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ABSTRACT

The adult ovary is covered by a simple mesothelium that is an uncommitted phenotype with epithelial and stromal characteristics. The aging ovarian surface bears the morphological signature of repetitive ovulatory rupture and repair, a process always preceded by the localized production of high levels of estradiol. With time, surface invaginations and inclusion cysts become more common. Furthermore, older ovarian surface epithelium (OSE) exhibits cell layering and columnar-shaped cells suggesting focal metaplasia may occur with age. Approximately 90% of ovarian cancers arise from OSE. Common sites for the development of ovarian epithelial cancer are within inclusion cysts and in surface invaginations where OSE tends toward metaplasia. During transition to the cancerous state, OSE acquires complex epithelial characteristics aligned to Müllerian ductal epithelia, and loses its stromal characteristics.

The main hypothesis for this thesis is that periods of elevated ovarian estradiol contribute to aberrant differentiation of OSE causing it to acquire the phenotypic characteristics of Müllerian duct epithelia.

To test this hypothesis, 7-10 month old Swiss Webster mice were administered two subcutaneous injections of estradiol valerate (EV) given 14 days apart. Blood and ovarian tissue estradiol levels were measured by radioimmunoassay and showed a significant elevation in estradiol levels within 2 days of a single injection. Levels remained significantly elevated following second estradiol exposure, but the degree of elevation was approximately half that following initial exposure. Nevertheless, estradiol was found to accumulate in the blood and ovarian tissue over time. It was hypothesized that the older ovary may be more sensitive to estradiol’s mitogenic effects, and that elevated estradiol levels in the ovary would promote morphologic alteration that included OSE hyperplasia, and metaplastic changes typical of differentiation toward the Müllerian phenotype. This was initially investigated with histology, BrdU immunohistochemistry and morphometry. Two days after EV treatment the ovary showed increases in surface area and volume. Ovarian constituent
volumes revealed this response was primarily due to hypertrophy of OSE and possible OSE hyperplasia. Morphological analysis showed abundant evidence of metaplasia in OSE.

A second EV injection did not produce these changes. It was proposed this could be due to the ability of OSE to differentially regulate estrogen receptor (ER) expression, thereby limiting estradiol’s effects on OSE. Histomorphometric alteration to OSE would likely involve cell-cell junctions and influence the expression of E-cadherin, a cell adhesion molecule and marker of OSE transition to Müllerian epithelia. Immunohistochemistry and immunofluorescence with confocal microscopy was used to show normal expression of ERα, ERβ and E-cadherin in older OSE and neighbouring oviduct. Expression of ERα and ERβ was predominately nuclear but both receptors also localized to cytoplasm, suggesting nuclear-cytoplasmic receptor shuttling. Differential expression of ER subtype and degree of colocalization appeared to differ with OSE cell shape, and between OSE and oviduct. Qualitative analysis suggested there was a large down-regulation of ERα in OSE and stroma, and ERβ in OSE after first EV injection. Strong ERα expression in OSE (but not stroma) and weak ERβ expression was evident following second estradiol exposure. Semi-quantitative analysis of ERβ protein expression by immunofluorescence profiling revealed an 11-fold decrease in ERβ expression had occurred after first EV injection (P<0.0001). In contrast E-cadherin expression increased after first and decreased following second estradiol exposure. These results suggest a role for estradiol in the aberrant differentiation of OSE.
ACKNOWLEDGEMENTS

When my boys were small, my favourite bedtime story I read them (yes, my favourite) was *The Little Engine that Could*. So when times got very tough during this PhD, there was always that little voice somewhere saying 'I think I can, I think I can... ' Now finally at the top of this PhD mountain I’m thankful I kept chugging! My grateful thanks to my supervisor, Dr. Peter Hurst, a man who knows first-hand, what its like to be around determined perimenopausal females who won’t take “no” for an answer (the mice of course)! Thank you for allowing me the scope to work with the ideas presented in this thesis and for your on-going support, Peter, especially near the end.

I am also grateful to the Department of Anatomy and Structural Biology for their support throughout the years. In particular the fabulous technical advice and assistance I have received from Andrew Mc Naughton (confocal work) and Robbie Mc Phee (graphics and poster) and all the cheerful postgrads for some great chats. I also want to thank Vicki Livingstone for her statistical guidance, and Dr. Steve Assinder for his help with the tissue estradiol RIA. To the wonderful lab and office mates I have had, Holly, Anne, Carthika, Mark, Jet, Rachael, Jossie, Louise and Pan. Thanks so much for all your caring and supportive words and deeds. Thanks abounding for the early help I had with histology in HSU, in particular the wonderful support I received from Diane Potter. To Gareth (special thanks), Dave and Nancy, my PhD committee members, so grateful for your gentle handling of my PhD progress.

Last, but by no accounts least, I want to thank my long-suffering and loving husband, Troy, and my two amazing sons, Simon and Andrew. Thanks so much you guys for putting up with me and being proud of me, it means so much! To my wonderful Mum, Shirley, and my Dad, Doug (in heaven) and all my brothers, Lindsay, Martin, Trevor and Philip. Hope I make you proud too. The final words must rest with my wonderful sister-in-law, Terri, who passed away on June 23rd 1985 after a courageous two-year battle with ovarian cancer. In Terri’s words “Ya gotta have a project” and then just sit back and “enjoy”.

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<tr>
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<th>Definition</th>
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<tbody>
<tr>
<td>aFGF</td>
<td>acidic fibroblast growth factor</td>
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<tr>
<td>ActRIIB</td>
<td>activin receptor II A</td>
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<td>ActRIIB</td>
<td>activin receptor IIB</td>
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<td>AEC</td>
<td>3-amino-9-ethylcarbazole chromagen</td>
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<td>AFP</td>
<td>α-fetoprotein</td>
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<td>ALK</td>
<td>activin receptor-like kinases</td>
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<td>anti-müllerian hormone</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>profile area</td>
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<td>androgen receptor</td>
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<td>arginosuccinate synthetase</td>
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<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<td>B/BO</td>
<td>bound/unbound fraction</td>
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<td>body mass index</td>
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<td>bone morphogenic protein</td>
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<td>BRCA</td>
<td>breast cancer tumor suppressor gene</td>
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<td>BrdU</td>
<td>bromodeoxyuridine</td>
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<td>cancer antigen 125</td>
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<td>DNA</td>
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<td>dpc</td>
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<td>DPX</td>
<td>dibutyl phthalate with xylene</td>
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<td>extracellular matrix</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid disodium</td>
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<tr>
<td>EG-VEGF</td>
<td>endocrine-gland vascular endothelial-derived growth factor</td>
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<td>epidermal growth factor</td>
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<td>Ems</td>
<td>empty spiracles</td>
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<td>Description</td>
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<td>EMT</td>
<td>epithelial mesenchymal transition</td>
</tr>
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<td>endoC</td>
<td>endothelial cells</td>
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<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>estrogen response element</td>
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<tr>
<td>ERK</td>
<td>extracellular signal related kinase</td>
</tr>
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<td>ERKO</td>
<td>estrogen receptor knockout</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GnRH</td>
<td>gonadotrophin releasing hormone</td>
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<td>hCG</td>
<td>human chorionic gonadotrophin</td>
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<td>HDLs</td>
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<td>HRE</td>
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<td>immunoglobulin</td>
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<td>interleukin 1</td>
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<td>intraperitoneal</td>
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<td>JAK/STAT</td>
<td>janus kinase signal transducer and activator of transcription</td>
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<td>KGF</td>
<td>keratinocyte growth factor</td>
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<td>KL</td>
<td>kit ligand</td>
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<td>KRAS</td>
<td>Kirsten rat sarcoma viral oncogene</td>
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<td>LBD</td>
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<td>low malignant potential</td>
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<td>müllerian inhibiting substance</td>
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<td>MMP</td>
<td>matrix metal proteinases</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>Nb</td>
<td>northern blot</td>
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<td>ND</td>
<td>nuclear domain</td>
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<td>P450scC</td>
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<td>radioimmunoassay</td>
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<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RTK</td>
<td>tyrosine kinase receptor</td>
</tr>
<tr>
<td>SA</td>
<td>southern analysis</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
</tr>
<tr>
<td>SDF-1</td>
<td>stromal cell derived factor 1</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SER</td>
<td>smooth endoplasmic reticulum</td>
</tr>
<tr>
<td>SERM</td>
<td>selective estrogen receptor modulator</td>
</tr>
<tr>
<td>SMAD</td>
<td>mothers against decapentaplegic and caenorhabditis elegans protein</td>
</tr>
<tr>
<td>Sox-9</td>
<td>sry-like high mobility group box 9</td>
</tr>
<tr>
<td>Sq-Rt-PCR</td>
<td>semi-quantitative RT-PCR</td>
</tr>
<tr>
<td>SRY</td>
<td>sex-determining region of the Y chromosome</td>
</tr>
<tr>
<td>StAR</td>
<td>steroid acute regulatory protein</td>
</tr>
<tr>
<td>STS</td>
<td>steroid sulphatase</td>
</tr>
<tr>
<td>SV40T</td>
<td>simian virus 40</td>
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<tr>
<td>TA</td>
<td>tunica albuginea</td>
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<tr>
<td>TC</td>
<td>theca cells</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
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<tr>
<td>VASP</td>
<td>vasodilator stimulated phosphoprotein</td>
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<tr>
<td>Vv</td>
<td>volume-weighted mean volume</td>
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<tr>
<td>w/v</td>
<td>weight per unit volume</td>
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<tr>
<td>Wb</td>
<td>western blot</td>
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<tr>
<td>Wnt-4</td>
<td>wingless protein 4</td>
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<tr>
<td>ZP</td>
<td>zona pellucida</td>
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CHAPTER 1: The Ovary

1.1 The anatomy of the adult mouse ovary

The adult mouse ovary is a spherical shaped structure measuring approximately 2-3 mm in diameter. It is positioned high on the posterior abdominal wall, in close proximity to the kidney. Bilaterally, the ovary communicates with one horn of the duplex uterus via the oviduct (Fallopian tube), which in turn is continuous with the rete system of the ovary. The ovary of a 1 month-old mouse is close to sexual maturity, since ovulatory cycles begin in the mouse from approximately 5 weeks of age (McGee and Hsueh, 2000). At this time, the ovarian cortex, separated from ovarian surface epithelium by basement membrane and connective tissue, contains follicles of all sizes from primordial to pre-ovulatory. Follicles are also located more centrally and little stroma is seen between follicular structures. As the animal ages the amount of stroma increases, and this may be related to follicular degeneration and resorption (Peters, 1969). Each ovarian cycle is polyovulatory and takes 4-5 days to complete, beginning with proestrus and progressing through estrus (when ovulation occurs), metestrous and diestrous. A fully developed capillary and lymphatic system occupies the central medullary region of the adult ovary, giving a lattice-like appearance to this region. A sac, the ovarian bursa, surrounds the entire structure. The bursal membrane is analogous to the broad ligament in the human and is continuous with an ovarian mesentery (Li et al., 2007). Each ovulatory cycle produces a variable number of oocytes that are ovulated into the bursal space between the surface of the ovary, covered by ovarian surface epithelium, and the bursal membrane. The bursal space becomes swollen with estrogen-rich fluid exuded from the follicles following ovulation. Recently, it has been found that the estrogen in bursal fluid regulates a bursal lymphatic stomata, of which little is still known, to homeostatically control the ovarian microenvironment (Li et al., 2007). Lymphatic stomata are small openings found in the mesothelium of serous membranes. The visceral lining of the bursal membrane is representative of such a mesothelium, and is continuous with the ovarian surface epithelium found adjacent to the hilus of the ovary. Figure 1.1 shows the mouse ovary and its relationship with near-by organs and vasculature. In life, the bursa
is embedded in a large amount of adipose tissue that provides support and connects the ovary to surrounding structures.

**Figure 1.1** Anatomical position of an adult mouse ovary (right). The ovarian bursa has been removed (from Anatomy of the Laboratory mouse by Margaret J Cook, 1965)
1.2. Embryological origins and development of the ovary

1.2.1. Genesis of the bipotential gonad

Gender is determined at the time of fertilisation in mammals by the presence or absence of the Y chromosome in the fertilising sperm. This chromosome contains the \textit{SRY} gene (sex-determining gene of the Y chromosome), and its expression causes the differentiation of specific cell lineages, culminating in the organisation and formation of the male testis (Yao, 2005). The male phenotype further requires the developing gonad to be exposed to hormones such as Anti-Müllerian hormone (AMH); also known as Müllerian Inhibiting Substance (MIS), testosterone, and Insulin-like 3 (Insl3). Secretion of these hormones facilitates regression of the Müllerian (paramesonephric) ducts, drives development of the Wolffian (mesonephric) ducts giving rise to the male ductal system, and facilitates testicular descent into the scrotum (Cederroth et al., 2007). In contrast, absence of \textit{SRY} gene expression suppresses secretion of these hormones and results in differentiation of the bipotential gonad into the female phenotype, giving rise to the uterus, oviduct, and ovaries that remain intra-abdominal (Cederroth et al., 2007). Organogenesis of the ovary however, may not simply be a passive process. Recent evidence indicates development of the female gonad may require a finely tuned interaction between somatic and germ cells during the embryonic period, with the female germ cell actively inhibiting testis-specific events such as formation of the testis cords, by virtue of entering meiotic division (Yao, 2005).

The developing gonad is first recognised as a rudimentary thickening along the cranioventral region of the mesonephros, one of three early embryonic kidneys. The gonadal anlagen is a mixture of somatic cells arising from three tissue types; mesenchymal, mesonephric, and coelomic epithelial (Byskov, 1986). Somatic cells from these donating tissues intermingle early on with small numbers of primordial germ cells (PGC) that have migrated to the gonad from their extra-gonadal site of origin (Byskov and Hoyer, 1994). In the mouse embryo at day 9 post fertilisation (E9) (length of gestation = 21 days), nephrogenic cells are observed to arise from mesenchymal tissue sandwiched between the presumptive aorta and developing somites in contact with
coelomic epithelium; an epithelial layer which lines the coelom of the body cavity and covers the organs that form beneath it. These cells begin the process of differentiating into the ductal system consisting of the Wolffian duct (mesonephric tubules) and rete testes in the male. In mouse ovary, invading mesonephric cells and tubules form the Müllerian duct and rete system consisting of three portions: the extra-ovarian, connecting, and intra-ovarian rete (Byskov, 1986).

During the process of cell differentiation, some nephrogenic cells escape this fate and migrate toward the coelomic epithelium. It is thought that simultaneously, coelomic epithelial cells actively migrate toward the mesonephros also, contributing to the bulging gonadal (genital) ridge (Byskov, 1986). This structure becomes recognisable in mouse at E 10.5 and by the fourth week of gestation in humans (Byskov and Hoyer, 1994). A rapid transition has thus occurred from what was formerly loose mesenchymal tissue, to an organised compact body of mesenchymal, mesonephric-derived, and coelomic epithelial cells, with PGC scattered amongst them. In humans, the fifth week of embryogenesis marks completion of the formation of gonadal blastema and the structure becomes known as the indifferent gonad (Byskov and Hoyer, 1994). This time correlates with 12.5 days post coitus (dpc) in mice.

Early observations to investigate PGC migration to the gonad were based on conventional histological techniques, and germ cells were sometimes difficult to distinguish from other nearby somatic cells. From the 1950s however, it was established that PGC demonstrated a high degree of alkaline phosphatase activity in their large cytoplasm (Chiquoine, 1954). Thus tracing a migration of PGC to the gonad became much easier when this characteristic of the cell was used for identification. Prior to alkaline phosphatase labelling, histological use alone in the identification of PGC may have contributed to the disparity in the reporting of germ cell origin. More recently, the study of germ cell migration has involved tracking PGC to the genital ridge in vivo using Green Fluorescent Protein (GFP), directed by the truncated Oct-4 promoter gene, expressed specifically in germ cells (Anderson et al., 2000). Oct4-GFP becomes incorporated into germ cells at approximately the same time as alkaline phosphatase and use of this marker has proved additionally valuable in establishing
early migratory patterns of PGC from their time of their induction between E 6.5-7.5, corresponding to the start of gastrulation (Starz-Gaiano and Lehmann, 2001).

Migration of PGCs has been shown to occur shortly after the 10th day of fetal life in mice (Everett, 1943), and is thought to continue until 12.5 dpc whereupon having colonised the gonad, germ cells differentiate into oogonia or spermatagonia according to embryonic sex. PGC migration to the presumptive gonad is via the embryonic mesoderm of the primitive streak and visceral endoderm of the yolk sac, thence on to the hind gut endoderm and mesoderm of the dorsal mesentery, finally arriving at the coelomic epithelium of the gonadal ridge (Byskov and Hoyer, 1994). Evidence of inductive events linked to germ cell migration are now known to be apparent as early as 5.5 dpc in the mouse epiblast (De Felici et al., 2004), and are proposed to involve activity of members of the Transforming Growth Factor Beta superfamily: Bmp4, Bmp8b and Bmp2, on the proximal epiblast.

Ultrastructural studies have shown PGC of many species, including mice (Jeon and Kennedy, 1973, Spiegelman and Bennett, 1973, Clark and Eddy, 1975) (Zamboni and Merchant, 1973), rat (Eddy, 1974) (Eddy, 1975) and human (Fujimoto et al., 1977) (Kuwana and Fujimoto, 1983), possess pseudopodia-like extensions and are capable of migration using ameboid movement. Active translocation of PGC through the developing gut wall en route to the genital ridge occurs this way (Raz, 2004). Genetic profiling in zebrafish, drosophilia, and mice, have suggested that genes activated in both germ cells and their surrounding somatic tissues are required for successful PGC migration (Starz-Gaiano and Lehmann, 2001). Molecules believed to actively direct PGC progression along the migratory pathway to the genital ridge include the chemokine stromal cell–derived factor 1 (SDF-1) and its receptor protein CXCR4 in mouse (Raz, 2004). One recent in vitro study has questioned the requirement for stromal cells in directing PGC migration. Farini et al (2007) (Farini et al., 2007) placed undifferentiated germ cells into culture medium inside transwell chambers in which a positive gradient for a limited selection of putative chemo attractants including SDF-1α and TGFβ was created. They observed that without stromal support, PGC continued to exhibit a migratory pattern if Kit Ligand (KL), also known as Stem Cell Factor (SCF) was present. PGCs are known to express c-Kit, the receptor for KL and
KL itself is expressed in somatic cells along the migratory pathway and in somatic cells of the gonadal ridge (Matsui et al., 1990) (De Felici et al., 1996). Chemoattraction was not witnessed in culture when either SDF-1 or TGFβ were present alone, indicating that these proteins may work synergistically with stromal derived factors to promote PGC migration.

During migration to the gonad and for a short time thereafter, germ cells undergo rapid mitotic division. In the short span of just five and a half days (ED 8 - ED 13.5), some 10-100 PGCs become around 25,000 in number (Byskov and Hoyer, 1994). Once at the genital ridge, PGC lose their migratory abilities and become entrapped by coelomic epithelial cells covering the presumptive gonad (Byskov and Hoyer, 1994). They make contact with both these and other somatic gonadal precursor cells (SGP), and coalesce to form the gonad proper. Germ cells appear to be a critical component in the regulation, organisation and maintenance of ovarian structure. When they are absent from the XX gonad, ovarian follicles, considered the functional unit of the ovary, fail to form. Testis development in contrast, is not affected by the absence of germ cells (McLaren et al., 1984).

Between E10.5-E12.5 transient Sry gene expression leads to upregulation of Sox9 (Sry-like High Mobility Group box 9), which in turn stimulates Fgf9 (fibroblast growth factor 9) expression and leads to increases in prostaglandin D2 (PGD2) synthesis; all of which ensure development proceeds down the male pathway. This time corresponds approximately to 6-7 weeks gestation in humans (Parker and Schimmer, 2006). In the absence of Sry, Wnt4 signalling (Wnt4 is a putative upstream regulator of Follistatin) opposes the male pathway by disrupting Sox9 expression and development of the female gonad proceeds (Cederroth et al., 2007) (Kim et al., 2006). The names spermatogonia and oogonia hereafter describe sex-specific germ cell lines in the developing embryo following sex differentiation.
1.2.2 Differentiation of the ovary

Differentiation of the embryonic ovary is defined by three critical events:

1. **Induction of meiosis** in oogonia to establish oocytes
2. Encapsulation of oocytes by a single layer of flattened follicular cells signalling prophase 1 meiotic arrest and **onset of folliculogenesis**
3. **Differentiation of steroid producing cells** in the ovary (Byskov, 1986).

These three processes occur independent of the gonadotrophins, which do not control ovarian events until after birth.

Oogonia are seen to enter meiotic division around E13.5 in mouse and between 8 and 13 weeks gestation in humans (Parker and Schimmer, 2006). Thereafter they are referred to as oocytes. In contrast to early testicular development when structural compartmentalization is immediately evident, early structural organisation in the developing ovary does not become histologically obvious until formation of primordial follicles at 16 weeks gestation in the human embryo, and in mice, gestationally much later, around E18 (Parker and Schimmer, 2006). The formation of well defined mesonephric-derived germ cell cords that position oogonia within ovarian stroma, and are analogous to testis cords in the male; appears species dependant and related to whether germ cells undergo immediate meiosis following sex differentiation (human, rat, hampster, mouse) or delayed (pig, sheep, cow) (Byskov and Hoyer, 1994). Thus humans and mice, both exhibiting immediate (minimal delay) meiosis, do not form clear germ cell cords.

During the process of ovarian differentiation, mesonephric tissue begins to regress yet an ovarian-mesonephric connection persists throughout (Byskov and Hoyer, 1994). Gonadal cells become progressively organised into discrete functional areas. In humans the germ cell population occupy a defined cortical area, whereas in mouse germ cells may be distributed uniformly or in clusters throughout ovarian tissue, with adjacent oogonia connected by cytoplasmic bridges. Follicular assembly begins in the innermost regions of the ovary when the oocyte reaches diplonema and presumptive granulosa cells surrounding the oocyte become segregated from it by an intact basal lamina (Byskov and Hoyer, 1994). At this stage it is believed cytoplasmic bridges between germ cells disaggregate and concurrently a huge loss of oocytes occurs predominately by way of apoptosis (Lobascio et al., 2007). The reason for such a large
loss of germ cells in the pre-natal mammalian ovary is still to be fully explained, but may be related to a loss of shared organelles such as mitochondria, which cannot continue to be shared between oocytes once cytoplasmic bridges break down (Epifano and Dean, 2002). Alternative mechanisms have also been proposed for germ cell attrition during fetogenesis and early postnatal life in mice and include autophagy and extrusion through the OSE (Maiuri et al., 2007) (Wordinger et al., 1990, Rodrigues et al., 2009).

In many species the centralised portion of the ovary making up the intraovarian rete is composed of an infiltrating mass of mesonephric cells and germ cell (medullary/ovigerous) cords that enclose germ cells, and appear to physically push them out to the cortical regions of the ovary and toward coelomic epithelium. The cords communicate with both the medullary and peripheral regions of the developing ovary. For this reason some authors have disagreed on the origins of the somatic cell precursors of follicular cells surrounding oocytes during formation of primordial follicles; stating they arise from coelomic epithelium (Everett, 1943) (Allen, 1904) (Brambell, 1927), mesenchymal cells of ovarian stroma (Peters and Pedersen, 1967) or mesonephric cells contributing to the ovarian rete system (Byskov and Lintern-Moore, 1973) (Upadhyay et al., 1979). Formation of ovigerous cords during ovarian development represents a temporary facility for both the organisation and positioning of oogonia, however, and as previously alluded to is largely dependent on the timing of the onset of meiosis. Therefore the question as to where follicular somatic cells (FSC) are derived from in embryonic ovary continues to fuel debate. Additionally, until recently, techniques used to establish the lineage of follicular precursor cells in the ovary were restricted to the identification of morphological hallmarks - relative dye affinities in thin resin sections, or analysis of electron microscopic images. Recently these approaches have been accompanied by the use of a marker of mitotic activity, bromodeoxyuridine. Using this approach, researchers have established in sheep ovary, that oocytes (oogonia entered into meiosis) newly migrated to the cortex of the ovary acquire somatic follicular cells at a time when coelomic epithelial cells, but not mesonephric cells, are proliferating. This strongly implicates somatic cells enclosing oogonia to be mostly of ovarian surface epithelial (coelomic epithelial) origin (Sawyer
et al., 2002), at least in sheep, which show delayed meiosis. Thus, species-specific variation in the origin of FSC likely contributes to discrepancies in the literature.

Folliculogenesis is the name given to the process by which the female germ cell (oocyte) develops within somatic follicular cells to eventually become a fertilizable ovum. This part of the thesis will focus on those events in folliculogenesis that occur during embryological and fetal development (Rajkovic et al., 2006). Both paracrine and autocrine factors mediate events in early folliculogenesis and although demonstrating a degree of redundancy, they nevertheless act synergistically to promote growth of the oocyte and surrounding somatic cells (Kol and Adashi, 1995). Both the oocyte and adjacent somatic cells are mandatorily required for successful follicle assembly, however. In the oocyte, initiation of meiosis appears to play a key role from the outset of folliculogenesis, since inactivation or mutation of genes driving entry into meiosis results in extensive oocyte loss, inability to form follicles, and resultant sterility in the adult (Epifano and Dean, 2002). A second oocyte-specific initiator of folliculogenesis is the basic helix-loop-helix transcription factor, Figα (factor in germline α), detected at E13 in the mouse. This transcription factor is expressed only in female germ cells, and is thought to tightly regulate early follicular cell coupling to oocytes via its stimulation of zona pellucida proteins ZP1,2, and 3. ZP proteins are known to be constituents of the extracellular glycoprotein matrix cementing the oocyte to its surrounding granulosa (follicular) cells (Yao, 2005). When Figα is absent, primordial follicles fail to form in the ovary and global depletion of oocytes occurs shortly after birth (Soyal et al., 2000). Mammalian folliculogenesis is arrested when the oocyte reaches diplotene of meiotic division and is not recommenced until immediately prior to ovulation (Gosden et al., 1997).

Species in which germ cells undergo immediate meiosis are unable to carry out de novo steroidogenesis in the ovary to any significant degree until the formation of follicles. In the mouse ovary, cells with organelles necessary for steroidogenesis do not appear until just following birth (Pehlemann and Lombard, 1978) and their presence coincides with the onset of follicular growth (Peters, 1969). During compartmentalization in the ovary, the (presumably mesonephric-derived) cells contributing to granulosa cell and thecal cell populations develop in such a way that
only thecal cells possess the capacity for steroid synthesis *de novo* (Byskov, 1986). As early as post-natal day 8 in rodent ovary, 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 (3β-HSD) activity is present in theca, interstitial gland cells and granulosa cells of growing follicles, indicating rodent infant ovary actively produces steroids (Juneau et al., 1993). In human ovaries immunostaining for 3β-HSD is present in the cytoplasm of thecal cells surrounding primordial follicles, by 28 weeks gestation and by 34 weeks is also seen in interstitial cells of stroma (Dupont et al., 1992).

