert, L.E. (1981) Gonadotropin-binding sites in the Rhesus monkey ovary: role of the vasculature in the selective distribution of hCG to the preovulatory follicle. *Endocrinology*, **109**, 356–361.

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