Although a cohort of genes including Steroidogenic Factor 1(SF-1); expressed at E9 and a member of the nuclear hormone receptor superfamily, are known to be crucial for steroidogenesis in the indifferent gonad, much less is known about genes that mediate ovarian steroid production and development (Parker and Schimmer, 2006). Research continues into this area with *WNT4* (a glycoprotein which functions in a paracrine fashion to control development (Parker and Schimmer, 2006), follistatin, members of the Transforming Growth Factor (TGF) β family, *GDF9* and *BMP15*; and the transcription factor *FILα* most studied to date.

**1.2.2.1 Stromal development in the ovary**

The developing ovarian stroma forms a resilient connective tissue that originates from mesenchyme and remains when medullary/ovigerous cords regress. Ovarian stroma in the 12-20 week old human fetus consists of interstitial cells that are analogous to Leydig cells in the male testis and are steroid-secretory in nature, and hilar cells, (also steroid producing) that lie in close proximity to nerves and blood vessels entering the ovary at the hilus (Carr, 2004). In the E12.5 mouse ovary, mesenchymal tissue composed of these somatic cells surround clusters of germ cells and the gonad appears homogeneous. A thin compact rim of tissue, the presumptive tunica albuginea is present beneath ovarian surface epithelium (OSE) (Byskov, 1986). Tunica albuginea has been shown *in vitro* to fully enclose the ovarian stroma by E15 in mouse (McKay and Sith, 1989). Clusters of interstitial gland cells surround primordial follicles at E18 after germ cells have entered meiosis at E13.5 (Parker and Schimmer, 2006). These interstitial cells are endowed with extensive cytoplasmic accumulation of both smooth endoplasmic reticulum and house many tubular shaped mitochondria (Gondos, 1975).
Extracellular matrix (ECM) molecules are of critical importance to gonad differentiation and growth (Miqueloto and Zorn, 2007), however, this area of gonadal development during embryogenesis has received comparatively less attention in the literature. Structural proteins fibronectin, laminin, collagen types I, III and IV have all been detected in the extracellular matrix of rodent gonadal stroma from E12-E20 (Paranko et al., 1983) (Agelopoulou and Magre, 1987) (Paranko, 1987) (Gelly et al., 1989) (Wight, 2002). Moreover, Miqueloto et al. (2007) (Miqueloto and Zorn, 2007) have recently shown the presence of the proteoglycan and glycosaminoglycan molecules biglycan, perlecan and hyaluronan in the developing gonads of mice. These ECM molecules are involved in the binding of growth factors controlling cell proliferation and differentiation, specifically TGFβ and basic fibroblast growth factor (bFGF). They furthermore create paths of least resistance for migrating cells establishing gonadal compartments and controlling angiogenesis, and facilitate cell adhesion and survival. It is proposed that particular sets of ECM molecules are required for different compartments of the developing gonad, where it appears they have a role in epithelial-mesenchymal interactions controlling cell migration and early coelomic epithelial basement membrane integrity (Miqueloto and Zorn, 2007).

Research into signalling pathways in fetal ovarian stroma is an evolving field, but recently new light has been shed on activin signalling in the stroma of developing human ovaries. Activins and inhibins are members of the TGFβ family of proteins known to influence many events inherent in the processes of cell proliferation, differentiation, adhesion, apoptosis and motility (Dunker and Kriegstein, 2000). Other members of this family; bone morphogenic protein (BMP), anti-müllerian hormone AMH; also known as müllerian inhibiting substance (MIS), TGFβ1 and TGFβ2, have all been located in adult rodent ovary. Specifically, in adults, activins stimulate follicle-stimulating hormone (FSH) and inhibins, as the name suggests, are inhibitory to FSH release (Lin et al., 2003).

In the developing human ovary, activin subunits are present and are thought to contribute to germ cell proliferation and survival in an autocrine/paracrine manner. Activin signalling is through membrane-bound serine-threonine kinase receptors. Activins bind to a type II receptor (either ActRIIA or ActRIIB), which upon recruiting
and phosphorylation of a type I receptor activates a downstream signalling pathway, the SMAD pathway. SMADs are a family of signal transducers that move into the cell nucleus and promote transcriptional complexes of specific DNA binding affinity (Massague, 1998). Type I receptors are termed activin receptor-like kinases (ALKs) and several have been identified. Activin preferentially binds ALK4, whilst BMP and AMH are thought to selectively bind to ALK2 (Martins da Silva et al., 2004), and may work through the mitogen activated protein kinase (MAPK) pathway, rather than the SMAD pathway.

ActRIIB and ALK4 expression has been shown using immunohistochemistry to be present in stroma and pre-granulosa cells and oogonia of fetal human ovaries from 14-18 weeks gestation (Martins da Silva et al., 2004). ALK2 is exclusively localised to stromal cells during the same time period and expression appears to increase with increasing gestation. Concurrent with activin receptor expression is that of SMADs 2 and 3 in the nuclei of somatic stromal cells. Interestingly, immunohistochemical evidence of the opposing presence of the α-inhibin sub-unit and its receptor betaglycan is lacking up to 23 weeks gestation in human fetal ovaries (Martins da Silva et al., 2004) (Rabinovici et al., 1991) and late gestation in rhesus monkeys (Rabinovici et al., 1991). Moreover, Billar et al (2003) (Billiar et al., 2003) showed α-inhibin to be only minimally expressed in the pre-granulosa, granulosa and interstitial stromal cells of fetal baboon ovaries during the last half of gestation when both maternal and umbilical serum estradiol levels were highest (4.2 ± 1.0 and 0.59 ± 0.13 ng/mL respectively). Conversely α-inhibin expression was upregulated in ovaries deprived of estrogen by the administration of aromatase inhibitors.

Estradiol levels increase dramatically toward the end of gestation (Johnson and Everitt, 1995) and are correlated to low levels of the inhibins. Ovarian estrogen receptor (ER) expression has been documented as early as E10 in embryonic mouse gonad. By E15 ER is localised to Müllerian duct epithelia and mesenchyme in the female mouse (Greco et al., 1993) and by the 100th day of a 184 day gestation ER can be immunolocalized to ovarian epithelial and mesenchymal cells in baboon (Pepe et al., 2002). Early in gestation, activin may work to increase ER levels and enhance the actions of estrogen. Increasing estrogen may in turn suppress activin expression,
which could conceivably return ER to basal levels by birth of the infant. This novel interplay between activin and estrogen signalling has recently been reported in ovaries of pre-pubertal mice using an elaborate set of in vitro and in vivo methodologies (Kipp et al., 2007a) (Kipp et al., 2007b). Taken together, it is possible that during much of early human fetal ovarian development, activins, under either direct or indirect control by estrogen, are unchallenged in their role as drivers of cell proliferation involving mesenchymal-derived tissues. Activin-initiated proliferation early in ovarian genesis may therefore equally affect stromal cells alongside pre-granulosa cells, granulosa cells and oocytes contained therein, driving both folliculogenesis and stromal development in the developing ovary. In the latter stages of gestation, this role may be taken over directly by estrogen until ER levels fall precipitously perinatally in response to withdrawal of placental support. One added consideration is the abundant production of α-Fetoprotein (AFP) by fetal liver (Greco et al., 1993). The tissue expression of this serum protein differs between mouse and human, with human but not mouse, showing expression in mesonephros which gives rise to stroma and the ductal system. The role of AFP is unclear, but it has a high affinity for estradiol and is suspected as being a chaperone, possibly transporting estradiol into cells (Jones et al., 2001). Interestingly, AFP is expressed in primitive tumors in adult organs in a manner that reflects sites of AFP expression during embryonic and fetal development.

1.2.2.2 Development of the ducts of the female reproductive tract

During embryogenesis prior to sexual differentiation, mesonephric-derived tissue forms two separate ductal structures adjacent to one another; the Wolffian (mesonephric) and Müllerian (paramesonephric) ducts. Unlike the bipotential gonad, these ducts are unipotential and one structure is destined to regress depending on phenotypic sex. In the case of the female, the Müllerian ducts persist, and regression of the Wolffian ducts occurs in the absence of testosterone and MIH at approximately 51 days post conception (humans), around E13 in mouse. The Müllerian ducts are formed by E12 in the mouse (37 days gestation in humans). Their appearance is later than that of the Wolffian ducts (30 days gestation, humans, E9.5, mouse) and corresponds with the appearance of the bipotential gonad (Parker and Schimmer, 2006).
An important aspect of the developmental origins of the Müllerian ductal system highly relevant to this body of work; is that Müllerian epithelia are derived from longitudinal invaginations of coelomic mesothelium, the same mesothelium that gives rise to ovarian surface epithelium. Further Müllerian duct differentiation results in the cranial portions becoming the oviducts, and the most caudal regions fusing to become the uterus and superior part of the vagina by 10 weeks gestation in the human female (Johnson and Everitt, 1995) (Strauss and Lessey, 2004). As fusion of the Müllerian ducts proceeds, they move antero-medially and come to lie in front of the rectum. During this process they maintain contact with the coelomic wall, simply stretching out the mesenchyme and coelomic epithelial coverings that overlay them. In much the same fashion as a dorsal mesentery is formed, this event culminates in the formation of the embryonic broad ligament; portions of which enclose the oviducts (mesosalpinx) uterus (mesometrium) and ovary (mesovarian). In rodents, the Müllerian ducts fail to fuse, leading to the formation of two distinct uterine horns, constituting a ‘duplex’ uterus. There is also no formation of the broad ligament, yet uterine horns remain supported by remnants of mesentery (Hoar, 1978).

Genes that have been shown through mouse gene knockout experiments to be involved in ductal development include paired-box gene 2 (Pax2), the LIM homeo-domain protein *Liml*, (also known as *Lhx1*), *Emx2*, a mammalian homologue of the *Drosophilia* head-gap gene *empty spiracles* (*ems*), and Wnt/wingless protein 4 (*Wnt4*). A further evolutionary conserved family of transcription factors that have a critical role to play in the organisation of cells in the developing ductal system in both mice and humans is the *HOX* (transcriptional regulators of the homeobox family) genes. In the absence of expression of the above genes, the female reproductive tract fails to form. Furthermore, retinoic acid signalling and the expression of *Wnt7a* appears to be required for intact Müllerian duct formation in the female and the successful regression of the Müllerian duct in the male respectively. *Wnt* signalling may also indirectly play a role in establishing epithelial cell polarity during Müllerian duct morphogenesis (Kobayashi and Behringer, 2003). It is notable that both these genes are expressed in the female reproductive tract into adulthood and are regulated by estrogen and progesterone (Strauss and Lessey, 2004).
Morphogenesis of the female reproductive tract, however, does not require ovarian estrogen production, since experiments involving mice with gene perturbations for both known estrogen receptors, ERα and ERβ, show that in the absence of exposure to this hormone, normal development of the reproductive tract occurs (Lubahn et al., 1993) (Krege et al., 1998) (Couse and Korach, 1999, Couse et al., 1999) (Dupont et al., 1992). Biosynthesis of estrogens from androgens has nevertheless been shown in vitro in whole rabbit (Milewich et al., 1977) and human (George and Wilson, 1978) embryo, and rat fetal ovaries (Weniger, 1993). Furthermore, during human fetal development, both uterus and oviduct become exposed to high levels of placental estrogen, and by the seventh month of gestation the newly formed uterine glandular epithelium responds with extensive proliferation and hypertrophy, resembling estrogen-stimulated adult endometrium during the proliferative phase of the endometrial cycle (Hess et al., 2006). Oviduct responses include epithelial cell proliferation, differentiation, and ciliogenesis (Anderson and Hein, 1976). Okada et al (2002) (Okada et al., 2002) showed that developing rat Müllerian duct epithelia responded to estrogen exposure with significant increases in progesterone receptor (PR) mRNA expression. Since PR gene expression is induced by estrogens via ER, their study provided circumstantial evidence that fetal ER responds to trans-placental estrogens. However in rodents, estrogen was thought to be derived from corpora lutea in the ovary, since rodent placenta does not contain aromatase and was therefore presumed only capable of androgen synthesis from progesterone (Ben-Zimra et al., 2002). Recently published studies have confirmed that rodent placenta converts estrone to estradiol in situ (Nokelainen et al., 2000).

Furthermore, uterine and oviductal development appear to be critically regulated by exogenous estrogen, since pre-natal exposure to either estradiol 17β, diethylstilbestrol (DES) a potent synthetic estrogen, or Tamoxifen, a selective estrogen receptor modulator (SERM) structurally similar to DES; results in teratogenic and carcinogenic effects in developing female reproductive tracts of rodents and humans (Block et al., 2000) (Akbas et al., 2004) (Greco et al., 1993) (Diwan et al., 1997). Estrogen is thought to produce these effects via alteration to expression patterns of Wnt7a and Homeobox genes (HOX), Hoxa9 and Hoxa10 through ERα (Strauss and Lessey, 2004).
1.2.2.3 Development of ovarian surface epithelium (OSE) and early expression of E-cadherin

During early embryogenesis the basement membrane underlying presumptive OSE is discontinuous (Byskov, 1986) (Byskov and Hoyer, 1994) (Miqueloto and Zorn, 2007) and it is not until sexual differentiation of the gonad that mesonephric-derived tissue and coelomic epithelium destined to become OSE begin to be separated. As a consequence, isolated germ cells can become trapped within coelomic epithelium and have been seen within ovarian surface epithelium for some time after the initiation of gonadal sex differentiation (Jirasek, 1971). It was therefore originally believed primordial germ cells (PGC) originated from the developing OSE, and the term germinal epithelium was applied (Allen, 1904).

The belief that these resident OSE germ cells persisted throughout reproductive life and derived from cyclic proliferation of OSE, was proposed by Allen et al, 1923 (Allen, 1923) and supported by other early reproductive researchers in this field (Evans and Sweazy, 1932). From the 1920s to the 1950s it was a widely held viewpoint. It was not until the work of Zuckerman, in 1951 (Zuckerman, 1951) who undertook a quantitative analysis of oocyte number throughout the mammalian life-span, and others who followed using meiotic labelling strategies (Rudkin and Griech, 1962) (Borum, 1967) (Peters, 1969), that doubt was cast over the validity of this assumption, and it became universally accepted PGC instead represented a finite germ cell population that had migrated to the ovary, and via ovulation and atresia progressively became depleted. By menopause there are believed to be little to none left in the adult mammalian ovary, a phenomenon believed to drive the climacteric in the human female.

It is prudent to mention at this time that there has been a recent radical departure from this widely accepted dogma. Johnson et al (Johnson et al., 2004) (Johnson et al., 2005) conducted a series of elaborate experiments which challenged the sustainability of this belief, providing evidence to support their hypothesis that the adult mammalian female may retain the ability to replenish stocks of depleted oocytes, reportedly showing the persistence of presumptive germline stem cells in OSE of young adult mice. Byskov et
al (2005) (Byskov et al., 2005) and others (Gosden, 2004) (Greenfeld and Flaws, 2004) continue to rigorously defend the accepted dogma, largely arguing against the appropriateness of some of the techniques used by Johnson et al to support their ideas.

Embryonic OSE of mouse (Odor and Blandau, 1969), rabbit (Gondos, 1969), hamster (Weakley, 1969), guinea pig (Jeppesen, 1975), and human (Gondos, 1975), has been shown using conventional histology and electron microscopy, to be remarkably similar in its cellular characteristics. In all species mentioned, cytoplasmic processes and microvilli face outward toward peritoneum, adjacent cells are interdigitated and junctional complexes are evident between cells. Elongated mitochondria, cytoplasmic vesicles, vacuoles, numerous free ribosomes and rough endoplasmic reticulum are all seen. Interestingly, numerous germ cells are identifiable within or close to developing surface epithelial cells in the ovaries of all mammalian species cited. Lipid accumulations and isolated cilium are also reported. Nuclei are irregularly shaped with peripheral clumping of chromatin.

Human OSE at 24 weeks gestation exhibits a degree of cell stratification. Unlike mouse OSE (Odor and Blandau, 1969), it retains some continuity with sex cords and other somatic cells surrounding oocytes, raising speculation that in the human, some OSE cells may give rise to granulosa cells (Papačaki and Beilby, 1971). It is not until the third trimester of human pregnancy (weeks 24-40) that the surface epithelium becomes completely separated from the cortex by basement membrane, and thickening of the tunica albuginea occurs. Similar temporal trends involving OSE stratification and the segregation of stroma from definitive OSE by an intact basement membrane are seen in the gestational period of the guinea pig (Jeppesen, 1975), rabbit (Gondos, 1969), and mouse (Odor and Blandau, 1969).

OSE has been alluded to as sharing a common embryologic precursor of oviductal and uterine epithelium, the embryonic mesoderm. Furthermore, the urinary tract and adrenal cortex derive similarly from mesoderm. This primitive tissue further differentiates into a mesenchyme (which demonstrates a degree of pluripotency), and coelomic epithelium, a type of peritoneal mesothelium (Murdoch, 1996). Coelomic epithelium envelopes the gonadal ridge and following a degree of invagination around
the gonad, differentiates further to form ovarian surface epithelium. In human embryos of 7-9 weeks gestation, the cortex of the ovary consists of germ cells and pre-granulosa cells haphazardly organised. As a basement membrane and tunica albuginea are not uniformly present, ovarian surface epithelial cells merely overlay stroma, but nevertheless remain conspicuous due to their uniform arrangement. They are columnar in shape with regular oval nuclei (Gondos, 1975).

By 9-12 weeks gestation in human, an absence of tunica albuginea is still apparent, but the basement membrane, though discontinuous, is formed. OSE cells at this point maintain their vertical orientation and form a layer 1-2 cells thick. Nuclei are regular and mitotic figures a common feature. Between 12-16 weeks gestation, surface epithelium is clearly distinguished as it becomes segregated from loosely arranged sex cords housing oocytes. At this stage OSE cells retain their columnar shape but are seen to be some 3-4 cell layers thick. In addition, there is a degree of loss of polarity and cell irregularity in the basal-most layers. Gondos et al (1975) extended their light and electron microscopic study to include aborted fetuses up to 20 weeks gestation.

Between 16-20 weeks gestation a marked proliferation of OSE cells was apparent, with cells in arranged in multiple layers. Papillary projections were seen. Tunica albuginea had developed considerably, yet discrete areas of communication remained between OSE and stroma. Most intriguingly, OSE cells were described as 'jumbled' at this stage in fetal development in humans, with varying orientations. Nuclear changes – infolding, nuclear pleomorphism, prominent nucleoli and irregular coarse clumping of chromatin were additionally reported both at light and electron microscopic level.

The phenomenon of OSE proliferation during embryogenesis and fetogenesis appears best documented in mice (Davies et al., 1999) and humans (Gondos, 1975), with studies on OSE responsiveness during fetogenesis in other mammalia particularly lacking. These studies may prove to be of great importance, because they reveal the existence of factors that may be influential during other stages in the reproductive lifespan, and in the progression of OSE toward neoplasia. Additionally, the observations made in such studies support hypotheses contained within this thesis.
which propose links exist between repeated exposure to estrogen and challenges to OSE integrity preceding oncogenesis.

First, human and rodent fetal ovaries are capable of steroid hormone synthesis (Goldman et al., 1966) (Jungmann and Schweppe, 1968) (Gondos, 1975) (George and Wilson, 1978) (George et al., 1979, Weniger, 1993) (Greco et al., 1993) (Vaskivuo et al., 2005). Furthermore, early expression of estrogen receptor occurs in fetal mouse (Davies et al., 1999) (Greco et al., 1993), rabbit (George et al., 1979) (Greco et al., 1993) and human ovary (Gondos, 1975) (Gould et al., 2000) (Vaskivuo et al., 2005). Secondly, fetal OSE exposed to high levels of estrogen produced by the placenta (human) and corpora lutea (rodent) and any other contribution from the immature gonad; responds with cell stratification, hypertrophy, metaplasia, papillary formation, and alteration to cell polarity. Thirdly, cellular changes observed in proliferative OSE during this time bear a striking similarity to those seen in surface epithelial neoplasms (Gondos, 1975), yet despite this period of exposure to high endogenously derived sources of estrogen, fetal OSE remarkably retains a capacity to be refractory to estrogen’s possible oncogenic effects. In fact by term, OSE becomes quiescent, returning to a monolayer of cuboidal cells in the human newborn (Auersperg et al., 2001) and by postnatal day 3 in the rodent (Rajah et al., 1992). The reason for this has not been elucidated, but may be related to the eventual formation of the basement membrane underlying developing OSE. The (previously) discontinuous nature of the basement membrane would have provided areas of direct contact between stroma and epithelium for a considerable portion of time during embryologic and fetal life.

As previously alluded to, fetal stromal interstitial cells are endowed with extensive cytoplasmic accumulation of both smooth endoplasmic reticulum and mitochondria. Histochemical analysis has shown stromal interstitial cells possess the enzyme 3β-hydroxysteroid dehydrogenase required for steroid hormone synthesis as early as the sixteenth week of human gestation (Gondos, 1975). Moreover, both aromatase and 17β hydroxysteroid dehydrogenases (17HSD) necessary for the conversion of androgens to estrogens have been localised to stroma in human fetal ovaries from 17 weeks gestation (Vaskivuo et al., 2005). A study using fetal mouse ovary found that although 3βHSD mRNA transcript was detectable, that of P450scc, and P450c17 were
minimally expressed in ovary taken at days 13, 15, 17 and 20 dpc (Greco and Payne, 1994). This result may reflect the delayed onset of folliculogenesis in mice when compared to humans. Nevertheless it should be affirmed that the authors qualitatively assessed enzyme expression with use of a single RT-PCR experiment, and did not further employ realtime PCR to assess fold changes of these genes relative to the housekeeper gene, an arguably more sensitive measure of relative levels of gene expression.

Moreover, individual compartments within the developing murine ovary such as stroma may well be the sites for estrogen synthesis, but potentially occupy a very small volume of whole fetal ovary. Therefore any expression of the steroidogenic enzymes required for estradiol synthesis should be considered as evidence for such a potential in fetal ovary. Fetal ovarian stroma may therefore represent a putative source of estradiol and may exert a paracrine influence on OSE proliferation in the developing mammalian ovary. The question remains as to what happens to this large accumulation of OSE cells in order to allow OSE to return to a monolayer prior to birth. It can be hypothesized that once the basement membrane forms a continuous barrier between OSE and stroma, estradiol or an estradiol-like molecular inducer of epithelial hyperplasia would exert its influence to a lesser degree, thereby allowing OSE to return to the quiescent state.

The observation in the early 1980s that human fetal OSE and peritoneum (both derived from coelomic epithelium) differed in expression of the cell surface glycoprotein and epithelial differentiation marker, CA125 (Kabawat et al., 1983), led to the postulation that OSE (which lacks CA125) retains a degree of pleuripotency that enables it to alter its phenotype in response to changing physiological and environmental conditions (Auersperg et al., 2001). Thus one alternative mechanism is the conversion of OSE cells back to a mesenchymal phenotype, in other words, they become stromal cells.

A third possibility is that a marked increase in the rate of apoptosis in fetal OSE cells occurs toward the end of the third trimester. Evidence of apoptosis in OSE of fetal mice is lacking, and apoptosis occurs to a very limited degree or not at all in adult
However *in vivo* and *in vitro* studies involving adult OSE from animals other than mice have shown a role for prostaglandins (sheep) (Ackerman and Murdoch, 1993), progesterone (monkey) (Rodriguez et al., 2002), and the gonadotrophins (sheep) (Murdoch, 1995), (human) (Pon and Wong, 2006); alongside a number of growth factors including epidermal growth factor (EGF), (human) (McClellan et al., 1999), tumor necrosis factor (TNF)-α (sheep) (Murdoch and Lund, 1999), and ovarian hepatocyte growth factor (HGF), (rat) (Hess et al., 1999), in apoptosis involving OSE. Interestingly HGF induced apoptosis of OSE *in vitro*, only in the absence of the synthetic fibronectin-like extracellular matrix protein, pronectin, and raises the possibility that OSE may be unable to undergo apoptosis if separated from underlying basement membrane. Apoptotic cells lose contact with their neighbours and those cells lining cavities are sloughed into the lumen of the cavity (Murdoch, 1995). Thus a fourth possible fate for excessively produced OSE cells during fetal life may be one of exfoliation into the peritoneal cavity and subsequent degradation by macrophages.

Cell to cell adhesion plays a pivotal role in whatever fate becomes OSE cells during early pre-natal development. Cell coalescence, migration, morphogenesis and differentiation, communication, disaggregation and the activation of cellular signalling pathways; all require the involvement of cell adhesion molecules. One family of cell adhesion molecules known to have a prominent role to play in the differentiation of the gonad is the cadherins. There are three forms of cadherins, epithelial or E-cadherin, neural or N-cadherin and placental or P-cadherin; each named for the tissues throughout which they are predominately distributed. The cadherins form a family of calcium-binding trans-membrane glycoproteins, with E-cadherin considered to be the prototype classic cadherin (Kobielak and Fuchs, 2004). They possess both an extracellular cadherin (EC) binding domain that mediates the calcium-dependent binding of cadherins on adjacent cells to form adherens junctions, and a cytoplasmic binding domain. The cytoplasmic domain of E-cadherin couples to the catenins. The catenins are intracellular proteins essential to forming links between E-cadherin and the actin/myosin cytoskeletal network within the cell, thereby establishing stable cell-cell junctions. The domain structure of E-cadherin is shown in Figure 1.2.
E-cadherin domain structure (from Kobielak and Fuchs; Nature Reviews, 2004)

E-cadherin contains both an extracellular cadherin (EC) and a cytoplasmic cadherin domain. EC binds calcium, enabling it to undergo homodimerization at the cell membrane. E-cadherin dimers on neighbouring cells interact at the EC domain and concurrently induce integration of their cytoplasmic actin filaments. Stabilization of intercellular adhesion is dependent on the cytoplasmic domain of E-cadherin successfully binding β-Catenin, which in turn binds α-catenin, a molecule central to the recruitment of several cytoskeletal proteins including filamentous (F) actin, α-actin, and the actin-binding proteins; ajuba, viniculin, myosin and vesatin, as well as VASP, the vaso-dilator stimulated phosphoprotein family of F-actin elongating proteins. Seals between adjacent cell membranes and the structural integration of actin filaments across whole epithelial sheets, is reliant on the intimate association of these cytoskeletal proteins and the presence of p120 catenin, a catenin thought to have a role to play in E-cadherin turnover, at the cell membrane.
During embryogenesis, disturbed regulation of cadherin-mediated cell junctions causes severe developmental abnormalities, and mutations of adherens junction proteins are associated with progression to carcinogenesis and metastasis in later life (Kobielak and Fuchs, 2004). The exact nature of the extracellular cadherin-cadherin binding remains to be fully elucidated, but the arrangement must be fluid to afford adherens junctions a large degree of plasticity not seen in occludens junctions, of pivotal importance to cell migration during embryogenesis. E-cadherin is expressed in blastomeres of mouse embryos from the point of cleavage (Takeichi, 1988). Initially all cells express E-cadherin, but as cell differentiation progresses, some cell and tissue types lose expression. Most notable is the lack of expression observed in neural and mesodermal tissue unless they have differentiated into epithelia. Epithelial components of the urogenital system, including Müllerian duct epithelia and OSE are two such examples. Expression of E-cadherin in developing ovary, and particularly in OSE, has not been extensively studied. However Ryan et al (Ryan et al., 1996) obtained protein isolates from porcine ovaries, performed SDS-PAGE, immunoblotting, and Northern blot analysis and were able to analyse E-cadherin protein and mRNA expression in fetal ovary. During fetal development, high levels of E-cadherin expression were found in ovary, with levels diminishing markedly in mature animals (pre-pubertal, cyclic or pregnant).

When pooled OSE protein isolates were analysed, there were significantly higher levels of E-cadherin in adult porcine OSE samples than samples obtained from fetal and neonatal ovaries, and E-cadherin expression was furthermore concentrated in OSE compared to granulosa or theca cells of healthy follicles (Ryan et al., 1996). Interestingly, these researchers pointed out a potential limitation in their work, noting that an unexpected finding of E-cadherin in porcine thecal cells (non-epithelial in nature) raised the potential for contamination of this sample by other cell types. OSE, like theca, represents a very small proportion of ovarian tissue, thus results obtained from pooling protein isolates in this study should therefore be regarded with a degree of caution.

A second study examined E-cadherin expression in the developing mouse gonad using immunohistochemistry (Mackay et al., 1999). Immunostaining for E-cadherin in the
11.5 dpc gonad was noted in the epithelial cells of the mesonephric duct tubules, with expression becoming weaker toward the ventral margins of the tubules. This phenomenon most likely reflects the observation that the appearance of E-cadherin during development is indicative of epithelialization of mesenchymal cells, something that in the mesonephric tubules would proceed in a dorsal to ventral fashion. Post gonadal differentiation, E-cadherin expression followed a similar distribution to that of the pig embryo, with immunoreactivity persisting for Müllerian duct epithelia and epithelia of mesonephric derived tubule systems, all of which had undergone mesenchyme-to-epithelial transition. E-cadherin expression in OSE of immature mice has been shown to be upregulated by estrogen, (MacCalman et al., 1994), so it is possible that in the latter stages of gestation in mammals when estradiol levels are elevated, E-cadherin expression in OSE may be observed.

To conclude, this chapter of the thesis has presented a synopsis of the anatomy of the adult mouse ovary, and has provided an overview of the development of the ovary and female ductal system during embryonic and fetal life. The following chapter will aim to provide a detailed review of the functioning adult ovary throughout reproductive life. Emphasis will be on OSE, the role of estrogen, and the epidemiology and current hypotheses relevant to the development of ovarian cancer.
CHAPTER 2: The mature ovarian surface epithelium: phenotypic adjustment to physiologic events

2.1. Mature ovarian surface epithelium: a mixed epithelial mesenchmal phenotype

The mature ovarian surface epithelium has a role in transporting materials to and from the peritoneum in humans and the bursal space and peritoneum in mice. It undergoes repeated rupture and repair at the site of ovulation following menarche, and is separated from underlying ovarian stroma by basement membrane and a dense collagenous connective tissue layer, the tunica albuginea (white coat). The tunica is responsible for the white external appearance of the ovary. These two connective tissue components are barrier-like, restricting to some extent the diffusion of materials between stroma and OSE and visa versa.

OSE is a modified peritoneal mesothelium and has been described as having an inconspicuous histological appearance with few distinguishing features (Auersperg et al., 2001) (Leung and Choi, 2007). This description relates to the OSE comprising a monolayer of relatively uninteresting-looking epithelial cells that are generally squamous to cuboidal in shape, but are occasionally low pseudostratified and columnar (Wischnitzer, 1965) (Papadaki and Beilby, 1971, Anderson et al., 1976) (Blaustein and Lee, 1979). In reality, OSE is distinct from other epithelia of either ectodermal or endodermal lineage (breast and intestine respectively serve as examples), since it persists as a mixed epithelio-mesenchymal phenotype (Auersperg et al., 1994) (Kruk et al., 1994) (Auersperg and Woo, 2004). This is a reflection of its mesodermal heritage and the ability embryonic coelomic epithelium has to differentiate along many different pathways, in particular Müllerian. Adult OSE thus retains an uncommitted pleuripotent phenotype, which when stimulated by specific physiological events, may undergo epithelial-mesenchymal morphogenesis and demonstrate features normally associated with other mesenchymal-derived epithelia (Wong and Leung, 2007).

Clues as to OSE mesenchymal potential include its expression of the epithelial cytoskeletal intermediate filament, keratin (sub-types 7, 8, 18 and 19), and the mesenchymal-associated intermediate filament, vimentin in humans (Auersperg et al., 2001). Additionally, both OSE and extraovarian peritoneum have the ability to secrete
stromal collagens (types I and III) (Auersperg and Woo, 2004). Despite sharing embryologically linked ultrastructural characteristics, OSE and its neighbouring peritoneal epithelium differ with respect to many other characteristics (Blaustein and Lee, 1979). Morphological and histochemical differences between the two epithelia emphasize the uncommitted phenotypic nature of OSE. For example OSE cell shape is squamous to low columnar compared to the entirely squamous nature of peritoneal cells. Additionally, OSE has cilia, cytoplasmic lipid droplets are present, and the epithelium produces mucin and sulphated acidic and neutral proteoglycans not seen in peritoneal cells. Two other differences between these epithelia are particularly noteworthy and include the presence of 17-β hydroxysteroid dehydrogenase in the peripheral regions of OSE cells, indicating a role exclusively for OSE in steroid synthesis. Electron microscopy additionally reveals finger-like cytoplasmic interdigitations anchoring peritoneal epithelial cells into their supporting stroma. Such anchoring between OSE and ovarian stroma does not occur in human ovary (Blaustein and Lee, 1979) and may explain the remarkable fragility of mature human OSE, which can be exfoliated from the basement membrane simply by rubbing with a gloved finger (Gillett et al; 1991).

The integrity of OSE is aided to some extent by interdigitations of adjacent plasma membranes. Cell-cell junctions include desmosomes (Gillett et al., 1992), tight junctions, and gap junctions (Grazul-Bilska et al., 1997). Points of cell-cell attachment constitute functional syncitiums that provide both cell-cell adhesion and communication via the transfer of low molecular weight molecules. The junctional characteristics of OSE are common to many mammals including rat (Mayerhofer and Garfield, 1995), hamster (Ribeiro et al., 1983), sheep (Grazul-Bilska et al., 1997), mouse (Wischnitzer, 1965) and human (Papadaki and Beilby, 1971) (also reviewed by Auersperg, 2001) (Auersperg et al., 2001). Adult mammalian OSE secretes integrins (Kruk et al., 1994) (Cruet et al., 1999) (human) and both neural and epithelial cadherins (Sundfeldt et al., 1997) (Davies et al., 1998) (human) (Fleming et al., 2007) (mouse) (Ryan et al., 1996) (pig) (Auersperg et al., 2001, Wong and Auersperg, 2002) (human). Furthermore, human OSE secretes components of basement membrane; laminin, and collagen IV, alongside proteases and gelatinases involved in the
breakdown of matrix proteins (Kruk et al., 1994), presumably at ovulation, but possibly also in wound healing and tumor progression involving OSE.

2.1.1 The role of the ovarian surface epithelium in follicular maturation and ovulation

Perinatally, the mammalian ovary is endowed with what is still widely accepted to be a finite number of primary oocyte enclosed within follicular cells. These small follicles constitute the ovarian reserve (Gougeon, 2004), and in the rodent consist only of the smallest (primordial) follicles formed by day 3 of age (McGee and Hsueh, 2000). At this stage the follicle and its contained oocyte may remain quiescent; part of a resting pool of follicles, undergo degeneration, or further grow and develop - a process known as initial recruitment (McGee and Hsueh, 2000). During initial recruitment, most follicles are lost to atresia. That is to say they degenerate due to a loss of specific survival factor(s) that may be intraovarian or extraovarian, depending on the stage of development the follicle has reached.

In rodents, small growing follicles have up to 20 surrounding granulosa cells (GC) that form a single layer. Medium-sized follicles may have up to 200 GC and are multilayered, and large follicles could have in excess of 600 GC exhibiting multiple layers, and are notable for their formation of a fluid-filled antrum (Pedersen and Peters, 1968). Human follicles grow in the same manner but are progressively classified as transitory or intermediary (oocyte is surrounded by a mixture of flattened and cuboidal GC, primary (one layer of cuboidal GC surrounds the oocyte), secondary (two or more GC layers), preantral (> two GC layers and no antrum formation), antral, and finally selectable/ pre-ovulatory or Graafian (Gougeon, 2004). The latter represent the very largest follicles, which in the mouse ovary take approximately 60 days to grow and measure 500-800 μm in diameter (McGee and Hsueh, 2000), and in the human take some 220 days or roughly 8 menstrual cycles, and measure 2-5 mm in diameter (Gougeon, 2004).

In mammalian species, follicles leave the resting pool continuously (Gougeon, 2004), so that at any one time, follicles may be seen at various stages of development within
the ovary. Approximately 99.9% of ovarian follicles present at birth are destined to undergo atresia due to apoptosis (programmed cell death), and will never become ovulatory. Follicular degeneration may occur at any time during follicle development but is more likely to happen at antral stage or beyond, due to the follicle's increasing reliance on the gonadotrophins for survival (Markstrom et al., 2002). Follicles that have progressed up to antral stage are present in the human female just prior to birth. Rodent ovaries do not have follicles of this maturity until the third week of post-natal life when serum gonadotrophin levels become sufficiently elevated, even though follicle stimulating hormone (FSH) receptors on GC may be found by post-natal day 7 (McGee and Hsueh, 2000). In a series of labor intensive experiments involving blood sampling at 5 minute intervals for a total of 5 hours using an automated blood-sampling technique, Urbanski et al were able to show the onset of afternoon pulsatile luteinising hormone (LH) release occurred between peri-pubertal days 30-38 in female rats (Urbanski and Ojeda, 1985).

Once puberty is reached in mammals, increases in the amount of circulating FSH during each reproductive cycle acts to rescue a cohort of growing follicles from an atretic fate. These follicles are said to then undergo cyclic recruitment (McGee and Hsueh, 2000). A few will become large, pre-ovulatory follicles and provide a major source of the cyclic estrogen secretion during reproductive years. In the largest of these follicles, levels of estradiol in follicular fluid are reported to be in the micromolar range (Belin et al., 2000) (Gougeon, 2004). This compares to serum estradiol levels of approximately 20-60 pg/mL (Young, 1987) in women during the follicular stage of the ovulatory cycle and similarly low levels are seen in mice (Fleming et al., 2007).

Puberty in the human female is defined as the period of first becoming capable of reproducing, and is marked by the menarche or first menstruation (Witchel and Plant, 2004). In contrast, non-primates do not menstruate, but sexual maturity may be defined as first ovulation and/or first estrus behaviour. Moreover, the surge in pituitary gonadotrophins immediately preceding first and subsequent ovulations, is essentially the same in all mammals. In the rodent, puberty occurs around day 34 of post-natal life (McGee and Hsueh, 2000). Mice are polyovulatory, thus multiple large follicles are selected to ovulate with each estrous cycle. It may be postulated that in polyovulatory
mammals with high fecundity, greater physical stress is placed on OSE at ovulation as
enlarging follicles compress OSE from beneath, possibly causing ischemia. However
there is evidence for the existence of a genetically-determined difference in set point
for negative feedback signalling affecting hypothalamic control of gonadotrophin
secretion, and therefore the numbers of follicles selected to ovulate in these animals
(McNatty et al., 1986) (Spearow, 1986). This is indication of an internal regulatory
mechanism to protect OSE from ischemia. Furthermore, it is now known that
significant increases in the intrafollicular pressure of the dominant follicle do not occur
during the hours prior to ovulation (Espey and Richards, 2006, Motta and Van
Blerkom, 1975). Therefore ischemia may not have as deleterious effect on OSE as the
large dose of estrogen, found in the liquor folliculi of follicles, which is discharged
onto OSE during each ovulation.

Nevertheless, as ovulation approaches, the cells of the surface epithelium immediately
overlying the dominant follicle flatten, lose microvilli, and take on the shrivelled
appearance of necrotic cells. A high degree of vacuolization occurs in their cytoplasm
and they begin dissociating from underling basement membrane and tunica. In sheep,
OSE cells at the point of follicular rupture furthermore display many features of
apoptosis such as nuclear pyknosis, cytoplasmic shrinkage, and the dissociation of
junctional complexes (Murdoch, 1996).

The term ‘apoptosis’ was applied by Kerr and colleagues (1972) (Kerr et al., 1972)
when making the distinction between programmed (natural or physiological) cell death
and accidental cell death (necrosis). Apoptosis represents a cascade of events that
requires activators, effectors and negative regulators, the end product of which is a
contained fragmentation of the cell’s nucleus, such that an inflammatory response in
surrounding tissue is avoided (for review refer Huppertz et al; 1999, Fadeel et al;
2005) (Huppertz et al., 1999) (Fadeel and Orrenius, 2005). Apoptosis is highly
conserved across species and has a pivotal role in the differentiation of the organism
during embryogenesis. It is also fundamental to the maintenance of tissue homeostasis.
The latter may depend on the efficient clearance of cells following cell death, as much
as the control of mitosis and therefore cell proliferation (Fadeel and Orrenius, 2005).
Ovarian surface epithelium changes prior to ovulation are followed by those of the underlying tunica albuginea, theca, and granulosa cells lining the presenting wall of the pre-ovulatory follicle (mural granulosa). Mural granulosa thin precipitously, and all contributing tissues begin to disengage from each other. This process appears aided by the accumulation of extracellular fluid that infiltrates between basement membrane and surface epithelial cells, and has been seen on electron microscopy to transform intercellular spaces into expanded channels, pushing neighbouring OSE cells away from each other, and lifting them off the surface of the ovary (Motta and Van Blerkom, 1975). Progressively, OSE cells are sloughed from the surface (Espey, 1967) (Espey and Richards, 2006) (Motta and Van Blerkom, 1975) (Murdoch, 1996), while underneath, tunica and thecal-derived fibroblasts elongate and assume motile-like morphology. It is possible the fluid accumulation observed by Motta and Van Blerkom is linked to the inflammatory-like process of ovulation, which involves an increase in follicular vascularization. Changes to vasculature include increased fragility and permeability of blood vessel walls, resulting in extravasation of fluid from their lumen into the intercellular space. Dramatically increased follicular synthesis of estrogen prior to ovulation may act synergistically with inflammatory mediators to promote this localised edema, and hasten detachment of OSE from the surface of the ovary (Motta and Van Blerkom, 1975).

Since OSE sloughs from the site of follicular rupture before ovulation, and ovulation has been shown to occur in rabbits where OSE has been removed by scraping (Rawson and Espey, 1977), there is a general consensus that surface epithelial cells do not play an active part in ovulation (Espey, 1967) (Motta and Van Blerkom, 1975) (Bjersing and Cajander, 1974b) (Bjersing and Cajander, 1974b). Recent research in sheep has shown OSE cells to produce plasminogen activators (PA) (Murdoch and McDonnel, 2002); serine proteases involved in several types of tissue degradation, yet ovulation occurs in the absence of these enzymes also. These proteases are thought to be released from cytoplasmic granules, presumably l里斯omes, in OSE cells (Bjersing and Cajander, 1974a). It is likely that OSE-derived PA influence ovulatory events by inducing the activity of matrix metal proteinases (MMPs) that include collagenases, gelatinases, membrane-type MMPs and stromelysins (Espey and Richards, 2006). These enzymes are involved in breakdown of extracellular matrix (ECM) including the
tunica albuginea, theca and basement membranes of the follicular wall and OSE. PA production dramatically increases in the presence of two inflammatory mediators linked to ovulation; Tumor Necrosis Factor (TNF-α, and the cytokine interleukin1 (IL-1)β (Yang et al., 2004). Other inflammatory mediators with a role to play in the OSE response to ovulation are the prostaglandins and leukotrienes. Derived from arachidonic acid, these products of cell membrane phospholipids may be induced by any perturbation to the cell membrane including the activity of mitogens (Simmons et al., 2004). In intact ewes, expression of prostaglandin endoperoxidase, an enzyme that catalyses the conversion of arachidonic acid to prostaglandins, is increased in apoptotic OSE cells adjacent to the follicular apex at ovulation. The non-steroidal anti-inflammatory drug, indomethacin, blocks both prostaglandin and TNFα-induced apoptosis of apical OSE cells overlying the dominant follicle and has also been reported to inhibit ovulation (Ackerman and Murdoch, 1993), albeit at supra maximal doses (TNFα-induced) (Murdoch and Lund, 1999).

Apoptosis in apical OSE immediately overlying the ovulatory stigma in sheep is seen leading up to ovulation but prior to sloughing of OSE. It is succeeded by necrosis and likely contributes to the departure of OSE from the ovarian surface. Bystander cells, cells circumjacent to the ovulatory follicle have also been reported to show both oxidative and DNA damage, but usually undergo spontaneous repair by way of activation of enzymatic anti-oxidant defense mechanisms linked to the synthesis of progesterone (Murdoch et al., 2001) and may be regulated by vitamin E (Murdoch and Martinchick, 2004).

The complexities of interactions between OSE and stroma during ovulation are still to be fully elucidated, however the indisputable endpoint is rupture of the mature follicle at its most apical region in response to the mid-cycle LH surge, because this is where it is structurally weakest. Following rupture, adjacent surface epithelium proliferates to repair the ovulatory wound and accommodate the formation of new corpora lutea. Following human chorionic gonadotrophin (hCG) induction of ovulation, DNA synthesis has been shown concomitantly to peak during this period and remains increased for some days thereafter, eventually returning to basal levels without further stimulation (Osterholzer et al., 1985a).
Studying BrdU incorporation rates in cyclic rats, Gaytan et al., 2005, reported OSE cells overlying dominant follicles as entering a proliferative phase between proestrus and estrus, with approximately 1/3rd of total OSE proliferation related to follicle growth, and 2/3rds linked to ovulation repair and formation of corpora lutea. Furthermore, this study showed that ovulation-induced proliferation was not limited to the edges of the rupture site and did not require disruption of OSE integrity. Interestingly, no differential expression of estrogen receptor activity was reported throughout the estrus cycle regardless of whether OSE was quiescent or proliferating, although only ERα was studied. Moreover, progesterone receptor was not expressed at any stage of the cycle. Authors concluded the pattern of proliferation seen in this study likely reflected cyclically driven microenvironmental changes in underlying ovarian structures (Gaytan et al., 2005). These of course may include the paracrine effects of growth factors and the steroid hormones, estrogen and progesterone.

The process of ovulation has thus far been linked to OSE cells changing their morphology and progressively disengaging as ECM components alter or degrade. They become part of an inflammatory-like response, undergo increased rates of both proliferation and apoptosis, and slough from the surface of the ovary. However when OSE cells are placed in culture, they are additionally capable of assuming a fibroblast-like morphology, mimicking the characteristics of stromal fibroblasts and increasing cell motility (Auersperg et al., 1984). This feature of cultured OSE is presumably related to its uncommitted phenotype, and the process is known as epithelial-mesenchymal transition (EMT) (refer 1.2.2.3). EMT is hypothesized to be a mechanism that confers advantages during post-ovulatory repair, since it may limit the proliferative response of OSE at this time. Furthermore, EMT may represent a homeostatic mechanism for OSE cells that become trapped within ovarian stroma during ovulation. It has been proposed that the alternative; a failure of OSE to undergo EMT during ovulation, may lead to inclusion cyst formation within ovarian stroma (Auersperg et al., 2001). Inducers of EMT in vitro include Epidermal Growth Factor (EGF), collagen (Salamanca et al., 2004) (both present at the site of ovulation in vivo) and TGFβ, an autocrine regulator of OSE growth (Berchuck et al., 1992).
2.1.2 Pregnancy and the ovarian surface epithelium

Fetal OSE undergoes increasing amounts of proliferation during pregnancy and it may be proposed this occurs due to high circulating levels of estradiol (refer 1.2.2.3). Pregnancy is a time when maternal serum levels of estrogen rise dramatically in mammals (Johnson and Everitt, 1995), so the pregnant ovary may represent a third period when OSE undergoes hyperplasia. Gaytan et al, 2005, included in their study, BrdU labelling of OSE of pregnant rats, and reported gestational OSE as comprising extremely flat cells with only occasional BrdU label (Gaytan et al., 2005). They concluded this was due to the inhibitory effects of progesterone, which during pregnancy in rodents is produced in equally high amounts (up to 300mg/day) (Johnson and Everitt, 1995) to estrogen. The decrease in gonadotrophins and the complete lack of ovulation-induced perturbation to OSE during pregnancy cannot be discounted as contributing to these observations.

In vitro and in vivo observations of OSE during pregnancy are lacking, yet paradoxically Papadaki and Beilby (Papadaki and Beilby, 1971) reported on the OSE from ovaries of pregnant humans in the 12th week of gestation some 37 years ago using electron microscopy. They reported a cuboidal epithelium that essentially looked like that of non-pregnant women of gestational age, with some exceptions. There was very little interdigitation of plasma membranes linking adjacent epithelial cells, thus increasing the distance between cells, and forming more numerous intercellular channels. Additionally, the basal membrane of OSE cells became deeply in-folded and forged into underlying tunica. These researchers also commented on an increase in intracellular lipid inclusions in the OSE of pregnant women. Due to the paucity of information on OSE during pregnancy, it cannot be concluded that OSE does not undergo periods of proliferation at this time.

2.1.3 The older ovary

Mammals of advanced reproductive years have ovaries that increasingly display characteristics typical of reproductive senescence. Middle-age in mice (7-10 months) approximately matches the perimenopausal period in women, which begins around 4-8
years prior to the menopause (Treloar, 1981), the average age of which is around 51 years in women (Wise et al., 1999), and ends one year following last menses. Significant endocrinological, biological and clinical features are a hallmark of this period, a time coincidental with advanced depletion of ovarian follicles, so that by the menopause (last menstrual period in humans) ovarian follicular reserve is near exhausted (Faddy et al., 1992). In much of the literature, reports on the neuroendocrine and reproductive changes marking the perimenopausal transition have become blurred with those of the menopause. This is in part due to the overlapping of definitions provided for the terms 'pre-menopause', 'perimenopause' and 'menopause', which have lead erroneously to the overlapping of data, both biological and epidemiological (Prior, 1998). Neuroendocrine changes that accompany the transition to infertility in aging mammals are essentially the same in humans and rodents (Wise et al., 1999) (Danilovich and Ram Sairam, 2006). They include rising FSH levels, decreases in fertility and fecundity and the lengthening of ovarian cycles that become increasingly erratic. In humans, estrogen levels have been reported to rise within this period (Wise et al., 1999) (Shifren and Schiff, 2000) (Djahanbakhch et al., 2007). This loss of precision and synchronization of neuroendocrine signals is accompanied by changes to the structure and function of the gonad.

A decline in follicles within human and rodent ovaries culminates in larger amounts of stromal tissue with age. The surface of the ovary becomes increasingly irregular, forming protuberances, involutions and deep clefts (Clow et al., 2002) (Auersperg et al., 2001). OSE cells take on a columnar shape, especially in surface invaginations and cysts. Squamous and cuboidal cells are still abundant, their respective shapes in humans possibly signifying cells which have, or have not been, involved in post-ovulatory repair (Gillett et al., 1991). Stratification of OSE increases in the aging ovary and may be seen in cysts as well as on the surface of the ovary. Epithelial inclusion cysts occupy both cortical and hilar regions of the ovary, and appear to develop as a direct consequence of age rather than ovulation number per se (Fleming et al., 2007). Such inclusion cysts stain positively for E-cadherin, (and CA125 in humans) (Auersperg et al., 2001). Older mouse OSE has been reported not to express E-cadherin unless located in the rete ovarii, near the OSE-mesothelial juncture, or in inclusion cysts where papillary processes are a feature (Fleming et al., 2007). This E-
cadherin expression almost parallels that seen in humans, where OSE is reported as being devoid of E-cadherin expression, unless the epithelium lies in clefts (Sundfeldt et al., 1997) or lines inclusion cysts (Auersperg et al., 2001).

The older ovary additionally shows age-related alteration to gene expression. A recent study by Zimon et al. (Zimon et al., 2006) using microarray to compare gene and protein expression profiles between 6 week old 8 month old mice, cited increases in the levels of genes encoding inflammatory and oxidative stress response factors. There was also upregulation of the mRNA for steroid hormones and associated growth factors, concurrent with enzymes considered protective for the ovary from carcinogenesis.

2.2 Hormones acting on ovarian surface epithelium

2.2.1 Steroidogenesis in the ovary: an overview

Steroid hormones are lipids that have a common precursor, cholesterol, derived from acetate in body tissues and found abundantly in cell membranes (Johnson and Everitt, 1995). They comprise four fused sterol rings, with each carbon in the fused rings assigned a number and every individual ring, a letter. Substituent molecules that are bound to an asymmetric centre on any particular carbon ring are further designated as α, if they project below the plane of the ring structure, or β if they project above. Modifications to the ring structure of the steroid backbone and associated side chains define the class of steroid hormone generated, and substitutions at carbons 3, 7, 11 and 17 alter significantly the biological activity and metabolism of the hormone. Furthermore, receptors for the hormones distinguish between stereoisomers and will bind them accordingly. For instance, 17β-estradiol is recognized as the active form of the hormone and is bound by the receptor, while 17α-estradiol is inactive and is not bound. Naturally–occurring steroid hormones gain their nomenclature from the saturated ring structures of the parent compound and are divided into cholestanes (cholesterol is an example), pregnanes (natural progestins [progesterones], glucocorticoids, and mineralocorticoids serve as examples), androstanes (testosterone is an example), and estranes (estradiol provides an example). Additionally, gonanes
represent a class of synthetic progestins that include desogestrel, norgestimate and gestodene, found in third generation combined oral contraceptive preparations (Johnson and Everitt, 1995).

The synthesis of sex steroid hormones from cholesterol involves a series of sequential steps that invoke cleavage of side-chains, reorganization of olefinic bonds, and the addition of hydroxyl groups. The normal pathway taken is from cholesterol to pregnanes, then on to androstanes and arriving finally at the estranes (Strauss, 2004). Cells capable of steroid synthesis have ultrastuctural characteristics that enhance (and are indicative of) their steroidogenic activity such as mitochondria, abundant smooth endoplasmic reticulum (SER), numerous cytoplasmic lipid droplets, clathrin-coated pits housing receptors for lipoproteins, lysosomes, and microvilli. The substrate for steroid synthesis in these cells may be obtained in three ways: from blood cholesterol in the form of high-density and low-density lipoproteins, (HDLs) (LDLs), by de novo synthesis from acetate in SER, and via cytoplasmic lipid droplet accumulation. All pathways are stimulated by the trophic hormones (Strauss et al., 1981) (Grummer and Carroll, 1988) (Strauss, 2004) (Grummer and Carroll, 1988).

Conversion of cholesterol to pregnenolone in inner mitochondrial membrane is the first step in steroid synthesis. It is the rate-limiting step, requiring the enzyme cytochrome P-450scc (side chain cleavage). Upon stimulation from trophic hormones, steroid acute regulatory protein (StAR), required for the transport of cholesterol to the inner mitochondrial membrane, increases the availability of substrate required for side-chain cleavage and therefore is a potent regulator of steroidogenesis. The cytochrome P450 gene constructs are designated CYP, followed by a number, where the numeral refers to the carbon number where the enzyme is acting, thus P450scc is a product of the gene CYP11A1. Other members of the Cytochrome P450 enzyme family are involved in hydroxylation (P450c17 [CYP17], P450c21 [CYP21B], P450c11B [CYP11B1], P450c11AS [CYP11B2] and aromatization P450arom [CYP 19] reactions that alter the sterol backbone and facilitate steroid hormone conversion (Strauss, 2004).

Pregnenolone is converted to the sex steroids; progestagens, androgens and estrogens, in the SER (Johnson and Everitt, 1995). Another family of enzymes, the
hydroxysteroid dehydrogenases (HSD) catalyse bi-directional reactions and are involved in both the biosynthesis and inactivation of steroid hormones. They exist in several different isoforms (Penning, 1997) and cooperate with steroid sulphotransferases, reductases and steroid sulphatase to regulate the level of bioactive hormone in target tissues (Strauss, 2004). The localization and expression of the major enzymes required for ovarian steroidogenesis are summarized in Figure 2.1.
Figure 2.1 Key enzymes and pathways of steroid hormone biosynthesis and their location in the ovary (adapted from Essential Reproductive Medicine, Carr et al, 2004).
In the human ovary, the primary source of cholesterol substrate for steroid hormone production is LDL, whereas rodents preferably endocytose and bind HDL (Grummer and Carroll, 1988). Binding of LDL (or HDL) requires the trophic hormones, LH and FSH to activate the second messenger, cyclic adenine monophosphate (cAMP), which in turn increases the synthesis of mRNA for lipoprotein receptors, and additionally stimulates the formation of cholesterol esters (Gwynne and Strauss, 1982) (Carr et al., 1982) (Carr, 2004).

Steroidogenesis in the cyclic ovary requires the cooperation of at least two cell types that are stimulated to synthesize their product by independent upstream mechanisms. The synthesis of estradiol in the ovary is an excellent example of such cooperation. In the theca, pregnenalone undergoes sequential enzymatic conversion to androstenedione. However, theca lacks P450arom so cannot complete the final conversion to estrogen. Instead, adjacent granulosa cells do this using 17β HSD1, completing the conversion through to estradiol (Strauss, 2004). Note in Figure 2.1 this reaction is irreversible.

Furthermore mammalian theca and cells of the corpora lutea, the theca lutein, act together with granulosa lutein cells of corpora lutea, to produce sizeable amounts of both progesterone and estradiol. Steroidogenesis in the ovary is thus regulated by three factors; the availability of substrate, the relative amounts and type of enzymes in each tissue/cell compartment, and the actions of FSH on granulosa cells (for estrogen synthesis) and LH on thecal and luteal cells (pregnolone synthesis and progesterone synthesis respectively) (Carr, 2004). Following the menopause, peripheral estradiol levels in the blood are the result mainly of contribution from the adrenals and the peripheral aromatization of androgens to estrogen in adipose tissue and skin. Localized production of estradiol in tissues correlates with aromatase expression and in the case of progression to carcinoma of breast and uterine endometrium, aromatase activity is often excessive and inappropriate (Bulun et al., 1999).

The degree to which OSE is capable of steroidogenesis has only in recent years received attention, due largely to its unresolved role in ovarian epithelial cancer, and emerging epidemiological evidence linking estrogen to this deadly disease (refer 2.3).
In vitro evidence for aromatase activity has been demonstrated in human OSE using reverse transcription polymerase chain reaction (RT-PCR) (Imai et al., 1994), immunohistochemistry (Kitawaki et al., 1996), and RT-PCR in combination with aromatase assay. Using the latter technique, aromatase activity in OSE was found to be regulated at both the transcriptional and the translational level (Okubo et al., 2000). Immunohistochemical localization of aromatase function in OSE has been reported as cytoplasmic in both normal and carcinogenic ovaries obtained from post-menopausal women (Kitawaki et al., 1996), and aromatase activity has been seen in stromal cells adjacent to ovarian carcinoma involving OSE (Kaga et al., 1996). More recently using a combination in vivo and in vitro approaches, Cunat et al; 2005, examined aromatase expression in ovarian cancer cell lines and normal ovarian tissue using laser capture microdissection. Although only 2 ovaries (both from post-menopausal women) underwent analysis following microdissection, these researchers were able to verify using Real-time RT-PCR that in situ, normal human OSE has a six-fold higher expression of aromatase when compared to stroma (Cunat et al., 2005).

In keeping with ovulation representing an inflammatory event (Espey, 1994), and the established role of OSE in steroid inter-conversion, OSE in culture also expresses mRNA for HSDs required for the synthesis of progesterone, cortisone, testosterone and 17β-estradiol (Rae and Hillier, 2005). Moreover, normal OSE obtained from pre and post-menopausal women undergoing surgery for benign gynecological disease was found capable of the de novo synthesis of both estrogen and progesterone in confluent culture (Ivarsson et al., 2001b). Human OSE in culture also expresses steroid sulphatase, an enzyme required to convert estrone-3-sulfate and dihydroepiandosterone-3 sulphate (DHEA-3-sulfate) to estrone and DHEA respectively. Estrone sulphate (ES) is produced by the peripheral aromatization of adrenal androgens and is the most abundant circulating estrogen conjugate. Once the sulphur group is removed, estrone binds with low affinity to estrogen receptor. However this process makes more estrone available for conversion to estradiol via 17β HSD (Rae and Hillier, 2005). Estradiol is the most biologically active estrogen and binds with high affinity to its receptor.
Finally, older mouse ovary (refer 2.1.3) has been shown by aging-specific gene array to have significantly increased mRNA levels of aromatase, progesterone receptor, and estrogen sulphotransferase (EST), an enzyme that catalyses the conjugation of estrone to a sulphur group producing ES – essentially doing the reverse of steroid sulphatase. However, the up-regulation of the genes for these steroid-related entities in the older ovary was not followed by an increased expression at protein level using Western blot and immunofluorescence (Zimon et al., 2006). In particular, EST protein levels, localized predominantly to OSE, were significantly reduced. This finding may indicate that ovarian aging has an effect on the stability of proteins, and underpins the importance of establishing the in vivo activity of the functional protein.

2.2.2 Major hormone receptors on ovarian surface epithelium and their ligands

Gonadotrophin releasing hormone (GnRH) is a peptide hormone produced by neurosecretory cells in the hypothalamus (Johnson and Everitt, 1995). An abundance of literature supports a mechanism for these cells that when stimulated, they release GnRH in a series of pulses into the hypophysial portal vessels. Upon binding to receptors on the gonadotrophs of the anterior pituitary, GnRH facilitates the pulsatile release of the gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). GnRH has been shown to exist in two distinct forms; GnRH-I and GnRH-II, and although traditionally associated with the hypophyseal-pituitary axis, mRNA for the GnRH-1 receptor subtype has also been localized to human OSE (Wong and Leung, 2007, Choi et al., 2001).

Both GnRH and GnRHR mRNA are synergistically regulated in a biphasic manner, when exposed to a GnRH analog, demonstrating first a down-regulation, followed by an up-regulation of both receptor (R) and ligand. High doses of GnRH analog result in down-regulation of the receptor while low or pulsatile doses result in an up-regulation (Kang et al., 2000). A biphasic pattern of GnRH/GnRHR regulation has been reported in human ovarian granulosa-luteal cells in response to GnRH (Peng et al., 1994) and is likely receptor-mediated, since co-treatment with a competitive GnRH antagonist abolished this effect in monkeys (Leal et al., 1989). The overall effect of activation of GnRHR on human OSE cells in vitro has been reported as one of growth inhibition, as
confirmed by tritiated thymidine assay, moreover GnRHR is postulated to regulate its own levels on OSE (Kang et al., 2000). The expression of GnRHR is also regulated by ligands; estrogen, progesterone, testosterone, activins, inhibins and gonadotrophins.

FSH and LH receptors are G-protein coupled receptors expressed on granulosa cells of developing follicles (FSHR), theca cells surrounding early developing follicles, and on theca and granulosa cells during late follicular development (LHR) (Richards and Farookhi, 1978). Both play essential roles in follicular maturation and corpora luteal function. Female mice lacking fshr exhibit phenotypes that reflect extremely low levels of circulating estrogens, underpinning the role of FSH through FSHR as a major stimulus for the induction of aromatase activity in granulosa cells (Danilovich et al., 2000) (Danilovich et al., 2001), and therefore estrogen production. mRNA for FSHR and LHR, is expressed strongly on OSE both in vitro and in vivo (Zheng et al., 1996) (Parrott et al., 2001), and both pituitary and chorionic gonadotrophin ligands have been reported to stimulate proliferation of normal OSE from rabbit (Osterholzer et al., 1985b), cow (Parrott et al., 2001), human (Syed et al., 2001), (Choi et al., 2002) (Kuroda et al., 2001), and mouse (Davies et al., 1999). However some authors suggest activation of the gonadotrophin receptors results in decreased cell proliferation, induction of apoptosis, or no appreciable difference to growth (Wright et al., 2002) (Pon and Wong, 2006), (Ivarsson et al., 2001a). This discrepancy may be due to differences in the methods (in vitro; cell non-confluent/cell confluent or in vivo; subjects used /age of subjects) and doses of gonadotrophin administered.

Androgen, progesterone and estrogen receptor are sex steroid hormone receptors (Johnson and Everitt, 1995). They are members of the classical steroid hormone nuclear receptor superfamily which includes receptors for other steroid hormones, vitamins A and D retinoids, thyroid hormones and a large amount of hormone receptors for which there is no known ligand, the orphan receptors (Evans, 1988) (Laudet et al., 1992) (Kumar and Thompson, 1999). Nuclear hormone receptors are ligand-activated transcription factors able to transduce extracellular signals to regulate transcriptional responses in target cells. They traditionally reside in the nucleus or cytoplasm of the cell and in the absence of ligand are sequestered by inhibitory heat shock protein (hsp) complexes and are inactive. Lipophilic ligands for the receptor
diffuse across cell and nuclear membranes to bind their cognate receptor, disengaging it from hsp. This induces a conformational change in the receptor, facilitating its dimerization and nuclear translocation. Binding of the ligand/receptor complex to specific hormone response element (HRE) within target gene promoters then occurs in the cell’s nucleus to regulate gene expression (Couse et al., 2006). All members of the sex steroid receptors consist of functional domains. The first is an ND or A/B domain, a DNA-binding or C domain, a hinge or D region, and a ligand binding or LBD domain. Estrogen receptors further possess an F domain of unknown function (Couse et al., 2006).

It is now recognized that the classical sex steroid target gene activation described is not the only way sex steroid responsive tissues may be regulated. Target genes in hormone responsive tissues can be activated by alternative ligands binding to the plasma membrane in a mechanism involving cross talk between second messenger systems, and kinase signaling cascades. Phosphorylation and subsequent activation of the sex steroid receptors may thus occur without the need for HRE (Couse et al., 2006). Additionally, there is increasing evidence for the existence of a cell-membrane receptor to account for the rapid, (referred to as non-genomic) actions of steroid hormones, since both classical and crosstalk mechanisms are deficient in explaining hormonally activated responses in cells that occur within seconds to minutes (Losel et al., 2003). This currently much-debated mechanism of steroid receptor activation may potentially explain the immediate OSE response to estrogen.

The complementary DNA (cDNA) for dog, guinea pig, frog and mouse androgen receptor (AR) was cloned in 1991 (He et al., 1990), and all steroid receptors including AR have been localized to OSE of rat (Adams and Auersperg, 1983) and human (Karlan et al., 1995) in cell culture. Androgens; dehydroepiandrosterone (DHEA), androstenedione (A4), testosterone (T) and 5α-dihydrotestosterone (DHT), induce and maintain male phenotypia, and the type and degree of activation of AR impacts on the extent of virilization both in males and females. The presence of androgen receptor (AR) mRNA has been demonstrated in normal cultured human OSE from postmenopausal patients using using RT-PCR (Lau et al., 1999). Furthermore, OSE expression of AR protein has also been demonstrated using Western blot and
immunohistochemistry (Chadha et al., 1994) (Edmondson et al., 2002). In the latter study primary cultures of normal human OSE stained for AR with varying levels of intensity. Stromal AR staining in contrast was uniformly intense, as was staining in adjacent oviductal epithelium. Following administration of a specific AR analog that bound and activated AR without undergoing metabolism to estrogen, it was established that activation of AR results in significant inhibition of cell death and an increase in DNA synthesis (Edmondson et al., 2002). These results contrast those of Karlan et al, 1995, who reported androgens to have no effect on rates of OSE proliferation.

Progesterone is essential for the establishment and maintenance of pregnancy. Furthermore, it is an important regulator of ovulation and lutenization of ovarian follicles during the estrous/ovarian cycle. Progesterone receptor (PR) has been successfully cloned in many species including rat (Park-Sarge and Mayo, 1994), chicken (Conneely et al., 1986) (Jeltsch et al., 1986), rabbit (Loosfelt et al., 1986), human (Misrahi et al., 1987) and mouse (Schott et al., 1991) and exists in two isoforms: PR-A and PR-B. Both products of a single gene, these receptors perform different functions. PR-A (preferentially expressed in murine reproductive tissues) functions mostly as a strong ligand-dependent transcriptional inhibitor of steroid hormone activity, where PR-B acts as a transcriptional activator of progesterone-responsive genes (Giangrande et al., 2000) (Li et al., 2003). Mice lacking progesterone receptor fail to ovulate and exhibit severe reproductive tract dysgenesis and inflammation (Lydon et al., 1995).

Studies have localized PR expression in cultured human OSE cells (Lau et al., 1999) (Ivarsson et al., 2001b) (Li et al., 2003), OSE of both pre-menopausal and post-menopausal women (Akahira et al., 2002) (Lindgren et al., 2004), and in samples obtained from women with primary epithelial ovarian cancer (Lee et al., 2005). Working through PR, progesterone inhibits OSE cell growth by inducing apoptosis in vitro, a process mediated via the Fas/FasL signaling pathway leading to downstream activation of caspases 3 and 8 (Syed and Ho, 2003). In vivo, progesterone induces marked apoptosis and is associated with increases in the expression of TGF-β2/3 on primate OSE (Rodriguez et al., 2002). Interestingly, both PR isoforms have recently been reported as being differentially expressed in mouse OSE during the estrous cycle.
with PRA being the dominant isoform in ovary, but only PRB expressed in OSE (Gava et al., 2004). Most recently, estrogen has been identified as capable of inducing down-regulation of PR (Mukherjee et al., 2005).

Estrogens act to regulate female reproductive organ development, differentiation, growth, maturation, and function. In the female, estrogen reproductive target tissues include mammary gland, placenta, uterus, vagina, oviduct, and the ovary itself.

Estrogen also has effects on non-reproductive tissue such as bone, skin, and the cardiovascular and central nervous systems. During reproductive life, the ovary produces three estrogens; estrone (E1), 17-β estradiol (E2) and estriol (E3). Of these, E2 is the most abundant and the most potent circulating estrogen in pre-menopausal women. Estrogen’s effects are mediated by two estrogen receptors, ERα and ERβ. The alpha form of the receptor was cloned from humans in 1985 (Walter et al., 1985) and has since been isolated in several species, including mice (White et al., 1987). Nucleotide sequencing of the cDNA clone in mouse revealed the full length ERα to consist of 599 amino acids. This corresponds to 595 amino acids in humans. ERβ (549 amino acids in rodents, 530 amino acids in humans) was cloned ten years later from rat prostate by Kuiper and colleagues, (Kuiper et al., 1996) and subsequently from human leukocytes (Mosselman et al., 1996) with mouse homolog cloned the following year (Tremblay et al., 1997).

Both ERα, which has a predominant mRNA transcript of approximately 7 kb, transcribed from the ESR1 gene, and ERβ (ESR2) have been found to have variant transcripts in different species, and variants of ESR2 co-exist with the wild-type form (ERβ-1) within tissues (Couse et al., 2006). ER-β1 has recently been recognized as the obligatory partner in the formation of heterodimers with co-expressing ERβ variants in situ, and is the only stand-alone functional ERβ (Leung et al., 2006). It displays a preference for forming heterodimers with variants ERβ-4 and β5, which are both found in ovary. The variants appear to act as enhancers of ERβ-1 activity. Heterodimerization of ERβ-1 with a particular variant may be dependent on the relative availability of the variant sub-type and may explain tissue-specific responses to ER activation.
The sequence homology between ERβ and ERα in their DNA binding domains is 96%, while the ligand-binding domain (LBD) differs between the two receptors within the same species, sharing less than 60% sequence homology (Couse et al., 2006). This likely confers some of the diverse effects of activation of either receptor by the same ligand. For instance, both receptors bind diethylstilbestrol (DES) and E2, however ERβ binds E2 with slightly less affinity than ERα. ERβ on the other hand, binds certain phytoestrogens (plant estrogens) such as coumestrol and genestein and the endogenous metabolite of androgen metabolism, 5α-androstane-3β,17β-diol, with higher affinity than ERα (Kuiper et al., 1997) (Kuiper et al., 1998).

It is well established that the coexpression of ERs both in vitro and in vivo can lead to heterodimerization of the two subtypes (Cowley et al., 1997) (Pace et al., 1997, Tremblay et al., 1999) (Li et al., 2004) and that in these partnerships, ERα dictates the functions of the heterodimer in genomic signaling pathways (Li et al., 2004). Therefore, when investigating the distribution and effects of a specific receptor protein for ER, it would appear crucial to determine that the chosen antibody does not recognize and interact with variants within a subtype or show cross-reactivity between subtypes.

ER expression patterns are conserved across mammalian species and both receptor subtypes are co-expressed in reproductive tissues, however both interspecies and within species differences have been reported in ER expression patterns. For instance, in mice and rats, ERα expression is reported in theca and interstitial cells in ovarian stoma but is weak, often not reported in granulosa cells of follicles. In contrast, humans, (Hillier et al., 1998) (Brandenberger et al., 1998, Lau et al., 1999) (Jakimiuk et al., 2002) (Li et al., 2003), monkeys, (Chaffin et al., 1996, Duffy et al., 2000) cows, (Van Den Broeck et al., 2002) sheep (Juengel et al., 2006), and hampster (Yang et al., 2002) consistently express ERα in granulosa cells, usually of growing follicles. Table 2.1 lists recent publications detailing ER expression patterns in the mammalian reproductive tract. The list is not exhaustive and publications prior to the cloning of ERβ in 1996 have been omitted.
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<th>Reference</th>
<th>Species</th>
<th>Reproductive status</th>
<th>Imaging technique</th>
<th>Tissue processing</th>
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<td>CI</td>
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While differences in the pattern of estrogen receptor expression may be expected when comparisons are made across species, the same cannot be said for differences existing within species. The most extensively researched species is the rat, yet there is still disagreement on whether granulosa cells of growing follicles express ERα. It is likely the incongruent nature of ER expression within species relates to the use of several different methodological approaches, differing stages of the ovarian cycle when ovarian cells or tissue were obtained and in the case of immunohistochemistry, the choice of antibodies, immunoreagents, and their working concentrations.

One potentially confounding factor that should be considered when a purely molecular approach is employed to establish ER expression in ovarian tissue is that levels of ER mRNA do not always correlate with levels of translated immunoreactive protein (Brandenberger et al., 1998) (Jakimiuk et al., 2002). Taking ovarian surface epithelium expression of ER as an example, and with reference to Table 1.1, it can be seen that expression is located frequently on OSE of rat using immunohistochemistry, whereas cell culture and RT-PCR have chiefly been used to show evidence for these receptors in humans (Hillier et al., 1998), (Lau et al., 1999) (Li et al., 2003). A notable exception to this is the immunohistochemical study conducted by Lindgren et al (Lindgren et al., 2004). Whether the staining patterns of both ER were similar or different in human OSE is not known, since this one study did not show images of the two receptors on OSE simultaneously.

Recently cultured mouse OSE has been reported to express ERα (Symonds et al., 2006), and both ERα and ERβ have been located in cultured cells using Real Time PCR in conjunction with Western blot (Kipp et al., 2007b). There are however, no studies of both ER subtype expression using immunohistochemistry in adult mouse OSE. Indeed, it has been concluded that OSE of adult rat and mice lack ERβ immunoreactivity (Couse et al., 2006).
2.2.3 Growth factors and regulatory pathways acting on OSE

2.2.3.1 Growth factors with a proliferative or survival influence on OSE

Aside from hormones, the ovary is an abundant source of growth factors and cytokines. Both are thought to be key regulators in the response of OSE to its environment and act synergistically with hormones. OSE expresses receptors for epidermal growth factor (EGF), both in vivo and in vitro. EGF is a powerful mitogen, especially when in combination with hydrocortisone (Salamanca et al., 2004). It is also found in stroma, developing and atretic follicles, theca, corpora lutea, and in blood platelets. The distribution of both EGF and its receptor in the ovary appear to change as a function of follicular maturation and regression (Reeka et al., 1998) (Maruo et al., 1993). EGF and its homologs; amphilregulin and transforming growth factor alpha (TGFα) cause OSE proliferation in an autocrine/paracrine manner in bovine and human OSE (Doraiswamy et al., 2000) (Jindal et al., 1994).

Additionally, EGF is proposed to have a role in differentiation, since it promotes epithelial-mesenchymal transition of OSE cells in cell culture (Reeka et al., 1998). Links have therefore been drawn between EGF and post-ovulatory repair mechanisms involving OSE. It should be recognized, however, that under culture conditions OSE will spontaneously lose epithelial markers, acquire mesenchymal markers, and assume a fibroblast-like morphology (Auersperg et al., 2001).

At least six other growth factors have been shown to stimulate OSE proliferation and/or promote survival in normal ovary. Keratinocyte growth factor (KGF), a member of the fibroblast growth factor superfamily, is produced by mesenchymal-derived stroma. Like most growth factors acting on OSE, KGF mediates interactions between OSE and stromal cells, as well as between theca and follicular granulosa cells (Parrott et al., 2000). Other members of this family; basic fibroblast growth factor (bFGF) and acidic fibroblast growth factor (aFGF), have produced increases in growth rates and papillae formation in rabbit OSE cells in culture (Pierro et al., 1996). Platelet-derived growth factor (PDGF) is secreted by platelets in the clotting process and exists in three isoforms (AA, AB, BB) that bind to two receptors (PDGFlα and β). \textit{In vitro}, PDGF has
been shown to increase tritiated thymidine incorporation in normal human OSE in a
dose-dependent manner involving both receptor subtypes (Dabrow et al., 1998).
PDGF induces expression of vascular endothelial derived growth factor (VEGF) in
endothelial cells (Wang et al., 1999), and an endocrine gland-specific VEGF has been
found in ovarian issue (EG-VEGF) (LeCouter and Ferrara, 2002). Together, these
growth factors may influence vascular permeability and angiogenesis in ovarian
stroma, (where they are expressed in human ovary) thereby influencing OSE by
indirect means (LeCouter and Ferrara, 2002). Both normal and neoplastic OSE
undergo autocrine regulation by insulin-like growth factor (IGF-1) and it’s receptor
IGFR, with downstream effects that mediate growth, development, cell cycle
progression, differentiation, tumor formation and metastasis, and resistance to
apoptosis (Nicosia et al., 2003).

2.2.3.2 Growth factors that inhibit and differentially regulate OSE
proliferation

Possibly the best studied anti-proliferative regulator of OSE growth is TGFβ. The
TGFβ family represents a large structurally related family of polypeptide growth
factors that control a multitude of processes including cell proliferation, differentiation,
migration, adhesion, motility, and cell death. TGFβ1 regulates growth of
mesenchymal/stromal tissue while having an anti-mitogenic influence on OSE via the
induction of cell cycle arrest (Massague, 1998), rather than apoptosis. In cultured OSE,
TGFβ acts in an autocrine manner to both inhibit basal OSE proliferation and that
induced by EGF (Vigne et al., 1994). The mRNA for other members of the TGF
family; the inhibins and activins (ligands and receptors), have been found to be
expressed on OSE in cell culture, however only inhibin was expressed at protein level
(Welt et al., 1997).

Pigment epithelium-derived factor (PEDF) is another growth factor secreted by OSE
and has an inhibitory effect on cell proliferation. Its effect on OSE is reversed by
treatment with estradiol (Cheung et al., 2006). One growth factor with observed
differential affects on OSE is hepatocyte growth factor (HGF) and its cognate receptor,
Met. HGF has been localized to OSE of rat, human and cow (Wolf et al., 1991)
(Parrott and Skinner, 2000). In rat OSE, HGF differentially induces mitosis or apoptosis depending on the presence of basement membrane, with apoptosis not evident in the absence of available ECM (Hess et al., 1999). HGF has not been localized to mouse OSE using in situ hybridization and immunoblot (Yang and Park, 1995), for reasons that may be species specific. Interestingly mouse OSE expresses Met and may bind HGF that has diffused from adjacent stroma. Furthermore, levels of HGF fluctuate with the ovarian cycle, indicating hormonal up-regulation of this growth factor. Human chorionic gonadotrophin (hGC) has been shown to regulate HGF in this manner in cultured rat OSE (Hess et al., 1999). Upregulation of HGF may also occur under the influence of estrogen, since stromal-conditioned media has been shown to increase ERα activity (Jiang et al., 2003).

2.2.3.3 Cytokines acting on OSE

Cytokines and growth factors are often referred to as one entity, since both are polypeptides. Cytokines are traditionally linked more to cells of the hematopoietic and immune systems. They exist as soluble extracellular proteins that regulate both innate and immunologically linked inflammatory reactions, cell growth, differentiation, development and repair, in order to promote homeostasis (Oppenheim and Feldmann, 2000). Cytokines of the Colony Stimulating Factor family and interleukins IL-1 and IL-6 have been found to be secreted by human OSE under culture conditions (Auersperg et al., 2001). IL-6 in particular, drives OSE proliferation (Marth et al., 1996). The ligand for the Kit tyrosine receptor, Kit ligand (KL), has additionally been localized to rat OSE and is proposed to work in a paracrine fashion to bind kit receptors found in theca, thereby stimulating OSE proliferation (Ismail et al., 1999). Macrophages, capable of locally secreting cytokines such as IL-1, IL-6 and tumor necrosis factor alpha (TNFα) infiltrate OSE in vivo, but only when OSE shows signs of metaplasia or lines epithelial inclusion cysts within the ovary (Gaytan et al., 2007). TNFα is a pro-inflammatory cytokine that is expressed on OSE and appears to be an inducer of argininosuccinate synthetase (AS), an enzyme regulator of vascular and ECM homeostasis (Szlosarek et al., 2007). The expression and activity of pro-inflammatory cytokines derived from macrophages on OSE draws attention to the hypothesis that ovulation is an inflammatory process.
2.2.3.4 Major signaling pathways

It is notable that the vast majority of growth factors and cytokines discussed herein work through tyrosine kinase receptors (RTK) on the cell surface and activate downstream signaling pathways. In the case EGF, the RTK is the ErbB receptor, of which there are 4. Binding to ErbB may activate 1 of 3 possible signaling cascades: the Ras/extracellular signal regulated kinase (ERK) pathway, resulting in increased proliferation and decreased apoptosis, the phosphatidylinositol 3-kinase (PI-3 kinase)/AKT pathway, resulting in cell survival and growth via the transcription of anti-apoptotic proteins and activation of other downstream initiators of protein synthesis; and the Janus kinase/Signal transducer and activator of transcription (JAK/STAT) pathway, promoting cell survival (Henson and Gibson, 2006). In contrast receptor serine/threonine kinases, activate SMAD to promote inhibition of proliferation involving OSE.

Growth factors and cytokines that respond to estradiol with proliferation of OSE include EGF, PDGF, Amphregulin, HGF (in the presence of ECM), Interleukins 1 and 6, and Kit Ligand, while TGFβ may inhibit estrogen-induced proliferation of OSE. The steroid hormone progesterone induces apoptosis of OSE and the signaling pathway involved is the Fas Ligand pathway (refer 2.2.2).

2.3 Ovarian cancer

2.3.1 Epidemiology of ovarian cancer: an overview

Ovarian cancer is the fourth leading cause of death in New Zealand women (Ministry of Health New Zealand Health Statistics, 2005) and the fifth leading cause of cancer-related death among women in developed countries (Choi et al., 2007). In 2005 190 New Zealand women succumbed to this disease, while 301 new registrations were also recorded (Ministry of Health New Zealand Health Statistics, 2005). A 2002 global comparison revealed New Zealand to have the second highest incidence of ovarian cancer in the world at 12.4 per 100,000 head of population (age standardized) (AIHW, 2006), second only to Northern Europe (13.3 per 100,000) in which Scandinavia ranked number 1 at 15 per 100,000. In contrast, Japan had the lowest recorded rate of
ovarian cancer at just 5 per 100,000 (Riman et al., 2004). Ovarian cancer incidence is also lower in African countries and amongst black populations, and in New Zealand the 5-year survival rate for ovarian cancer is lower in Maori compared to non-Maori/non-Pacific women (Jeffreys et al., 2005). A disturbing upward trend in the rates of ovarian cancer is apparent in Northern European countries, where cancer registries have tracked the occurrence of ovarian and other cancers for many years. The incidence of ovarian cancer in British women increased by 18% between 1975 and 2005, with an age-specific increase in incidence of 55% in women over 65 years of age during this time (cancer research U.K website).


Ovarian cancer is highly heterogeneous and one of the most complex of all human malignancies. Around 90% of all ovarian cancers however, are reported as arising from OSE and cysts derived from OSE (Scully, 1977) (Scully, 1995). Recently the fimbriated regions of the oviduct have also been implicated because tubular intraepithelial carcinomas of this region have been seen in women undergoing prophylactic salpingo-oohorectomy for BRCA mutations (Piek et al., 2001) and in women diagnosed with ovarian or peritoneal serous carcinomas (Kindelberger et al., 2007). However these tumors often coexist with OSE neoplasia. Increasing age and total ovulation number have both been implicated as factors contributing to OSE proliferation (Fathalla, 1971, Fredrickson, 1987, Clow et al., 2002) a feature traditionally linked to ovarian epithelial oncogenesis. Environmental factors proposed, but not proven to play a part in the genesis of ovarian cancer include infectious agents, use of talc on the perineum, asbestos, environmental pollutants, fat in the diet and smoking (Herbst, 1994). Reproductive lifetime events that offer the ovary protection from carcinoma are well documented epidemiologically, and include multiparity, lactation, hysterectomy or tubal ligation, and use of oral contraceptives during reproductive years (Riman et al., 1998). It has been reported that 5 or more years of oral contraceptive use is associated with a 30-50% reduction in ovarian cancer risk (Lukanova and Kaaks, 2005) and that the benefits of such use persist for 10 years following their cessation (Riman et al., 1998). In contrast, use of either combined, or
estrogen alone hormone replacement (HRT) for long durations once ovulations have ceased, statistically correlates with increases in ovarian cancer (Lacey et al., 2006). Anovulatory infertility, which includes luteinized unruptured follicle syndrome, is reported as being associated with increased susceptibility to ovarian carcinogenesis (Shoham, 1994) and is not associated with disruption to the surface epithelium. Ovulation induction in infertile women has additionally been linked to ovarian epithelial dysplasia (Nieto et al., 2001), a putative precursory status in the development of ovarian epithelial neoplasia.

A major known risk factor for ovarian cancer is a strong family history of ovarian and breast carcinoma, and to a lesser extent familial cancers of the colon and endometrium (Auersperg et al., 2001). Specific mutations involving the breast cancer tumor suppressor genes BRCA1 and BRCA2 increase a woman’s lifetime risk for ovarian cancer from 1-2%, to between 60-70%. BRCA genes are involved in DNA repair, control of the cell cycle, and apoptosis (Wong and Auersperg, 2003). BRCA germline mutations are linked to increases in cancer rates predominately of breast, ovary and prostate, yet have been observed in all tissues (Auersperg et al., 2001). This is suggestive of hormonal influences regulating BRCA gene function. Mice heterozygous for the Brca1 and the Brca2 mutations, although not displaying frank ovarian tumor development, have abnormal mammary and ovarian phenotypic responses to diethylstilbestrol (DES), an estrogenic compound (Bennett et al., 2000) and BRCA1 interacts with estrogen receptor (Wong and Auersperg, 2003), repressing ERα transcriptional activity (Zheng et al., 2001).

Although the gonadotrophins and androgens have also been implicated in studies to play a role in the development of ovarian cancer (Choi et al., 2002, Edmondson et al., 2002) (Syed et al., 2001), several independent epidemiological studies have suggested that long duration of unopposed estrogen use is associated with increased risk (Rodriguez et al., 2001), (Lacey et al., 2002), (Riman et al., 2002) (Folsom et al., 2004) (Mills et al., 2004, Moorman et al., 2005) (Pearce et al., 2009). In a small follow-up study of 908 women who had received oral Premarin (estradiol valerate) for climacteric symptoms, the risk for developing ovarian cancer was two to three times greater than expected based on researchers’ previous data. In this study, increased risk
was associated with the strength of the dose and not the duration of use or the total amount of estrogen taken. However of particular interest is that of the women who were highest risk for developing ovarian cancers, most had previously also taken stilbestrol (DES) during pregnancy in the years prior (Hoover et al., 1977), suggesting a cumulative risk may have existed.

Recently, Lacey et al (2006) reported that 10 years or more of unopposed estrogen therapy statistically correlated with ovarian cancer in women aged 50 -71 years (at baseline, 1995) who took part in the National Institutes of Health AARP Diet and Health Study (RR = 1.89, P = 0.004). This large study of 97,638 women, 214 of whom had ovarian cancer by the year 2000, also reported that five or more years use of either continuous or sequential estrogen plus progestin HRT was also significantly associated with ovarian cancer (RR = 1.82 and 3.09 respectively). Pearce and colleagues have in 2009 confirmed these findings using a large review of population-based case-control studies, cohort studies, and randomized trials, but have additionally found estrogen-alone hormone therapy to confer the higher risk of the two drug regimens (P = 0.004), with a relative risk over 5 years (RR (5)) of 1.22, ?< 0.0001 (Pearce et al., 2009).

Moreover, in a 2007 prospective observational study of 82,905 exclusively postmenopausal women, both current and past users (5 years or more) of postmenopausal hormone therapy demonstrated a significantly increased risk of developing ovarian epithelial cancer (RR = 1.41, RR = 1.52 respectively). In this study, risk was significant for unopposed estrogen (RR for 5 year increment of use = 1.25, P < 0.001), but not for estrogen plus progestin (RR for 5 year increment of use = 1.04) (Danforth et al; 2007). Studies finding increased risk of ovarian cancer related to the use of combined estrogen/progesterone hormone therapy are significant however, because evidence to date has pointed to a protective role for progesterone in the genesis of ovarian cancer (Rodriguez et al; 1998). Thus epidemiological data relating the type of formulation used, the dose and route of administration, and the timing and duration of use of hormonal therapy to the genesis of ovarian cancer are required and to date have been lacking. Very recently, however, a large Danish prospective cohort study conducted in women aged between 50-79 years of age found increased risk for
ovarian cancer amongst current users of hormonal therapy regardless of formulation, route of administration, or duration of use (Morch et al; 2009).

Interest in the hormonal etiology of ovarian cancer continues as new studies add weight to the proposal that estrogen is a major player. The most recent study literally adds weight to the estrogen hypothesis, linking women with a Body Mass Index (BMI) of $>30$kg/m$^2$, who have never used HRT and who do not have a family history of ovarian cancer, to a significantly greater risk of developing cancer of this organ compared with normal weight women (Leitzmann et al., 2009).

2.3.2 Classification, pathogenesis and current hypotheses on the genesis of ovarian epithelial cancer

Ovarian cancer tumors are named in accordance with the cell type they originate from, which in turn relates to the embryonic precursor cells. Tumors fall into three main groups: surface epithelial-stromal tumors (derived from coelomic mesothelium), sex-cord stromal tumors (derived from mesencyhme and mesonephros), and germ cell tumors (Chen et al., 2003, Lukanova and Kaaks, 2005). Epithelial cancers are further sub-divided into serous (accounting for approximately 50% of all cancers of the ovary and consisting of cells resembling oviductal epithelia), mucinous (5-10% of all cancers and comprising cells resembling endocervical/Müllerian and intestinal epithelia), clear cell (4-5%), endometrioid (10-25%, consisting of cells resembling the uterine endometrium), mixed (5% of all ovary cancers and consisting of two or more sub-types), and transitional cell or Brenner tumors (5% of ovarian cancers with cells that resemble the transitional epithilium lining the bladder). Furthermore, surface epithelial-stromal tumors are classed as benign if they are not aggressively proliferative and invasive, borderline or low malignant potential (LMP) if they are highly proliferative but exhibit no invasiveness, or malignant if they are proliferative and invasive (Chen et al., 2003). Tumors that are highly malignant and lack specific cellular differentiation, are termed undifferentiated, while tumors that are not designated a specific sub-type are referred to simply as adenocarcinomas.

Ovarian epithelial cancers are the leading cause of death from all gynaecological cancers in Europe and North America (Choi et al., 2007). Their lethality stems from
the fact that the vast majority of patients present with advanced disease and response to any therapy is poor (Herbst, 1994). The overall five year survival rate for ovarian epithelial cancer is 42% compared to 70% for cervical cancer, and 85% for endometrial cancer (Liu and Ganesan, 2002), (Ho, 2003), (Nicosia et al., 2003). The prognosis worsens with advanced stages of the disease. Once the tumor has spread within or outside the abdominal cavity, a mere 20% of patients will survive 5 years from the time of diagnosis (Friedlander, 1998).

In 30 years of increasingly intensive and novel approaches towards determining the etiology of this deadly disease, an understanding of its scientific basis remains poor and mortality rates continue to be extraordinarily high. There remains no reliable diagnostic indicator to serve as an early means for detection, since the majority of ovary tumors held for investigation in tissue banks derive from patients with advanced disease, and an animal model capable of mimicking these early changes has not been identified (Vanderhyden et al., 2004). Few animals naturally develop ovarian tumors, but those that do are older. Intriguingly, animals such as mice are polyovulatory yet rarely develop ovarian cancer. These observations may either indicate that animal models are too diverse to yield meaningful results in relation to the genesis of ovarian cancer, or they may as a result of their diversity, provide important information that so far has been overlooked.

To date some of the most valuable information relating to early changes in the ovary with progression to carcinoma have been obtained when women considered high risk for ovarian cancer have had their ovaries removed prophylactically. In a non-blinded study that compared the histologic appearance of these ovaries to the ovaries of women with no known risk factors for the disease, 85% had two or more, and 75% had three or more of the following histologic findings: surface epithelial pseudostratification; papillomatous outgrowth on the surface of the ovary, deep cortical OSE invagination, multiple papillary projections housing small cystic spaces (cyst adenomas), epithelial inclusion cysts, cortical stromal hyperplasia and hyperthecosis as well as corpora luteal and hilar cell hyperplasia. Interestingly, 30% of control subjects (mean age of 44.7 years) also demonstrated at least 2, and 10%, 3 of these same histologic features (Salazar et al., 1996). A second similar blinded study could not verify the presence of
all the above features in women with a mean age of 50-53 years, but found a significant increase in the presence of inclusion cysts and changes to nuclei of OSE cells in prophylactically removed ovaries (Wemess et al., 1999). Numbers of inclusion cysts did not correlate with parity and therefore may indicate that other mechanisms act to produce these cysts. Increases in the number of deep cortical invaginations and papilloma were also observed, but were in approximately equal abundance in control and at-risk ovaries. Unfortunately it was evident in this study that sampling was not random and was minimal (1-2 representative sections) from control ovaries. The authors state that the two independent pathologists examining the ovaries may have additionally applied the histological criteria used to assess the tissue to varying degrees.

Obtaining two-dimensional (2-D) information of biological worth from thin microscopic sections requires stringent independent observation and measurement. Much more information may be gathered relating structure to function and providing analysis of morphological change within a structure by translating 2-D data into 3-D quantities. Over recent decades, significant advances have been made in the quality and validity of such (stereological) methods (Mayhew, 1991). Where morphometry represents a two-dimensional quantitative method that uses a form of calibrated measure (a micrometer) to obtain a set of direct 2-D measurements from thin microscopic sections, stereology uses a test system, often composed of test points or lines laid over a known frame (or test area) to provide indirect estimates of quantities such as surface area, volume and constituent volumes, yielding meaningful and robust data. The latter technique requires a systematic randomized method of sampling, something that was not employed by Wemess and colleagues.

It is not yet established whether ovarian epithelial cancer occurs de novo or results from the progression of a dysplasia. If the latter applies, microscopic features identified in both normal older, and at-risk ovaries, may represent the earliest form of precursor lesion in epithelial ovarian cancers. It is now firmly established that OSE-lined clefts and epithelial inclusion cysts where OSE cells assume columnar shape, are preferential sites of both benign metaplastic processes and neoplasia (Auersperg et al., 2001). A greater vigilancy in the search for such precursors amongst pathologists, as Scully
alluded to in the 1990s (Scully, 1995) may therefore pay large dividends, not only for women at risk for ovarian cancer such as those 5-10% of women who carry the BRCA mutation (Wong and Auersperg, 2003), but also for the vast majority of women who develop sporadic ovarian carcinoma, and for whom at present there is no established causal mechanism.

Alterations to the ovarian surface epithelium that signal the propensity of OSE to undergo neoplastic transformation do not occur in isolation. They are accompanied by significant changes in the expression of proteins and other regulatory molecules on OSE. For instance, E-cadherin, considered the major intercellular adhesion molecule in most epithelia is expressed in human OSE, only where the epithelia shows signs of metaplastic progression (cortical inclusion cysts, invaginations and in cells of columnar shape). It is expressed constitutively in human oviduct, endometrium and endocervical epithelia, however, and is therefore a reliable marker of the normal Müllerian phenotype. When human OSE undergoes early neoplastic transformation, E-cadherin expression is upregulated. E-cadherin is thus considered to be an inducer of aberrant (Müllerian) epithelial phenotypia in OSE {Auersperg, 2001 #83}. The expression of P-cadherin (1.2.2.3) on OSE in response to pathogical changes is similar to that of E-cadherin (Auersperg et al., 2001). With advancing ovarian cancer, there is a loss of E-cadherin, with its reappearance coinciding with metastatic spread. Loss of ERβ expression relative to that of ERα also occurs as a result of OSE neoplastic formation (Brandenberger et al., 1998) (Lazennec, 2006). Alterations to other proteins with oncogenesis include loss of cellular retinol-binding protein 1 (CRBP1), a binding protein for vitamin A, and Dab2, a negative regulator of Ras- (RTK) -mediated cell growth and positioning. Mutations of the KRAS and cMYC oncogene, overexpression of BTAK/Aurora-A gene critical for chromosome separation and centrosome function (Cvetkovic, 2003), phosphatidyl inositol 3 kinase (PI3K) and its inhibitor PTEN, EGF-R and cFMS (colony stimulating factor) receptor have additionally been found. Concurrent with many of the above perturbations to gene and protein expression in OSE is the expression of CA-125 and mutations of P53 tumor suppressor gene (Auersperg et al., 2001).
The literature in recent publications reflects the opinion of a growing number of researchers in the field of epithelial ovarian cancer, that when sampling ovaries for the presence of possible precursor lesions, the adjacent oviduct should not be overlooked. In summary, three observations underpin this. First, ovary, oviduct, and uterus all derive from embryonic coelomic epithelium and therefore share a common Müllerian heritage. Secondly, women who have undergone tubal ligation and hysterectomy have decreased risk of developing ovarian cancer (Riman et al., 1998). Finally, there are an increasing number of studies where at risk women with known BRCA germline mutations undergoing prophylactic bilateral salpingo-oophorectomy, have been discovered to not only have occult ovarian tumors, but also occult carcinoma of the oviduct (Salazar et al., 1996) (Hartley et al., 2000) (Olivier et al., 2004). It should be emphasized that both ovarian and oviductal precursor lesions and occult tumors were discovered only at microscopic level.

Currently five hypotheses have been formulated to account for the development of an ovarian cancer:

1. The incessant ovulation hypothesis
2. The hormone hypothesis (gonadotrophins and steroid hormones)
3. The germ cell depletion hypothesis
4. The inflammatory response/oxidative-induced DNA damage hypothesis
5. The resistance to EMT hypothesis

The first three of these hypotheses are discussed in the following chapter of the thesis in relation to the choice of the mouse model used in this study. The inflammatory response/oxidative-induced DNA hypothesis has been suggested in the work done by Murdoch et al., 1999, 2001, where OSE cells exposed to inflammatory mediators over successive rounds of ovulation in sheep undergo irreversible oxidative DNA damage, pre-disposing them to mutation and subsequent oncogenesis. Both Vitamin E and progesterone can inhibit this progression (Murdoch and Lund, 1999) (Murdoch et al., 2001) (McDonnel et al., 2005).
Finally, the inability of OSE cells to undergo EMT has been proposed by Auersperg and colleagues. It provides a mechanism to explain the formation of epithelial inclusion cysts, as OSE cells, trapped in stroma or pinched off from OSE during the process of ovulation, retain their epithelial phenotype and become embedded in stroma. The formation of such epithelial inclusions may provide a microenvironment for OSE cells that favours access to growth factors such as EGF and prolonged exposure to an estrogen rich milieu. The ability of OSE to undergo EMT is significantly reduced with progression to the malignant state (Auersperg et al., 2001, Auersperg and Woo, 2004).

2.4 Overview of research and hypothesis for study undertaken

Epidemiological studies paradoxically point to increased risk of ovarian cancer in older women exposed to exogenous hormones in the form of HRT, while use of combined oral contraceptive preparations in young women during their reproductive years decreases ovarian cancer risk markedly. Older ovaries have undergone numerous ovulations, and every ovulatory event induces successive rounds of breakdown and repair of the OSE. Possibly of greater importance, however, is that each ovulation is preceded by the local production of high levels of endogenous estradiol. A broad hypothesis underpinning this thesis is that the perimenopausal period represents a critical period in the older ovary, much like embryogenesis, when additional exposure to exogenous estradiol may serve to augment the ovary’s natural exposure to this hormone and produce changes representative of early indicators of oncogenesis involving OSE.

This thesis describes and discusses the results of a series of experiments in mice that have been designed to be of an investigative nature. They attempt to elucidate the nature of the older OSE response to exogenous estradiol, and investigate the possibility that estradiol induces the expression of some estrogen-responsive proteins, while inhibiting others in the aging ovary. Further to this, the experiments aim to investigate to what extent these same proteins act synergistically to produce their effects on OSE, and if such effects correlate to those seen in OSE with progression to ovarian cancer.
The broad aims of these experiments were therefore to:

1. Expose the ovaries of older virgin mice to estradiol treatment over an extended time frame in vivo.

2. Investigate the hormonal concentrations of estradiol in the blood and in ovarian tissue during estradiol treatment to determine whether ovarian changes observed in vivo coincided with elevated levels of estradiol.

3. Determine if depot injections of estradiol valerate given to older mice specifically induce hyperplastic and metaplastic OSE change by conducting detailed light microscopic, histochemical, morphometric and stereological analyses of control mouse OSE, and comparing these findings to OSE from estradiol-treated mice following each hormone injection.

4. Complete a comprehensive stereological analysis of ovarian volumes and ovarian constituent volumes in control and treated mice to assess the effects of depot estradiol treatment on the whole ovary and on remaining ovarian structures.

5. Develop immunohistochemical protocol and use light and confocal microscopy techniques to investigate the location, distribution and differential expression patterns of ERα and ERβ estrogen receptor subtypes in ovaries of control mice, and make comparisons to ovaries of mice treated with estradiol valerate.

6. Use double and triple immunofluorescence label to enable concurrent localization of both ER subtypes in OSE of control mice and compare expression patterns to those in oviduct.

7. Use immunohistochemistry to study normal E-cadherin expression in OSE of control mice and compare this expression with that seen in oviduct.

8. Localize ERβ and E-cadherin in control OSE and oviduct using double label immunofluorescence and confocal microscopy.
CHAPTER 3: Using a mouse model to test the in vivo effects of a depot estradiol on the older ovary.

3.1 Introduction

A major hypothesis underpinning this thesis is that in older ovaries, ovarian exposure to estradiol will over time induce changes in OSE typical of early progression toward neoplasia. The development of a suitable animal model for the study of early events linked to ovarian cancer is crucial toward understanding the biological and genetic factors underlying the disease process, perhaps revealing early stage indicators or a suitable biomarker. Furthermore, an appropriate animal model will provide an important in vivo opportunity to trial intervention strategies aimed at circumventing or better treating the disease, thus offering some degree of hope to women at risk of developing ovarian cancer, who to date have been afforded none. Development of both in vitro and in vivo models has revolved around limited knowledge of the initiating events of ovarian cancer (Garson et al., 2005) and has therefore led to several different approaches. Herein a brief discussion of past and current in vivo models will establish the reason for the use of estradiol in the induction of perturbations to OSE and the choice of an older mouse model for this study.

One of the issues facing researchers attempting to develop an animal model of ovarian oncogenesis is that few animals spontaneously develop ovarian cancers. An exception to this phenomenon is the domestic hen, which has been shown to develop both ovarian adenocarcinoma and granulosa cell tumors of the ovary, and in addition displays relatively high rates of oviductal adenocarcinoma (Fredrickson, 1987). For this reason, it was initially believed an avian model may prove optimal to study the development of ovarian cancer. The observation that hens underwent extended periods of uninterrupted ovulatory cycles also formed the basis of the incessant ovulation hypothesis in the pathogenesis of ovarian cancer (Fathalla, 1971) and latterly the basis of a more recent study linking incessant ovulation in hens with accrual of DNA damage to ovarian surface epithelia (Murdoch et al., 2005). Frederickson’s large avian study revealed that although adenocarcinoma of the ovary occurred more frequently in hens, it was age-dependent, with tumors uncommon in hens less than two years old.
Furthermore, hens were shown to have a predisposition (possibly genetic or strain-dependent), to tumors involving the reproductive tract in general, with 45% of all hens in the study developing a form of reproductive pathology. Importantly, no correlation could be established between numbers of eggs laid and the frequency of ovarian tumors in hens (Fredrickson, 1987), thereby arguing against the incessant ovulation theory. Moreover, where the source of the great majority of human ovarian cancers is the surface epithelium of the ovary, those seen in the domestic hen were of ‘unknown derivation’ and exhibited marked variability in their morphology. Development of avian ovarian adenocarcinoma also reportedly showed no relationship to the hormonal milieu.

Spontaneous ovarian neoplasms have been reported to develop in mice (Tillmann et al., 2000) (Alison and Morgan, 1987) (Liebelt et al., 1987) rats, (Walsh and Poteracki, 1994) (Gregson et al., 1984), baboons (Moore et al., 2003), and monkeys (Kaspareit et al., 2007); and have been described in domestic animals such as pigs, horses, dogs and cats (MacLachlan, 1987). These studies not only reveal advancing age as a common theme in the occurrence of ovarian tumors across species, but also indicate that epithelial-derived neoplasms of the ovary occur more often in mice and domestic animals, than in other animals studied.

OSE has been implicated as the source of the majority of ovarian cancers (Scully, 1977) (Scully, 1995) and both rat and mouse OSE cells undergo spontaneous immortalization in culture. When engineered to transform in vitro, for instance with SV40 T antigen early genes, or permitted to undergo several passages in subculture these cells undergo spontaneous malignant transformation. Such cells can subsequently be xenografted into nude mice, and allow for the evaluation of the roles of oncogenes in neoplastic progression involving OSE (Vanderhyden et al., 2003). Alternatively, established cancer cell lines or OSE cells transiently cultured then via retroviral transduction, induced to express combinations of oncogenes, may be injected into the peritoneal space, or underneath the bursa in rodents. The limitations of these experimental approaches are two-fold. First, OSE cells are artificially induced to become (or already are) tumorigenic when they are introduced into the host, and second, the host is immunocompromised. Thus this approach with any animal model
cannot be used to clarify early events linked to oncogenesis, nor represent these events as they would unfold in an animal that has not undergone manipulation of the immune response.

In vivo models of germ cell depletion address the observation that the occurrence of epithelial invaginations and inclusion cysts, two putative markers of pro-neoplastic processes, increase with age (Clow et al., 2002) and heredity (Vanderhyden et al., 2003). OSE with stromal invasion and stromal interstitial hyperplasia, also deemed pre-neoplastic, additionally occur in experimental situations where mutations in mice result in defective follicular development and attrition of germ cells. (Murphy, 1972) (Murphy and Beamer, 1973) (Ishimura et al., 1986) (Danilovich et al., 2001). The development of ovarian tumors has been studied by inducing germ cell depletion in inbred and hybrid mouse strains with a number of approaches including xenobiotic chemicals known to be lethal to oocytes, X-irradiation, neonatal thymectomy; which results in an autoimmune response toward germ cells, advanced age, and other methods (for a review see Capen et al, 1995) (Capen et al., 1995) (Capen, 2004). Germ cell loss results in elevated gonadotrophins and it appears oocyte loss, increased levels of gonadotrophins and low circulating levels of estrogen are required to promote neoplastic growth of tubular adenoma in mice. Although the menopause is a time when both oocyte loss and increased levels of FSH occur in women, the intense stromal and OSE proliferation that leads to benign tubuloadenoma formation in oocyte depleted mice does not have an exact counterpart in women, however such neoplasms derive from OSE (Capen, 2004). A criticism of this model is that it assumes oocyte depletion in the ovary to be an all-or-nothing event. In other words, it does not account for the oocyte depletion that occurs throughout reproductive life, and ignores the potential importance that the natural acceleration of this process may have on the ovary during the perimenopausal transition.

Biskind and Biskind established in 1944 that elevated gonadotrophin levels lead to ovarian tumor formation in rats (Biskind and Biskind, 1944). Transgenic mouse models have been generated with disruptions to both LH and FSH receptor and both targeted gene disruptions result in ovarian pathologies that include benign neoplasms and cysts (Risma et al., 1995) (Risma et al., 1997) (Nilson et al., 2000) (Danilovich et
al., 2001). However observed neoplasms were of stromal, granulosa cell or sex cord origin and not from OSE. In these mice, sterility, chronically elevated levels of the gonadotrophins (and in some cases estrogen and testosterone) and increasing age were common factors. Connolly et al (2003) (Connolly et al., 2003) reported the first successful transgenic model of epithelial ovarian cancer using transgenic mice expressing the early region of Simion Virus SV40 under the control of a Müllerian inhibiting substance receptor promoter (tgMISIIRTAg). The promoter transcript is expressed in ovarian cancer lines and in ascites fluid taken from women with ovarian cancer, but is also expressed in normal mouse OSE. Unfortunately, newborn tgMISIIRTAg mice were found to already have tumors seen in adult mice, making this model inappropriate for the investigation of events linked to ovarian neoplasia (Garson et al., 2005). Targeted gene disruption has also recently been achieved by use of the Cre-loxP recombination system, which uses cre, a recombinase, and its associated binding site loxP, to selectively activate or inhibit genes in OSE. Cre-loxP is administered as an injection under the ovarian bursa in mice (Sauer, 1998). This approach, while producing an effect in what is essentially a microenvironment, has allowed for the modeling of ovarian neoplasia in immunocompetent hosts.

Finally, one line of in vivo investigation into ovarian neoplastic transformation involves studying the consequences of ovarian exposure to events and substances that occur naturally or may be introduced exogenously. Increased frequency of OSE stratification and invagination occur naturally with total lifetime ovulation number, while advancing age also correlates with increased frequency of inclusion cyst formation (Clow et al., 2002). Gonadotrophins will induce OSE proliferation in vivo (Davies et al., 1999, Hess et al., 1999) (Stewart et al., 2004) (Burdette et al., 2006) although FSH failed to stimulate proliferation in rabbit OSE (Bai et al., 2000). Pronounced OSE dysplastic changes including proliferation, surface invagination, papillary excrescences and inclusion cyst formation, have all been observed following the administration of testosterone, (Silva et al., 1997), estrone (Stein and Allen, 1942), DES (Silva et al., 1998) and estradiol (Silva et al., 1998) (Bai et al; 2000) (Stewart et al; 2004) (Gotfredson and Murdoch, 2007). In the case of estradiol, the most potent of the naturally occurring estrogens, proliferation of OSE and all other reproductive epithelia occurs in vivo at physiologic levels (Gaytan et al; 2005) (Stewart et al.,
Furthermore, estradiol is routinely prescribed for younger women in combined oral contraceptive preparations and older women in the form of hormone replacement therapy. For this reason, estradiol was considered to be the best candidate to study hormonally driven changes in OSE. It is noteworthy that no studies of hormonally induced OSE dysplasias have specifically looked at these changes as they occur in the older ovary. Elevated FSH and later, LH, decreased inhibin B production, increased variability of ovulatory cycles and accelerated follicle depletion, are common to both rodents and humans entering the period of reproductive senescence, (Wise et al., 2002) suggesting the choice of an older mouse model to be a suitable one. In a pilot study for this experiment, it was observed that OSE of 7-10 month old mice, age-equivalent to mouse middle age, responded with more cell stratification and frequency of papillae than younger OSE, suggesting that older ovarian surface epithelium was more sensitive to estradiol. An epidemiological consideration was that most ovarian cancers occur in older women, with only 15% occurring prior to the menopause, (Vanderhyden et al., 2003) and that estrogen HRT given to women during the perimenopausal transition and after menopause for relief of climacteric symptoms, significantly increases the risk of ovarian cancer (Rodriguez et al., 1995) (Lacey et al., 2002) (Lacey et al., 2006). Finally, no studies have addressed whether the ovarian response to estradiol is the same with first and subsequent exposure. To facilitate such a study of older OSE, an esterified estradiol preparation, Estradiol Valerate (Schering) was chosen, that when delivered subcutaneously should provide the same depot effect reported for women using similar forms of hormone replacement.

In summary: In order to address the first three aims of this thesis, an in vivo model was developed to facilitate the study of changes to OSE during elevated and prolonged periods of estradiol exposure in older ovaries. Secondly, definitive links between estradiol exposure and ovarian epithelial dysplasia were required to confirm that the changes seen in the ovary were likely to be due to the effect of the estradiol, and not to the indirect stimulatory effects of another hormone. Radioimmunoassay (RIA) was chosen for this purpose due to the availability of an assay with a high level of specificity and sensitivity.
3.2 Methods

3.2.1 Animals

Female virgin Swiss Webster mice, 7-10 months of age, were group housed 5 per cage under standard conditions of 12.00 hour light/dark cycle, with temperature and humidity control. Mice had access to pellet food and water *ad libitum*. Ethics approval was obtained from the University of Otago Animal Ethics Committee for all animal manipulations (application numbers 35/02, and 69/07). To establish whether mice had normal ovarian cycles prior to commencing experimentation, vaginal cytology was assessed daily in all mice for 4 consecutive estrous cycles.

3.2.2 Experimental design and estradiol treatment

This study was designed to assess the ovarian response to both a first and a repeat depot injection of estradiol given over one month (Figure 3.1). On day 0 of the experiment, animals were weighed and randomly assigned to 8 treatment groups, (n=5-7 mice per group). To achieve randomization, Graphpad Software (www.graphpad.com) was used to generate random numbers between 1 and 10 *ad infinitum*, and animals assigned to estradiol treatment groups if they drew an odd number, or control groups if they drew an even number. This gave all mice a 50% chance of being in either group. The process was repeated until the animal allocation for both groups was attained. Additionally, four mice each received either no intervention (NI) or a subcutaneous (SC) injection of castor oil vehicle (CO) and were sacrificed immediately to act as controls for the CO excipient. Of the other mice, experimental animals received a SC injection of estradiol valerate (EV), 10 μg body weight in CO, which provided the depot effect, while control animals received equivalent volumes of CO. A second EV injection was delivered to remaining mice on day 14, when it was estimated serum estradiol levels would reach a nadir. After sacrifice of NI and CO animals on day 0, mice were culled at the following time points: Day 2 (48hrs following first EV), Day 13, Day 16 (48 hrs following second EV) and finally on day 28. Vaginal cytology was assessed weekly throughout the experiment, and body weights recorded. Prior to being culled, mice were weighed and vaginal cytology repeated.
Figure 3.1
Experimental Design. Large arrows indicate timing of EV injections, while small arrows indicate the day mice were culled and samples obtained.
As the experimental drug protocol used in this study represented the first of this type in mice, the dose of EV and the decision as to when to deliver the second injection of the preparation, was based on available pharmacokinetic data outlining the half life of the drug \textit{in vivo} to be 4-5 days, maximum serum concentrations between 2 and 5 days post administration and pre-treatment levels 15 days post administration (Dusterberg and Nishino, 1982). This data was considered alongside that from a rat study in which a single intramuscular injection of EV (10\(\mu\)g/g body weight in sesame oil) resulted in immediate cessation of estrous cycles and gonadotrophin levels reaching a nadir by 11 days post injection, thereafter gradually recovering to basal levels (Brawer et al., 1986).

\subsection*{3.2.3 Blood and tissue collection for estradiol RIA}

Mice were killed by ip injection of pentobarbitone sodium 60mg/mL (0.1mL) and blood withdrawn for estradiol assay using a 24-gauge needle and ventricular puncture through the anterior chest wall under deep anaesthesia. After overnight refrigeration at 4\(^\circ\)C, blood was centrifuged at 1500x\(g\) to remove serum from pellet. Serum was stored at -20\(^\circ\)C until assayed. One ovary was snap frozen in liquid nitrogen and stored at -80\(^\circ\)C for ovarian tissue estradiol assay. Heart tissue was additionally snap frozen to provide a quality control for extraction efficiencies.

\subsection*{3.2.4 Estradiol extraction and radioimmunoassay}

Estradiol extraction (Appendix D) was performed overnight at 4\(^\circ\)C using 70% methanol. Excess aqueous phase was removed by Centrivap Concentrator, (Uniscience), and residue frozen at -20\(^\circ\)C prior to assay. Estradiol extraction was undertaken on two occasions for tissue estradiol analysis. To calculate extraction efficiencies, 50 pg/mL of estradiol was added to extract derived from heart tissue, which contains estrogen receptor but is not an estrogen-producing tissue. Heart tissue was chosen in addition for its complete absence of pericardial fat in the mouse, as fat contains estrogen synthesized from the peripheral aromatization of androgens.
Preliminary trials determined levels of estradiol were very low in cardiac tissue, often lower than the minimum detection limit of the assay (0.6 pg/mL), confirming the choice of cardiac tissue as an appropriate one for recovery estimates. Tissue estradiol assays therefore included analysis of heart tissue estradiol concentration performed in duplicate, with and without the added estradiol. Results generated from the tissue were used to check for percent recovery of the hormone. Extraction efficiencies of 82% and 86% were subsequently recorded for the two separate extractions.

Radioimmunoassay of estradiol 17β uses a procedure with principles common to competitive binding assays. Briefly, competition exists between a non-radioactive and a radioactive antigen for a fixed number of estradiol binding sites provided by the addition of an antibody; in the case of this assay, a rabbit anti-estradiol antiserum. The amount of radioactive estradiol antigen bound to the antibody is inversely proportional to the concentration of the free or unlabeled estradiol present. Separation of the free and bound fractions of the hormone is achieved with the use of a double antibody system, where the addition of the secondary antibody; goat anti-rabbit gamma globulin, results in a precipitation reaction that ultimately allows a measure of the amount of bound radioisotope labelled hormone. According to the free hormone hypothesis, it is the unbound hormone that is available to bind to estradiol receptors in target tissues and initiate estrogenic activity (Mendel, 1989). This assay was performed using the DSL-39100 3rd Generation estradiol kit (Diagnostic Systems Laboratories Texas, USA) according to manufacturer’s instructions (Appendix D). Standard curves were generated during a series of trials to investigate the appropriate dilutions for both serum and tissue estradiol assays. In serum assays the optimal dilutions for control samples for each assay were 1:4 and 1:2 respectively, while in EV treated animals; due to serum estradiol levels reaching high extremes, a dilution factor of 1:40 was used. In each case the diluent consisted of a serum zero standard lacking estradiol. Due to the small size of the mouse ovary sample, both homogenization of the tissue to provide sufficient elutant, and subsequent reconstitution of the hormone required relatively greater amounts of reagents than that used with larger tissue specimens, for instance with rat ovary. This had the effect of pre-diluting the samples. Therefore for ovarian tissue RIA, final optimal dilutions were 1:1 for control samples and 1:4 for EV treated samples in zero standard. Standards and controls were run in duplicate (or triplicate
where reagents permitted) for all assays, with unknowns in duplicate. An example of a standard curve from one of the assays is included (Figure 3.2). Interassay variation coefficients were estimated by calculating the mean estradiol concentrations generated by replicates of both low (10 pg/mL) and high (30 pg/mL) quality controls supplied in the RIA kit. A standard deviation was then calculated for the combined mean of low and high controls and the standard deviation divided by this mean and expressed as a percentage difference when compared against the same assay at a different time point. These calculations gave satisfactory inter assay variation coefficients of 8.02% (serum) and 8.57% (ovary). Intra assay variation coefficients were unable to be performed due to insufficient amount of quality control samples in the RIA kit that were required to generate the data, however duplicate sample results demonstrated high reproducibility within assays.
Figure 3.2
Estradiol standard curve. Individual standard curves were generated for each radioimmunoassay to measure serum and ovarian tissue estradiol concentrations. The x-axis shows standard concentration, which ranged from zero to 150 pg/mL, while the y-axis shows specific binding of the sample (B) divided by the unbound sample and is expressed as the % binding. As the amount of I-125 bound antigen is inversely proportional to the amount of unbound (free) hormone, then the maximal B/BO value is associated with the detection of the most minimal levels of estradiol in the standard/sample, while the minimal B/BO value is associated with the detection of the highest levels of estradiol in the standard/sample.
3.2.5 Uterine weight

Immediately following sacrifice, the abdominal cavity was opened and the uterine horns identified deep to the intestines and large amounts of adipose tissue. Once identified, both uterine horns were traced to their distal point of origin at the uterine cervix where they were dissected free. At their proximal end, the ovaries were seen within the bursal membranes positioned lateral to each uterine horn. Each ovary communicated with the uterus via a short oviduct segment. The ovaries, bursa, and proximal regions of the uteri were carefully stripped of surrounding fat, connective tissue and blood vessels, and retrieved from the abdomen. Once out of the animal, the uteri were divided from the ovaries and bursa, briefly stripped of remaining fat, and weighed using a Mettler Toledo AG204 electronic scale prior to immersion in fixative, paraformaldehyde 4% (w/v) in PBS. Uteri, alongside vaginal opening and vaginal cytology smears showing vaginal epithelial cornification, are evidence of the in vivo biological activity of estradiol (Dusterberg et al., 1985).

3.2.6 Ovary weight

Ovaries were dissected free of as much fat as was practical without damage to OSE, and weighed prior to fixation.

3.2.7 Statistical analysis

Analysis of RIA was conducted using Assay Zap software (version 2.0 Biosoft; Cambridge, U.K). All quantitative analyses are presented as mean ± SEM. Statistical significance was determined by two-way ANOVA and Bonferroni post test or t test using GraphPad Prism 4, version 4.0c statistical software (GraphPad Prism Inc; San Diego, California). Confidence interval was 95%. P < 0.05 was considered statistically significant.
3.3 Results

3.3.1 Vaginal cytology

Vaginal smears were obtained for 4 consecutive estrous cycles prior to the commencement of the experiment and revealed that although animals were cycling, some had prolonged estrous and/or diestrous smears, often lasting 2-3 days. Additionally, smears were taken 24 and 48 hours after the commencement of treatment on day 0, and thereafter every 4 days. Control animals displayed the same cyclic behaviour throughout; while EV treated mice had predominately estrous smears within 24 hours, with a few animals showing proestrous smears that became estrous within the following 24 hours. Thereafter, EV treated mice had persistent estrous smears and large rafts of cornified epithelial cells could be identified. Occasionally, small numbers of leukocytes could be seen surrounding cornified cells. The appearance of leukocytes bore no relationship to the length of time since last estradiol exposure, and was found to be associated instead with invasion of bacteria, indicating that in some animals EV treatment increased the frequency of vaginal infection.

3.3.2 Animal weight

Random assignment of mice to treatment groups resulted in a degree of variability in the weights of the animals on day 0 of the experiment. Control animal weights ranged between 35.2 – 57.8 g (mean 46.5g) and 29.6 – 61.8 g (mean 52.4 g) in EV treated mice. As this experiment required hormonal manipulation in older mice for a month long period, alterations to body weight were possible. A metabolic study of rats treated with estradiol valerate subcutaneously over an 11 day period revealed that treated animals gained weight, but at a slower rate than control animals (Dusterberg and Nishino, 1982). Results from the current study revealed no significant differences in body weight occurred between groups from prior to commencement of the experiment to the time of sacrifice (Fig 3.3). However an analysis of body weight within each group showed significant weight loss had occurred in treated mice between days 2 and 13 (following one EV injection) and days 2 and 16 (following two EV injections), with mice reattaining weights comparable to their own baseline levels and those of controls by day 28 (Fig 3.4).
Figure 3.3

Animal body weights obtained prior to the commencement of the study and at the time of sacrifice (which represents combined weights of animals sacrificed on days 2, 13, 16 and 28) were compared. There was no significant difference in body weight between the groups. Results are means ± SEM.

Figure 3.4

A comparison within animal groups revealed significant weight loss occurred in treated mice between day 2 and day 13 and day 2 and day 16 (P<0.05). By day 28, animal weights were similar to baseline recordings at day 0. Days of EV injection (2 and 14) indicated by arrowheads. Results are means ± SEM.
3.3.3 Uterine weight

The combined uterine weight (Fig 3.5) was obtained from mice at each sample time point. Forty-eight hours following the first estradiol injection (day 2), uteri were noticeably thickened and their increased density could be felt when cutting through the cervical stump with a scalpel blade. The denseness and rigidity of the uteri obtained from EV treated mice was a consistent finding throughout the experiment, with uterine weights approximately twice that of control uteri by day 28 following two depot estradiol injections.

3.3.4 Ovary weight

Preserving the integrity of OSE during resection and removal of adipose tissue was a priority for this study. As a consequence, some adipose tissue was inevitably left in situ and therefore small differences in the weights of ovaries may not have been discovered. The combined weight of ovaries taken from mice at each sample time point is shown in figure 3.6. No differences in ovarian weight were seen. Ovarian weight was additionally calculated as a percentage of total body weight, and revealed no significant differences within or between animal groups.
Figure 3.5

Combined uterine horn weight was significantly heavier in EV treated mice than controls at all time points (P < 0.05 days 2, 13, and 16; and P < 0.01, day 28). Arrows indicate day of EV injection. Results are means ± SEM.

Figure 3.6

Ovaries were weighed and the weights from each ovary expressed as a combined weight. There were no significant differences between treated and control animals at any time. Arrows indicate day of EV injection. Results are means ± SEM.
3.3.5 Serum and ovarian tissue estradiol levels

RIA was used to determine the effect of depot estradiol on serum and ovarian tissue estradiol concentrations at each time point (Fig. 3.7A). NI mice showed no significant differences in serum or ovarian tissue estradiol levels compared to oil injected controls, indicating CO did not alter steroid binding. Control mice had consistently low serum estradiol (range 13.88 - 43.9 pg/mL). Levels in EV mice were significantly elevated above control mice 48 hours after first EV injection (day 2), but were similar to controls by day 13. Forty-eight hours following second EV injection, (day 16) serum estradiol levels in treated mice were half those on day 2, but at this time, and at day 28, were still significantly elevated above controls.

Figure 3.7B depicts changes in ovarian tissue estradiol concentration at sample time points. Average estradiol levels in control ovaries ranged between 66.19 and 117.1 pg/mg of tissue over the course of the experiment. Notably, two animals had high levels of endogenous estradiol (>330 pg/mg). Ovarian tissue estradiol concentration rose steeply in treated mice by day 2, in tandem with serum levels, falling by day 13 to 37% of the values recorded on day 2. However, unlike serum, ovarian estradiol concentration remained significantly elevated above controls at this time. Second estradiol exposure again led to an increase in ovarian estradiol concentration within 48 hours. Similar to the pattern observed on day 16 with serum levels of the hormone, ovarian estradiol concentration rose to approximately half that seen on day 2, yet remained significantly higher than controls. Ovarian levels remained elevated in treated mice at day 28.
Figure 3.7A

Serum estradiol levels in treated mice were 36 times control values on day 2 following first EV injection (P < 0.0001), decreasing by day 13 to levels comparable to controls. 48 hours after second EV, levels were half that following first injection but were significantly elevated above controls (P < 0.05). At day 28 levels remained elevated (P < 0.01). Arrows indicate day of EV injection. Results are means ± SEM.

Figure 3.7B

Ovarian tissue estradiol levels followed a pattern similar to serum levels. By day 2, ovarian estradiol concentrations were more than 11 times higher in treated mice (P < 0.01), and were 3 times higher than controls at day 13 (P < 0.05). A second EV injection elevated tissue estradiol concentrations to 50% of that recorded in response to first EV (P < 0.05). Levels were significantly higher than controls at all time points however, including the final sample on day 28 (P < 0.05). Arrows indicate day of EV injection. Results are means ± SEM.
3.4 Discussion

The 7-10 month old Swiss Webster mice used in this study were representative of middle age in mice, and control animals retained estrous cyclicity. Notably however, cycles were variable in the amount of time spent in estrus and diestrus, with some animals showing estrous smears and/or diestrous smears lasting 2-3 days. Such variability within cycles is likely due to their aging neuroendocrine status (Wise et al., 1999) (Wise et al., 2002) and is reminiscent of the neuroendocrine-driven changes to ovarian cycles observed in perimenopausal women (Brann and Mahesh, 2005). In middle-aged rodents, there is both a delay and an attenuation of the LH surge, and there is alteration to pulsatile secretion of LH (Nass et al., 1984) (Cooper et al., 1980) (Wise, 1982). Moreover, there is an accompanying increase in FSH concentrations, evident especially during the secondary FSH surge that occurs in rodents in estrus (DePaolo, 1987). These age-dependent alterations to the pituitary regulation of ovarian cycles are considered to reflect changes in GnRH secretion during the transition to acyclicity, and attenuated steroid feedback for the LH surge (Brann and Mahesh, 2005). A sequential loss of neuroendocrine responsiveness first to estradiol and then to progesterone during reproductive senescence has been demonstrated (Tsai et al., 2004), although no corresponding changes to hypothalamic expression of ERα or ERβ mRNA have been found (Wilson et al., 2002). Interestingly, middle-aged rats have been observed to have significant elevations in circulating estradiol levels, which may contribute to altered positive feedback (Nass et al., 1984). Although levels of the gonadotrophins were not assayed as part of the current study, the observed deviations from normal cyclicity in control animals are possibly indicative of the aforementioned hormonal changes, and signal a progressive decline in hypothalamic and pituitary control over ovarian function during middle age. These observations thus support the choice of 7-10 month old mice as a suitable model with which to examine the in vivo effects of depot estradiol treatment on the older mammalian ovary.

In this experiment random assignment of mice to treatment groups resulted in a surprisingly small variation of only 1.7 g in the average weight of mice between groups. It was possible that if mice became stressed by interventions that included restraint to deliver oil-based subcutaneous injections, they would lose condition. The
data analysis reveals that over the first two weeks from the time of initial intervention, this was especially the case with EV treated animals (Fig. 3.2). EV treated mice lost weight from day 2 to day 16, but later regained weight and by day 28 were near baseline. In contrast, control animals maintained a relatively stable body weight over time.

It was evident from the outset that older mice were fatter than their younger counterparts. At the time of sacrifice it could be seen that much of this fat resided in the abdominal cavity. It is well documented that the transition to the menopause in women increases the central distribution of adipose tissue and risk of cardiovascular disease, which is rarely seen in women prior to the menopause (Tchernof et al., 2000). This suggests a hormonal mechanism for the regulation of central adipose accumulation in women, and there is convincing evidence for the existence of estrogen receptor in adipose tissue. Several studies have demonstrated the binding of labeled estradiol to both nuclear and cytoplasmic receptors in adipose tissue of rats, (Wade and Gray, 1978) (Gray et al., 1981) sheep, (Watson et al., 1993) and humans (Anwar et al., 2001). Furthermore, the extent of binding was markedly reduced by the administration of exogenous estradiol in ovariectomized and adrenalectomized rodents (Rebuffe-Scrive, 1987). Estrogen HRT in postmenopausal women has been shown to decrease abdominal and intrapelvic fat deposition, an effect that is ameliorated by the addition of testosterone (Mattiasson et al., 2002) (Davis et al., 2000). Taken together, results from these studies would suggest a possible role for the administration of exogenous estradiol in the weight loss seen in EV mice following treatment. Interestingly, a second estradiol injection was not associated with the same degree of weight loss afforded by initial exposure to the hormone.

The uterotrophic effects of estradiol are well documented in several species and involve cellular hypertrophy and hyperplasia, and increases to the amount of tissue bound fluid (Allen et al., 1939). Rodents administered subcutaneous estradiol show marked uterine growth, and a 4-fold increase in uterine weight has been reported in rats within 7 days of a single 0.3 μg dose, with uterine growth increasing over time (Fawcett and Deane, 1951). The increases in weight were attributed to greater height of uterine epithelium and accompanying increases in the size and number of endometrial
glands. Interestingly, the underlying stroma also showed significant hypertrophy of smooth muscle, considerable fluid accumulation, and an abundance of mitotic figures (Fawcett and Deane, 1951). These effects have been shown to be mediated by estrogen receptor in vivo, and ER is present in epithelial and stromal uterine compartments in adult uteri (Cooke et al., 1997). There is strong evidence to suggest that the in vivo effects of estrogen on uterine epithelium are mediated through stromal ER and not epithelial ER and are specific to ERα. Using ERKOα mice, Cooke et al showed that in recombinant mouse uterine tissue lacking stromal ERα, uterine epithelial proliferation could not be stimulated, even if epithelial ERα were present. The influence of stromal ERα on uterine epithelial hyperplasia is further supported by in vitro studies, where estradiol is not mitogenic for isolated uterine epithelial cells, but when stromal and epithelial cells are co-cultured, E2 significantly increases epithelial DNA content (Inaba et al., 1988) and if grafted in vivo, mitogenesis results (Cooke et al., 1986). Furthermore, uterine ER exhibits differential expression of ER subtypes with ERα expression stronger in benign hyperplastic processes, and diminished in atypical hyperplasia and carcinoma, while ERβ also diminishes during progression to endometrial carcinoma (Hu et al., 2005). The studies outlined thus provide support for the use of mouse uterus in providing a valid biological reference for the in vivo effects of estradiol in reproductive tissues, since evidence is provided linking uterine growth to estradiol exposure in mice, an effect which is mediated through ERα, and which requires the stromal compartment of the uterus.

Furthermore, estrogen’s effects on uterine tissue may be indirect via the paracrine activation of growth factors such as IGF and EGF (Gielen et al., 2007), a feature also linked to OSE proliferation. It can be proposed, therefore, that the mechanism of estradiol’s influence on uterine growth may have parallels in the ovary and underpins the importance of using in vivo techniques to assess the influence of estradiol on the ovary. In the present study, uterine weight was seen to be significantly elevated at each sample time point, but was more so at day 28. This result is in agreement with that of Fawcett et al (1951), and indicates the effects of estradiol on uterine epithelium may be cumulative.
The surface epithelium of the ovary contributes less than 1% of ovarian volume in normal human ovaries (Katabuchi and Okamura, 2003), so that OSE hyperplasia and hypertrophy, even if extensive, would be unlikely to have an influence on ovarian weight. Vaginal smears revealed treated animals to be in persistent estrus, since for the most part, they showed vast sheets of cornified epithelial cells. This was an indication of the anovulatory status of treated mice in response to EV, and further reflected the cessation hormonal feedback mechanisms. Such an ovarian state may lead to smaller ovaries as numbers of corpora lutea, and possibly large preovulatory follicles, declined. This was not reflected in ovarian weights, however, as no statistically significant differences were found between treatment groups. It may be concluded that in mice, where the diameter of the ovary is a mere 2-3 mm, and where retaining an intact OSE necessitated leaving at least some fat surrounding most ovaries, that weighing the ovary was not a sensitive enough indicator of changes to ovarian constituents contributing to the ovary mass. Therefore a stereologically based quantitative analysis of ovarian volume and ovarian constituent volumes was considered necessary.

An important part of this experimental design was aimed at addressing the hypothesis that changes which occurred in the ovaries of older mice over the course of the month long experiment were attributable to the actions of the exogenous estradiol administered, and not to the actions of another hormone. To ensure results reflected this, a commercial estradiol 17-β assay kit was used with a high affinity for estradiol and minimal cross reactivity to estrone and estriol, the two other naturally occurring antigens. Additionally, the assay had a very low detection limit of 0.6 pg/mL and an upper detection limit of 150 pg/mL. When samples were diluted to the requirements of the standard curve and a reliable standard curve generated, results could be satisfactorily obtained for both control animals with low basal levels of estradiol, and EV treated animals induced to have extremely high levels in the nanogram range. Furthermore, reproducibility of assay results was fundamental to accuracy. This was ascertained measuring interassay variation coefficients and ensuring these were within acceptable limits (< 10% variability is optimal).

The efficiency of estradiol extraction from tissue in each assay was also calculated by adding a known amount the estradiol 1-125 tracer to replicate tubes containing
reconstituted extract from heart tissue, and comparing the yield against the 50pg/mL standard replicates. These measures gave optimal validation of the assay data for these experiments in our laboratory. Baseline serum levels of estradiol have previously been reported in mice using the DSL39-100 3rd generation estradiol kit, and ovulating mice aged between 6-9 months were reported as having mean plasma estradiol levels ranging from 17.82-18.96 pg/mL. Older cycling 12 month-old mice had marginally higher serum levels of 24.49 pg/mL (Fleming et al., 2007). A mean estradiol level of 27.8 pg/mL was obtained across all time points in control mice in the present study and is comparable with those of Fleming et al.

Ovarian tissue estradiol concentrations have not been reported in mice, but levels of estradiol in human ovarian tissue are reported to be some 100 times that of serum (Lindgren et al., 2002) at normal physiologic values. It can be postulated that much of this estradiol comes from the follicular fluid of large pre-ovulatory follicles where levels of estradiol are in the micromolar range (Gougeon, 2004). The results of this experiment show basal ovarian estradiol levels to be substantially higher than serum levels at all time points. When mice were delivered their first injection of estradiol, serum and ovarian estradiol levels became significantly elevated within 48 hours. Although serum estradiol levels declined to near control concentrations by day 13, ovarian tissue levels remained elevated. A second estradiol injection (delivered on day 14) resulted once more in significant increases in serum and ovarian levels of the hormone, but surprisingly at this time, estradiol concentrations in both compartments were only half that observed on day two following first exposure to EV. Furthermore, although estradiol levels decreased in treated mice, serum and ovarian tissue estradiol remained significantly elevated at day 28, despite no further hormone administration after day 16.

Levels of circulating sex steroid binding globulin (SBG) are known to increase following estradiol administration in humans (Strauss, 2004), and may influence the amount of bound hormone in the blood, however adult murine liver does not make this protein (Janne et al., 1999). Changes to the amount of hormone bound to alternative plasma proteins may nevertheless contribute to the above findings of the present study. Additionally, hepatic up-regulation of the Cytochrome P450 (CYP) enzyme family,
responsible for the in vivo catabolism of estradiol, may have occurred following first EV injection. This would result in enhanced metabolism of estradiol and therefore less circulating hormone. Anaak et al, (2003), have reported elevated hepatic CYP3A9 in rats following estrogen treatment with estradiol benzoate (Anakk et al., 2003).

Finally, the results demonstrate for the first time that estradiol accumulates in a temporal fashion, both in the ovary and in the blood, following exogenous estrogen treatment. Interestingly the accumulation of estradiol in tissue has previously been reported in humans. High doses of estrogen (15mg/day) delivered to women has been seen to accumulate in ER-positive and normal breast tissue (Geisler, 2003). Estradiol is also retained in adipose tissue in humans, and in the brain, uterus and pituitary glands of rodents and squirrels (Gorzalka and Whalen, 1974). In post-menopausal women, estradiol valerate, specifically, has been linked to accumulation of estradiol in the plasma following repeat administration of the combined HRT drug Climodien (Zimmerman et al., 2000). The results presented herein raise the possibility that estradiol in the form of HRT may have cumulative effects that may sustain or further augment the effects of endogenous estradiol on OSE.
CHAPTER 4: Morphological and morphometric changes in the older mouse ovary in response to two depot estradiol treatments in vivo.

4.1 Introduction

A second overarching hypothesis in this thesis proposes that the period marking the transition to reproductive senescence in mammals represents a critical period where the ovary, and in particular the uncommitted OSE, is more sensitive to the influence of estradiol. It is postulated that this sensitivity is increased when older ovaries are exposed to additional estradiol from exogenous sources. This hypersensitivity may be expressed quantitatively in morphological and morphometric changes to the ovary. Secondly it is proposed these changes represent an exaggerated physiologic response to the hormone in the aging ovary, and that such a response will predominately involve changes to OSE.

Ambiguity exists in definitions of the perimenopause and the menopause in literature relating to reproductive senescence (2.1.3). As a result, data representative of hormonal changes during the menopausal to post-menopausal transition may have also been taken to be representative of the pre-menopausal to menopausal state (perimenopause) (Prior, 2005). This has led some researchers to question the accepted dogma, that during the perimenopausal period there is persistent decline in ovarian estradiol production due to dwindling follicle populations. An alternative view is that the perimenopausal ovary may, amidst marked anatomical and physiologic change, paradoxically increase levels of ovarian estradiol production by way of intermittent estrogen surges (Santoro et al., 1996, Prior, 1998) (Prior, 2005). Therefore this chapter begins with a table (Table 4.1), which documents morphological and other features related to the ovary, observed during developmental periods known to coincide with high levels of endogenous estradiol. The table highlights commonalities and differences in the response of the ovary to physiologically regulated estradiol levels at these times, and comparisons can also be made between features that exist in normal ovaries and ovarian epithelial neoplasms.
<table>
<thead>
<tr>
<th>Stage</th>
<th>OSE layers</th>
<th>OSE invagination</th>
<th>OSE papillae</th>
<th>Loss of OSE polarity</th>
<th>Chromatia aggregation</th>
<th>Presence of lacunae/cyst</th>
<th>Presence of microvilli or cilia</th>
<th>Lipid droplets ovarian tissue</th>
<th>Ovary E2 production</th>
<th>ER present OSE</th>
<th>Serum E2 levels</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal</td>
<td>Y - Y Y Y Y</td>
<td>Y YM - - -</td>
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<td>- - -</td>
<td>Y Y Y</td>
<td>-</td>
<td>High</td>
<td>Human</td>
<td>{Gondos, 1969 #93}</td>
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<td>- - -</td>
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<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>YM/C</td>
<td>Y</td>
<td>Negligible</td>
<td>-</td>
<td>High</td>
<td>Rabbit</td>
<td>{Milewich, 1977 #186}</td>
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<td>Y Y Y</td>
<td>Y</td>
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<td>Y</td>
<td>Baboon</td>
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<tr>
<td>Pregnancy</td>
<td>N N</td>
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<td>-</td>
<td>Y Y YM/C</td>
<td>Y</td>
<td>Negligible</td>
<td>-</td>
<td>High</td>
<td>Human</td>
<td>{Pepe, 2002 #108}</td>
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<td>-</td>
<td>-</td>
<td>Y</td>
<td>Sheep</td>
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<tr>
<td>Perimenopause/Middle age</td>
<td>Y Y</td>
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<td>Y Y Y</td>
<td>Y Y Y</td>
<td>Y Y Y</td>
<td>Y Y Y</td>
<td>High</td>
<td>Human</td>
<td>{Pepe, 2002 #108}</td>
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<tr>
<td>Menopause</td>
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<td>-</td>
<td>Y</td>
<td>Mouse</td>
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<tr>
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<td>Y Y</td>
<td>Y Y Y</td>
<td>Y Y Y</td>
<td>Y Y Y</td>
<td>Y Y Y</td>
<td>Y Y Y</td>
<td>Low</td>
<td>Human</td>
<td>{Pepe, 2002 #108}</td>
<td></td>
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</tr>
</tbody>
</table>

Table 4.1
Age stage associated morphological features common to normal mammalian ovaries and ovaries showing a cancer of the ovarian surface epithelium. A dashed line indicates researchers did not comment on this feature. Y-Yes N-No M-Microvilli C-Cyst.
Throughout fetal development and pregnancy, and during the transition to, and the time after reproductive senescence, there are several features common to the normal and the cancerous ovary. OSE cell layering, papillary processes, loss of cell polarity and cyst formation, represent some of the morphological features seen in these natural physiological states. Moreover, ovarian exposure to high levels of both locally-derived and, (apart from the post-menopausal state), high circulating levels of endogenous estradiol, are also common to both the normal and cancerous states. Furthermore, both neoplastic and normal OSE express estrogen receptor. It may be postulated from these observations, that under normal circumstances, fetal ovaries and ovaries from women exposed to high circulating levels of estradiol during pregnancy, derive protection from some endogenous factor, since non-benign ovarian neoplasms are a rare event in pregnancy and fetal life (Machado et al., 2007). This does not appear to be true in older age, since by far the majority of ovarian epithelial cancers occur during transition to and after the menopause (Vanderhyden et al., 2004). Thus an additional hypothesis relating to the older ovary is that the effects of estradiol may be cumulative and result in a loss of both structural and functional plasticity in OSE. In order to address this possibility, there is first a need to provide a brief summary of in vitro and in vivo studies into the effects of estrogen on OSE.

Auersperg and colleagues developed cell culture techniques used to characterize OSE in the early 1980s (Auersperg et al., 1984). Experiments exposing normal OSE cells to estradiol in culture have since yielded inconsistent results. Where some researchers have failed to identify a role for estradiol in proliferation of cultured normal OSE from women, (Karlan et al., 1995) (Ivarsson et al., 2001a) monkeys (Wright et al., 2002) (Wright et al., 2005) and sheep (Doyle and Donadeu, 2008), others have reported the opposite to be true in human (Syed et al., 2001) and rabbit (Bai et al., 2000). Moreover, the former findings do not agree with in vivo studies. As far back as 1942, Stein and Allen reported that injections of estrone into the bursal space of normal and hypophysectomized mice resulted in marked mitotic proliferation of ‘germinal epithelium’ using colchicine label (Stein and Allen, 1942). More recently, in vivo studies exposing normal OSE to estradiol have used BrdU and 3H-Thymidine incorporation into dividing cells to assess rates of OSE proliferation (Bai et al., 2000) (Murdoch and McDonnel, 2002) (Gotfredson and Murdoch, 2007, Stewart et al.,
2004), and strongly suggest a role for estrogen in an OSE proliferative response. The discrepancies that exist in vivo may be explained in part by the degree of variability in culture media and culture methods used; methods chosen to quantify rates of OSE proliferation, and the species under investigation (Auersperg et al., 2001). In order to ensure that OSE proliferation is due to estradiol and not due to androgens, estradiol metabolites, or the gonadotrophic hormones, it is necessary to use investigative methods that allow for the elimination of these variables. Primary cell cultures of OSE and immortalized non-tumorigenic cell lines have traditionally provided valuable information in this respect, however both FSH and androgens have again produced mixed results when assessed for their role in OSE proliferation in vitro (Karlan et al., 1995) (Edmondson et al., 2002). Estradiol metabolites are linked to both induction of and protection from epithelial carcinogenesis (Lord et al., 2002), however little is known of the effects of estradiol metabolites on normal OSE. Both E1 and E3 have been shown to produce mitogenic effects on OSE, but possess much lower affinity for ER (Syed et al., 2001) (Stein and Allen, 1942). In the previous chapter the use of a highly specific and selective estradiol assay to definitively quantify levels of E2 in EV treated ovaries at each sample time point, partially addressed this variable. Further investigative methods will be discussed later in this thesis.

Increasing evidence supports a role for the ovarian ECM and stroma in the induction of OSE proliferation by estradiol and in OSE expression of ERα (Bai et al., 2000, Jiang et al., 2003). The contribution of the ovarian stroma to E2-induced OSE proliferation via ERα appears not to be as a direct result of the contribution of stromal estradiol, but rather to indirect effects of paracrine factors; IGF, EGF, HGF, and possibly SDF-1 (Hall and Korach, 2003) by E2 (Jiang et al., 2003, Auersperg et al., 2001) (Hess et al., 1999). Such a mechanism has been established for estradiol-induced proliferation of both uterine and breast epithelium (Cooke et al., 1997) (Haslam and Woodward, 2003). It has therefore been suggested that in vivo studies of the OSE response to estradiol may be preferable to in vitro studies (Stewart et al., 2004) and the middle-aged mouse model was chosen for these and other contributing factors (3.1).

Middle age in rodents can be regarded as the reproductive and neuroendocrine equivalent of the perimenopause in women (Wise et al., 1999) (Wise et al., 2002)
(Danilovich and Ram Sairam, 2006) (3.1). A morphological description of 8 month old mouse ovary has previously been undertaken (Clow et al., 2002), however the description was limited to the ovarian surface and cysts. Moreover, a systematic quantitative analysis of ovarian volume and ovarian constituent volumes in middle-aged mice has not been reported in the literature. It is therefore the aim of this chapter to present a comprehensive morphological and morphometric analysis of middle-aged mouse ovaries that may offer insight into the dynamics of ovarian structures after many years of estradiol exposure. This information will be used to determine changes to ovarian morphologic and morphometric parameters when additional estradiol is administered to the aging ovary in the form of two depot estradiol valerate (EV) injections over 28 days (3.2.2). Analysis will largely focus on OSE.

4.2 Methods

4.2.1 Tissue collection and preparation for morphology, morphometric and stereological analysis

Using the mouse model and experimental protocol outlined in the previous chapter (3.2.1-3.2.2), mice were culled at pre-determined time points (Figure 3.1). Two of these time points, day 2 and day 16, provided a 48 hour time point to study the more immediate effects of estradiol on ovarian structure following each EV injection. The remaining time points, days 13 and 28, represented the furthest time from each EV injection, and allowed for the examination of any delayed, attenuated or cumulative effects on the ovary.

At the time of sacrifice, one ovary was dissected free of surrounding adipose tissue with care taken not to disturb OSE. Ovaries were immediately placed in freshly prepared 4% (w/v) paraformaldehyde (Appendix A) in 0.1mol PBS $1^{-1}$ for 4-6 hours at room temperature. This period of fixation for mouse ovaries was previously identified as allowing for optimum antigen retrieval while preserving tissue morphology. Following three changes of 70 % ethanol, samples were processed overnight in and embedded in paraffin wax (Appendix C). Blocks of processed tissue were trimmed and sectioned using a Leica Jung RM 2025 microtome (Leica Microsystems, Germany)
and a disposable knife (Feather, Cell diagnostics, Dublin). Immediately the tissue became visible in the block, ovaries were serial sectioned exhaustively to facilitate stereological analysis (Howard and Reed, 1998).

During ovulation and repair and periods of OSE proliferation, OSE cells alter their shape. While a thin fibroblast-like morphology is assumed during ovulatory repair, OSE cells often become columnar during proliferative episodes. Additionally, proliferation results in less space on OSE for greater numbers of cells, and may well provide a catalyst for increases in the number of surface involutions and the formation of deep surface invaginations and papillae (Auersperg et al., 2001). Since the present study aimed to assess a dynamic OSE phenotype, and provide an accurate set of quantitative data on both the nature and the rate of OSE proliferation, a decision was made to section ovaries at 3 μm. At this section thickness the likelihood of sampling all OSE cells, from the smallest (2μm) to largest (~8 μm) cross sectional diameter would be high. Additionally, areas of cell layering would be optimally visualized for quantitative purposes.

To accurately and efficiently acquire unbiased 3-D information from a 2-D section through any structure, it is imperative to ensure that all areas of the structure have an equal chance of being sampled. To achieve this it is necessary to introduce randomness at all sampling stages. As previously stated (3.2.2), animals were randomized to groups. At the tissue level, randomness relates to the position of sections within the object of interest; in particular the first slice taken through the object, and to the orientation of the slices (Gundersen and Jensen, 1987). To eliminate orientation bias, ovaries were isotropically oriented within tissue blocks so as to be invariant with respect to direction and therefore sampling, and the first section chosen from a random starting position generated by a random numbers table. For example, beginning from a random starting point between 1 and 63 (number 21), every 21st section was set aside for analysis. This systematic sampling strategy yielded a sampling interval of 63 μm from the 63 sections generated (spanning ~ 3969 μm). Of these, a uniform, random subset of sections (n = 6-10) with a known distance between them (t), were used to generate data for analysis.
This technique, known as systematic random sampling, was proposed by Cavalieri in 1635 (Gundersen and Jensen, 1987) and eliminates sampling bias. It has more recently been reviewed by Mayhew (Mayhew, 1991) and Gundersen et al, 1999 (Gundersen et al., 1999) and is demonstrated in Figure 4.1. Optimization of this technique requires an understanding of the effect of sampling density on the introduction of systematic sampling and measurement (point counting) error. Therefore the number of samples observed and the distance between observations should ideally produce a small coefficient of error. A selection of at least 5-7 animals per group and between 6-10 slices per ovary was shown through prior analyses of variance (ANOVA) conducted at all sampling levels, to be optimal for the purposes of obtaining ovarian volumes and ovarian constituent volumes in this study (as recommended by Gundersen et al, 1999 (Gundersen et al., 1999) and Mayhew, 1991 (Mayhew, 1991).

Selected sections were briefly immersed in 30% (v/v) ethanol, floated onto a 42°C water bath for expansion of the embedded tissue and collected onto polylysine-coated slides. Tissue adherence was further enhanced by placing all slides on a 45°C hotplate for up to 30 minutes, followed by incubation overnight in a 37°C incubator.
Figure 4.1
Systematic random sampling (Figure from Mayhew, T.M 1991). The organ is sectioned into a series of randomly positioned (uniform random) slices. Slice orientation is arbitrary. The numbers represent a quantity in each slice, for instance a constituent volume or number. In this case the sum of these is 535. Taking every third slice with a random starting point generates three possible samples (a, b, c). As the numerical sum of one set =178, then 3 x 178 (534) represents an estimate of the quantity in the whole organ. Corresponding values for alternative sets would be 540 (b) and 531 (c).
4.2.2 Staining and visualisation: morphology and histomorphometry

Sections were de-waxed in two changes of xylene and rehydrated through a series of gradedethanols: 100%, 95%, and 70% (two changes for 2 minutes each) before rinsing in two changes of tap water. After rinses in distilled water, sections were stained with periodic acid-Schiff (PAS) (Appendix A). This histological stain oxidizes glucose residues creating aldehydes that react with the Schiff reagent to yield a purple-magenta color. It therefore selectively stains tissues with a high proportion of carbohydrate, glycoproteins or proteoglycans. In the ovary, PAS is a valuable tool for the identification of basement membranes and the zona pellucida surrounding oocytes within follicles. Staining of nuclei was achieved with Wiegers iron hematoxylin for 5-10 minutes at room temperature, while counterstaining was with methyl blue-picric acid mixture for 30 seconds (Appendix A). Visualization of ovarian structures for qualitative morphological analysis was achieved by capturing images of each section using a D10 BXF RT color Spot digital camera and software (Diagnostics Instruments inc.) on an Olympus AX70 Provis microscope, with re-projection onto a Philips flat screen monitor.

4.2.3 Stereological and morphometric analysis of cell and tissue features and BrdU immunohistochemistry

4.2.3.1 Ovarian volume and ovarian constituent volumes

Ovarian volume was estimated using the Cavalieri estimator (Howard and Reed, 1998). This methodology is underpinned by the assumption that the volume of any object may be estimated from a series of equidistant parallel sections that transect it, and are separated by a known distance (t) (outlined in 4.2.1). Volume can be simply obtained by summatting the areas of all cross section profiles through the object, and multiplying this by t. In practical terms, estimating ovarian volume using this method required capturing images of all sample sections using the digital camera and software linked to the Olympus AX70 Provis microscope as previously described. They were subsequently projected onto a flat screen using the 4x objective lens. The image was saved and printed on to A4 paper. Using a 20 mm point counting grid positioned at random over each image, the cross sectional area was estimated for every section,
cross sectional areas summated for all sections per ovary, and the summated area multiplied by the distance between the sections.

Following adjustments made for the final magnification of the printed image, a mean ovarian volume was obtained from all ovaries per treatment group at each sample time point except day 0 (3.2.2). The equation used to quantify ovarian volume was:

$$V_o = t \cdot ap \cdot \Sigma p_o$$

Where:

- $V_o$ = the volume of the ovary
- $t$ = the interval between section samples
- $ap$ = the area occupied by one point, $p$ landing on the ovary (converted to a final magnification)

Example:

$$V_o = 0.378 \text{mm} \times 20\text{mm}/90\text{mm} \times 220$$

$$= 18.48 \text{mm}^3$$

For the point area ($ap$) to take into account the final magnification of the image, a 1mm graticule was photographed using the 4x objective lens. The image was printed onto A4 paper and measured with a ruler. On A4 paper, 1mm (1000 µm) became 90mm. This gave the true size of the point area, when divided by the length of one side of the point counting grid (20 mm).

Figure 4.2 provides an example of the point counting technique used to estimate profile areas and volumes.
Figure 4.2

Estimation of profile areas and volumes with use of a point counting grid (Figure from Mayhew, 1991). The point counting grid is positioned at random over images. The number of points (indicated by a $P$) landing directly on the profile (6) is an unbiased estimate of the profile area ($ap$). Since sectioning through the profile was also systematically random, then the number of points landing on a constituent entity of interest (in the case shown, the cell’s nucleus), would also be an unbiased estimate of the fraction of the cell volume occupied by the nucleus. This is directly applicable to the measurement of constituent volumes of structures within the ovary.
Volumes of ovarian constituents were additionally assessed using this method and included corpora lutea, large (antral), small (pre-antral) and atretic follicles, blood and lymphatic vessels, cysts and stroma. Follicles were classed as atretic if they had the following morphology: granulosa cells undergoing disaggregation and apoptosis (pyknotic nuclei), evidence of oocyte degeneration and oocyte shrinkage with eccentric location within the follicle. When administering high levels of exogenous estrogen, it is possible that animals will be induced to ovulate due to positive feedback of the estradiol on the pituitary, an event that should produce a sizeable decrease in large follicle volumes and a corresponding increase in the volume of corpora lutea by the day 2 time point. However, endogenous levels of the hormone required to induce a spontaneous LH surge in women (350 pg/mL) (Brenner and West, 1975) are considerably lower than those produced by a single EV injection of 10μg/g body weight in these animals (1.3 ng/mL) and so the outcome may differ. The development of ovarian cysts following a single EV injection has also been reported in Wistar rats, but took 8-9 weeks to develop (Brawer et al., 1986). This same study commented on a significant decrease in numbers of healthy follicles within 16 days of EV treatment, but notably used 10μm thick sections and conducted 2-D measurements of follicles to provide 3-D information without regard to ovarian volume. The present study took a stereological approach to investigate if these trends were apparent in older mice receiving a repeat dose of EV.

4.2.3.2 Measuring mean OSE cell volume using the point-sampled linear intercepts method

During periods of exposure to high levels of estradiol, many reproductive tissues respond not only with hyperplasia, but also with hypertrophy and metaplastic change. Such responses are well documented for epithelial tissues of the uterus, oviduct, mammary glands, and vagina in humans and rodents (Brenner and West, 1975) (Rubin et al., 2007, Rimoldi et al., 2007). Initially they are adaptive processes, but may also lead to neoplasia (Rubin et al., 2007). To assess if hypertrophy of OSE cells occurs in response to EV, a volume-weighted mean volume (Vv) of OSE cells was obtained 48 hours following both EV injections using the point-sampled linear intercepts method.
described by Gundersen and Jensen (Gundersen and Jensen, 1985) (Gundersen et al., 1988).

Ovary sections were obtained by systematic random sampling (4.2.1). Control and EV treated mice (n=5/group) had single cell linear intercepts measured from each of 7 isolated areas of ovarian surface epithelium chosen at random with use of a sine weighted frame. At least 100 individual OSE cells were measured per ovary. OSE cells are small entities, and a cuboidal OSE cell in the (estrogen) unstimulated state measures approximately 5-7 μm in diameter. For this reason, measurement of the cells necessitated a two-step process. The ovary was first imaged using the 4x objective lens, whereupon a sheet of acetate with a circle depicting a full 360° was positioned centrally over the image. Using a random number's table, a number between 1 and 360 was obtained. This number corresponded to the area where cells would be measured and a landmark was taken. Next, the landmark was relocated using the 40x objective lens and the cell(s) of interest viewed with the image projected onto a Philips flat screen monitor (4.2.2). A selection sine-weighted frame with a test probe containing intercept points (Fig. 4.3a) was appended over the image and a second random number obtained between 1 and 97 using numbers arranged with a non-equidistant sine-weighted orientation (Fig. 4.3b). The intercept test grid was placed over the frame in such a way that one of the intercept lines passed through the bottom left corner of the frame and through the designated random number. Figure 4.3a provides an example, using the number 60. Guided by the intercept grid using the Spot analysis software, a line was drawn and a measurement taken across the chosen OSE cells. Each cell measurement was recorded onto an excel spreadsheet and the number cubed. The following calculation was applied: $V_v = \pi / 3. T^3$ where $T^3$ (the intercept length) is the mean value of the cubed lengths of isotropic lines drawn through random points landing on OSE cells.
Figure 4.3a
An example of a sine-weighted frame with a test probe containing intercept points appended over top of the frame.

Figure 4.3b
Compilation of a non-equidistant sine-weighted orientation for selecting 3-D isotropic directions on vertical sections (from Sorensen, 1991 (Sorensen, 1991).)
4.2.3.3 Measurement of ovarian surface area and frequency of ovarian surface involutions

In the previous chapter it was evident that a single injection of estradiol rapidly induced significantly high levels of the hormone, not only in the blood, but also in ovarian tissue. When endogenous estradiol levels are high during fetal development and during neoplastic progression, OSE exhibits multiple areas of cell layering, OSE epithelial papillae, inclusion cyst formation, loss of OSE cell polarity, and nuclear irregularities (Table 4.1). It may be postulated, according to the critical period hypothesis presented in this chapter, that during the perimenopausal period and middle age in the mouse, intermittent high dose surges of this hormone when combined with previous longterm exposure to estradiol, will result in a similar but exaggerated OSE response. Periods of rapid OSE proliferation could therefore be responsible for OSE invagination and deep crypt formation. In the process, some OSE cells may become trapped and unable to undergo EMT due to the rapidity of the event. To test whether depot estradiol treatment was capable of inducing such a response, ovarian surface area was measured using the same sections generated for morphometric analysis (4.2.1) and SPOT RT software in conjunction with the Olympus AX 70 Provis microscope. Images were captured using the 4x objective lens and displayed on a monitor. The perimeter of the ovary in each section was manually scanned, surface area measurement obtained, and measures averaged for each ovary. The relative frequency of deep surface involutions in each ovarian section was additionally recorded, providing a semi-quantitative analysis of this morphological alteration. To quantify frequency of ovarian surface involutions, a 2-step process was used as described (4.2.3.2) to obtain areas for random measures and to perform the measurement, with the exception that after obtaining a landmark to measure surface involutions (and with the inclusion criterion satisfied), either the 20x or the 40x objective lens was used to better visualise the area to be measured. A sine weighted frame and intercept test grid were not required.

Prior to each series of measurements to obtain surface area estimations or frequency of OSE surface involutions, microscope objective calibrations were checked using a 1mm
calibration slide with 10μm divisions and the 4x objective lens, followed by the 20x and 40x objective lenses and images captured and stored. Using the Spot RT analysis software for measures of distance and area and for each magnification using the calibration slide, a line was drawn for a predetermined distance (say 100 μm) on the image of the slide. Next an exact same distance was measured on an image of ovary using the same magnification. This was to ensure that measurements taken at each magnification were correct for that magnification. To obtain the relative frequency of ovarian surface involutions, using the computer mouse, a line was drawn from the apical portion of the innermost cells lining involutions, to the apical portion of the outermost cells flanking the entrance. A concavity > 122 μm was the minimum inclusion criterion. This distance was chosen since it represented approximately the size of a mouse antral follicle. Follicles of this size and above protruding from ovarian stroma caused progressively deeper convolutions in the ovarian surface and were proportionately more often associated with OSE invagination.

4.2.3.4 Measuring OSE depth

The formation of OSE papillae is the result of excessive OSE cell layering and provides a further indirect measure of OSE proliferation. Cell layering does not necessarily result in papillae formation however, and accurate analysis of the OSE proliferative response to estradiol would be enhanced with a thorough quantitative analysis of the degree of OSE cell layering. To facilitate this, each section was again captured by digital camera and software on the Provis microscope using the 4x objective lens, and displayed on a monitor. A transparent sheet of acetate onto which a circle with lines depicting a full 360 degrees was positioned centrally over the image as described in 4.2.1.3. Using a random numbers table to provide a number between 1 and 360, three OSE sites were assigned for measurement per section with the image re-projected using the 40x objective lens. Calibration of objective lenses was as previously described. Measurements were taken from the basement membrane to the apical surface of the outermost OSE cell. The basement membrane was identified using combined PAS/Weigert’s haematoxylin stain (4.2.2).
4.2.3.5 Measuring total ovarian surface epithelial volume

OSE growth may be due to hyperplasia and hypertrophy. Both will result in increases to OSE cell volume due to increases in the amount of intracellular material, but estradiol may additionally raise intercellular volume by increases in the volumes of extracellular fluid. Motta and Van Blerkom (Motta and Van Blerkom, 1975) identified fluid accumulation in the extracellular space immediately prior to ovulation (2.1.1). Although undoubtedly an inflammatory response to some extent, this phenomenon is coincident with high endogenous levels of estradiol, and a role for estradiol in the accumulation of extracellular fluid is well established (Tollan et al., 1992) (Stachenfeld and Keefe, 2002). Additionally, a measurement of total OSE cell volume (as opposed to measurement of the mean OSE cell volume) will include areas of OSE cell layering. To acknowledge these factors, the volume occupied by the entire surface epithelium was obtained by multiplying the mean surface area of the ovary by the mean OSE depth for all ovaries examined.

4.2.3.6 BrdU immunohistochemistry

BrdU immunohistochemistry to directly assess rates of OSE proliferation was carried out the day immediately following tissue collection onto slides to preserve antigenicity. Additionally, Superfrost Plus slides (Biolab Scientific U.S.A) were chosen to maximize tissue adherence.

Animals and ovaries were sourced and prepared as previously described (3.2.1-3.2.2). Twenty-four hours prior to the time of sacrifice, all mice received three ip injections at 8 hourly intervals of 5 mg BrdU mL\(^{-1}\) (Sigma Chemical Co, St Louis, MO), prepared in 0.9% (w/v) sterile saline (0.2mL). Ovaries were weighed and fixed in 4% (w/v) paraformaldehyde in 0.1M PBS as previously described (4.2.1). Subsequently they were embedded in paraffin wax and serial sectioned at 3 \(\mu\)m. Systematic random sampling throughout the ovary was used to obtain a subset of 5-8 sections per ovary for BrdU analysis. Sections were dewaxed in two 5-minute changes of xylene and rehydrated through a series of graded ethanols (4.2.2). After two rinses in PB (3 minutes each), antigenic sites were exposed with Proteinase K mL\(^{-1}\) (Sigma) in 0.1
mg/mL in 0.1mol Tris-EDTA buffer for 30 minutes at room temperature (RT). Enzymatic activity was stopped with the use of TBS (Appendix C) containing 4 mmol CaCl\textsubscript{2} L\textsuperscript{-1} for 10 minutes at RT and three 5-minute rinses in PB (Appendix C). DNA denaturation was achieved with incubation in 4mol HCl L\textsuperscript{-1} for 10 minutes at 37°C. The acid was neutralised with 0.1mol sodium borate L\textsuperscript{-1} for 10 minutes at RT, followed by three 5-minute washes in PB. Primary antibody incubation was performed overnight in a moist chamber at 4°C using monoclonal mouse anti-BrdU antibody (Dako, Carpinteria, CA) diluted 1:50 in dilution buffer (Appendix C).

Primary antibody was removed by three 5-minute washes in PBS prior to application of secondary antibody; biotin-conjugated goat anti-mouse IgG (Amersham) diluted 1:200 in PBS. Sections were incubated for 1 hour at RT. Three 5-minute PBS washes followed to remove traces of secondary antibody. Endogenous peroxidase activity was quenched using 0.3%(v/v) H\textsubscript{2}O\textsubscript{2} in methanol and a 10 minute incubation at RT. Three 1-minute washes in PB removed the peroxide solution. Signal amplification was achieved with streptavidin-biotin horseradish peroxidase (Sb-HRP) diluted 1:100 in sterile PB and incubation for 1 hour at room temperature. After two further washes in PB, sections were developed in 3-amino-9-ethylcarbazole AEC Chromagen (Sigma) according to manufacturer’s instructions. Counterstain was with Gills # 1 hematoxylin for 4 seconds followed by rinses in tap water and distilled water. Sections were mounted using 90% glycerol. Small intestine acted as a positive control for proliferation of crypt stem cells, while negative control slides received dilution buffer in the absence of primary antibody.

BrdU immunohistochemistry was undertaken over a period of approximately four months during which time the ambient temperature in the lab rose to >30 °C. An incidental finding was that as ambient temperature rose above approximately 23°C, background staining became increasingly problematic. This was resolved partially by decreasing incubation times performed at RT, in particular the proteinase K enzymatic step (decreased by 10 minutes) and the AEC developing step (decreased from 10 minutes to 7). The most effective remedy was simply to perform BrdU immunohistochemistry on cooler days.
**BrdU labelling indices**

OSE cells were visualised using the Olympus AX 70 microscope and images captured and stored on a digital camera using the 4x objective lens. To establish whether rates of OSE proliferation in control ovaries were dependent on OSE location relevant to underlying structures, and to assess if they were affected by stage of the estrous cycle, a sample of 5 ovary sections from 5 mice representing all stages of the cycle was examined. Labelled cells were counted from OSE overlying corpora lutea, large antral, small antral, small pre-antral and all atretic follicles. Other sites included areas where OSE was lifting away from the surface of the ovary and where OSE overlay superficial vascular structures and cysts. Numbers of labelled OSE cells were totalled for each entity and results expressed as means ± SEM. To follow, a comprehensive analysis of overall rates of OSE proliferation was conducted on randomized samples to determine if differences existed between control and EV treated ovaries. Total OSE cell number and BrdU-positive cell number were obtained from counts around the perimeter of each ovary section. Analysis included 8 sections per ovary from 6 mice per group. Labelling indices were expressed as the percentage of labelled cells, and counts were averaged for each ovary.

Unstimulated OSE cells in primary culture double in number every 32 hours (Karlan et al., 1995). Estradiol’s effects on OSE are primarily proliferative (Syed et al., 2001) (Bai et al., 2000) (Stewart et al., 2004) (Silva et al., 1998), but this effect may be indirect, since both small and large doses of estradiol have been reported to arrest OSE cell division in vitro (Karlan et al., 1995, Wright et al., 2002) (Wright et al., 2003) (Wright et al., 2005) but not in vivo. In vivo, the presence of stroma and basement membrane may positively influence the mitogenic effects of estradiol via growth factors (Auersperg et al., 2001). The use of exogenous estradiol using live animals could therefore potentiate increases in rates of OSE cell division. To accommodate this possibility, and to acknowledge the opinions of experienced researchers who have called into question the reliability of pulse labelling to accurately assess rates of cell division (Hirshfield and DeSanti, 1995) (Burdette et al., 2006), additional stereological methods to quantify OSE hyperplasia were employed (4.2.3.2 - 4.2.3.5).
4.2.3.7 Statistical analysis

All quantitative analyses are presented as mean ± SEM. Statistical significance was determined by two-way ANOVA and Bonferroni post test or t test using GraphPad Prism 4, version 4.0c statistical software (GraphPad Prism Inc; San Diego, California). Confidence interval was 95%. P < 0.05 was considered statistically significant.

4.3 Results

4.3.1 Morphology

4.3.1.1 Control ovaries

Untreated middle-aged mice had spherical shaped ovaries (Fig. 4.4a). The shape of surface epithelial cells was simple squamous to cuboidal covering larger structures such as large antral follicles and corpora lutea, cuboidal over smaller structures, and columnar near or in cell surface involutions (Fig. 4.4b). Isolated areas of stratified OSE were evident (Fig. 4.4c), but did not form papillae. Surface involutions when seen were usually shallower in control ovaries than in estradiol treated mice. Deep invaginations of OSE into ovarian stroma were rare, but were observed in the oldest ovaries. Hilar cysts (Fig. 4.4d) with collapsed profiles were seen, and appeared to become more common with increasing age, therefore more frequently seen in 9-10 month old mice than 7-8 month old mice.

It was apparent mice were ovulating as both fresh and regressing corpora lutea were observed in all but one control ovary. Additionally, large healthy antral follicles were common, whereas small follicles and atretic follicles appeared less common.
Figure 4.4

Morphology of the older ovary in control mice *Weigerts iron haematoxylin/PAS*

(A) Older mouse ovary viewed at low magnification
   Scale bar = 200μm

(B) Ovarian surface epithelium
   OSE cell shape was squamous and cuboidal overlying large structures,
   or columnar (arrows) in or near cell surface involutions. Scale bar = 25μm

(C) OSE stratification (arrow) showing two to three cell layers. Scale bar = 25μm

(D) Hilar cyst (arrow) showing typical collapsed profile. Scale bar = 200μm
4.3.1.2 *EV treated ovaries*

Forty-eight hours following initial EV treatment (day 2), ovaries had highly involuted surfaces with deep invaginations lined by cuboidal and columnar epithelium (Fig. 4.5a). Several areas of multiple cell layering were seen, notably close to and within ovarian surface involutions. Cells contributing to these areas of stratification commonly assumed random orientations. Epithelial papillary projections were noted and frequently, vacuolated areas that stained positive for PAS were seen (Fig. 4.5b). The latter structures were common to both control and treated ovaries and have previously been identified as the remnants of degenerating oocyte zonae (Kelly et al., 1984). Large cysts were present in 50% of EV treated ovaries, some with an epithelial lining several layers thick (Fig. 4.5c). Cilia were present on OSE cells on the surface of papillary excrescences adjacent to oviduct in one mouse (refer to Fig 4.7a for an image showing this structure). Prominent vascularity underlying the tunica albuginea, and areas suggestive of interstitial lipid accumulation were evident. By day 13 there was a marked decrease in ovarian surface involutions compared to day 2 (for quantification refer to figure 4.6a). Deep invaginations were evident in only one ovary but the ovary showed extensive OSE stratification within the cleft (Fig.4.5e). OSE cells were mostly squamous and cuboidal in shape, and cell layering much less apparent than on day 2. Forty-eight hours following second EV injection (day 16), ovaries had a degree of surface undulation once more. Surface involutions did not lead to the deep invaginations previously seen in the ovary 48 hours following first EV exposure.

Treated ovaries were elongated (Fig. 4.5d) and cell layering apparent, following similar patterns of distribution to day 2. OSE cell shape ranged from squamous to columnar. Papillary projections, some blood-filled and housing both somatic and germ cell types, were evident (Fig. 4.5e). Ciliated OSE was again seen on one ovary. Day 28 EV treated ovaries remained elongated (Fig. 4.5h) with varying degrees of surface involution and cell stratification. Some ovaries contained large corpora luteal-like structures that differed in appearance from normal corpora lutea. Constituent cells within these structures had an abundant pale-staining cytoplasm and what appeared to be lipid inclusions, distinguishing them from normal corpora lutea. Another interesting observation was the presence of translucent vesicles in oviductal epithelial cells (Fig. 4.5g), also suggestive of lipid accumulation.
Figure 4.5

Ovarian morphology of EV treated mice

_Weigert's iron Haematoxylin/PAS_

(A) Whole ovary showing deep surface involutions on day 2
Scale bar = 200μm

**Insert** shows surface involution at higher magnification. Scale bar = 50μm

(B) Epithelial cell layering and papillary projections (black arrows) suggestive of an early hyperplasic response. PAS vacuolated areas (white arrow) were frequently seen in treated and non-treated ovaries and represent residual zona pellucida of degenerate oocytes. Scale bar = 25 μm

(C) Non-hilar inclusion cyst with multiple epithelial cell layers
Scale bar = 50μm

(D) Day 13 EV treated ovary showing deep surface invagination with areas of multiple cell layering. This feature was unique to this ovary at day 13 since the incidence of cell surface invaginations was markedly reduced in ovaries at this time point.
Scale bar = 100 μm

(D') Area of cell layering seen in D at higher magnification

(E) Day 16 EV treated ovary showing typical elongated appearance, a common morphological feature observed at day 28 also
Scale bar = 200 μm

(F) Day 16 papillary projection containing both somatic and germ cell types (arrow indicates oocyte). Epithelial cells form the base of the projection Scale bar = 50 μm

(G) Oviduct from EV treated mouse day 28, showing multiple translucent vesicles (one indicated by an arrow) suggestive of lipid inclusion in luminal epithelial cells.
Scale bar = 25 μm

(H) Day 28 ovary. In this photomicrograph two images were taken using the x4 objective lens and were subsequently overlapped to illustrate the extent of elongation of the ovary. Scale bar = 200 μm
4.3.2 Morphometry

4.3.2.1 Frequency of ovarian surface involutions and ovarian surface area
Rates of OSE invagination and cell layering have been reported to increase with age and total ovulation number (Clow et al., 2002). The current study documented the frequency of ovarian surface involutions in EV treated mice and allowed for a comparison to ovaries of older untreated mice. First estradiol exposure resulted in a 10-fold increase in surface involutions compared to controls (Fig. 4.6a). As may be expected, this was accompanied by a 25% increase in ovarian surface area (Fig. 4.6b). There were no differences in these parameters thereafter, despite a second EV injection.

4.3.2.2 OSE depth measurements
OSE depth in treated mice was over two-fold that of controls by day 2 (P< 0.001) (Fig. 4.6c) but was similar to controls at day 13. Treated mice showed no significant differences in OSE depth when compared to controls 48 hours following repeat estradiol, but by day 28 OSE depth was significantly greater in treated mice once again, despite no further estradiol treatment (P< 0.05).

4.3.2.3 Total OSE volume
Total OSE volume (Fig. 4.6d), which included areas of OSE cell stratification, correlated with mean OSE cell volume, OSE depth, and ovarian surface area in treated mice, showing a 4-fold increase above controls by day 2. Total OSE volume was additionally significantly different between groups again at day 28.
Figure 4.6 Morphometry

(A) Frequency of ovarian surface involutions
By day 2 there was a 10-fold increase above controls in the average number of surface involutions (16 ± 6.43 (EV), 1.6 ± 0.81 (control), P =< 0.05). This difference was not observed thereafter. Arrows indicate day of EV injection. Results are means ± SEM.

(B) Ovarian surface area
Ovarian surface area was significantly greater in EV treated mice compared to controls by day 2 (P< 0.0001). Thereafter no difference in ovarian surface area was seen. Arrows indicate day of EV injection. Results are means ± SEM

(C) OSE depth measurements
OSE depth in treated mice increased significantly above that of controls by day 2 (P< 0.001), and at day 13 was similar to controls. No increases in depth occurred 48 hours after a second dose of EV (day 16). By day 28, however, OSE depth had begun to increase in EV treated mice again (P < 0.05). Results are means ± SEM

(D) Total OSE volume
Total OSE volume was greater in EV treated mice compared to controls by day 2 (P <0.0001) and at day 28 (P < 0.05). At all remaining time points no difference was seen. Arrows indicate day of injection. Results are means ± SEM
Figure 4.6 (A-B)
Figure 4.6 (C-D)
4.3.3 Stereology

4.3.3.1 Ovarian volumes and constituent volumes (Vv)

Ovarian volume and constituent volumes are listed in Table 4.2. Total ovarian volume did not differ between treated and control mice at any time point. A 3-fold increase in mean OSE cell volume occurred by day 2, indicative of a significant degree of cellular hypertrophy in the OSE of treated mice (P < 0.0002). This was not evident at day 16 following repeat estradiol treatment. By day 28, small follicles occupied more ovarian volume (P < 0.05), while the volume of corpora lutea within treated ovaries was less relative to controls (P < 0.05). There were no other significant differences in remaining constituent volumes within the 28-day period.
Table 4.2

Ovarian volume and ovarian constituent volumes (mm$^3$)
OSE volume data is expressed as μm$^3$

<table>
<thead>
<tr>
<th>Structure</th>
<th>Time point (day)</th>
<th>Control ± SEM</th>
<th>EV ± SEM</th>
<th>P value</th>
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<td>Stroma</td>
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* Significant P values shown only.
4.3.4 BrdU labelling indices

BrdU label was most intense in OSE overlying corpora lutea and large healthy antral follicles in control mice. Labelling was least over atretic, small antral and pre-antral follicles. These trends did not reach significance however, most likely due to the small sample size examined (n=4). Maximum numbers of labelled cells were recorded during the period from proestrus to estrus. In treated mouse ovaries, BrdU-labelled OSE was found predominately overlying large antral follicles and atretic follicles, but was equally intense in areas overlying blood vessels and stromal tissue. The lowest amount of BrdU label was seen in OSE overlying small antral follicles.

EV treated mice commonly showed extensive areas of OSE cell layering and papillary projections by day 2, 48 hours after receiving the first estradiol injection, yet few to no cells had retained BrdU label in these structures (Figure 4.7a and b). This observation was confounding, since after 6 months of data collection there were no clear trends evident in rates of OSE cell proliferation between control and treated mice. Subsequently a decision was made that BrdU labelling indices would be analysed for the day 2 time point only, and alternative methodologies further used to measure hyperplasia across all time points (refer 4.2.3.3-4.2.3.5). When the data from day 2 labelling indices was analysed, as anticipated there were no significant differences in rates of BrdU incorporation between control and treated groups (Figure 4.7e). To determine whether a difference in the total numbers of OSE cells between control and treated ovaries existed, labelled and unlabelled OSE cells from all 8 sections per ovary/group (n=6 per group) were summed. EV treated ovaries were subsequently found to have significantly more total OSE cells per sections counted (P< 0.05) (Figure 4.7f).
Figure 4.7 BrdU incorporation and BrdU labelling indices day 2

(A-B) OSE papillae and areas of OSE cell layering (arrows B) were common by day 2, but showed epithelial cells with little or no BrdU label. Cilia were seen on round papillary structures consisting of stratified OSE cells. These structures projected outward from the ovarian surface into the bursal space (arrow A). Proliferating granulosa cells in growing follicles (arrowhead figure B) provided an internal positive control for BrdU nuclear specificity. Scale bar = 25 μm (A) and 50 μm (B).

(C) External positive control (small intestine). BrdU label is present in proliferating cells of the intestinal crypts. Scale bar = 100μm

(D) Negative control. Small intestinal section in which primary antibody was excluded from the antibody dilution buffer. Intestinal crypt cells are unlabelled. Scale bar = 100μm

(E) BrdU labelling indices were recorded from 6 control and 6 treated mice (Labelled OSE cells were counted around the entire perimeter of each ovary section (n=8 sections per ovary) and expressed as a percentage of total OSE cells counted. No significant differences were found between EV treated and control animals using this method.

(F) Total number of OSE cells counted
OSE cell number (labelled and unlabelled) were counted around the entire perimeter of each ovary using the previously stated numbers of animals and sections. This revealed a significantly greater average number of total OSE cells counted/ 8 sections/ovary in EV treated mice P < 0.0001).
4.4 Discussion

Results presented in this chapter have been generated from methodologies developed to address the hypothesis that during middle age, the ovary exhibits hypersensitivity to estradiol. With reference to Table 4.1, it may be concluded that there are 11 ovary-specific phenotypic features associated with periods of high endogenous E2 exposure in vivo. The administration of exogenous estradiol in this study resulted in the observation of 10 out of 11 of these features within 48 hours of a single estradiol injection (Rambo and Szego, 1983).

OSE cell layering, papillae, deep surface invaginations and involutions, accompanied by increases in OSE depth, volume, and surface area, are all suggestive of significant OSE remodeling and proliferation. This was not confirmed with BrdU labeling indices, raising the possibility that the rate of OSE proliferation outstripped the availability of BrdU label. Evidence in support of this is that the great majority of cells contributing to stratification and lining surface invaginations did not incorporate BrdU. Furthermore, the first injection of BrdU was not administered until 24 hours following estradiol in accordance with the prescribed protocol (4.2.3.6). It is therefore proposed that in this study, proliferative changes to ovarian morphology may have occurred within 24 hours in response to a single estradiol injection. As a consequence, large numbers of OSE cells would have entered the S phase, (when BrdU is incorporated into DNA) very quickly, resulting in multiple cell divisions but minimal BrdU label. Significantly greater total numbers of OSE cells were counted in EV treated mice, lending weight to the proposed mechanism.

The classical model of E2 action involves a ligand-mediated activation of nuclear estrogen receptors, which interact with estrogen response elements (ERE) in the promoters of target genes, recruiting several coactivators to mediate transcriptional regulation. Transcriptional activation resulting in the generation of new estradiol-induced protein requires time (from 4 hours to many days). However, estradiol has also been shown to drive signal transduction events in estrogen-responsive tissues within seconds to minutes both in vitro (Pietras and Szego, 1975) and in vivo (Rambo and Szego, 1983).
Szegö, 1983), and may hasten progression from the G1 to the S phase of the cell cycle by regulating expression and function of the transcription factor, c-Myc, and Cyclin D1 (Doisneau-Sixou et al., 2003). In ovariectomized adult mice a single injection of estradiol has been shown to result in a synchronised wave of DNA synthesis and cell division in uterine epithelial cells commencing 6 to 9 hours following E2 and peaking at between 12-15 hours post E2 administration (Tong and Pollard, 1999). There is a possibility that the nature of OSE proliferation in vivo is very similar to that reported for uterine epithelium.

The outcome of this experiment thus emphasises the importance of using a cumulative BrdU labelling strategy for assessing rates of OSE proliferation in vivo when animals have been administered a known inducer of mitogenesis. A cumulative labelling approach has previously been used to investigate proliferation of mouse ovarian surface epithelium following a single gonadotrophin-induced ovulation (Burdette et al., 2006). In this study, BrdU was given ip at the same time the hormone was delivered. Immediately following the BrdU injection, animals had the label added to their drinking water, thereby enabling cumulative labelling. Using this method, Burdette et al were able to demonstrate a basal OSE proliferation rate (labelling index) of 45% over 60 hours in immature (25 day old) mice. When gonadotrophins were administered the proliferation rate rose to > 85% over the same time period. A similarly high labelling index (50%) has been reported in rats immediately after ovulation when BrdU was administered ip 1 hour prior to sacrifice (Gaytan et al., 2007). These results are in contrast to those where BrdU pulse labelling has been used in rodents, giving labelling indices of between < 0.2% - 1.3% during estrus (Fleming et al., 2007) to 7% across the entire estrous cycle (Gaytan et al., 2005). Indeed, there are numerous approaches to BrdU pulse labelling strategies and caution should be applied when attempting to make comparisons between in vivo studies.

A finding in common however, regarding rates of OSE proliferation, is that the majority of proliferative activity is associated with the lead up to ovulation, the ovulatory event itself, and the immediate post-ovulatory state (Gaytan et al., 2005) (Burdette et al., 2006). Proliferation is therefore increased in areas of OSE overlying larger antral follicles and corpora lutea, a trend also recorded in the present study.
Quantitative analysis of BrdU label relative to OSE location did not reach significance, however a larger sample size may have changed this result.

When serum and ovarian tissue estradiol concentrations were at the lowest recorded (day 13), OSE did not have the stratification and invaginations previously seen on day 2. Moreover, dependent morphometric parameters such as ovarian surface area, OSE volume and OSE depth were normal. Additionally, ovaries began to alter their shape from spherical to cylindrical. This morphogenesis continued, with ovaries becoming progressively less convoluted on their surfaces, and more elongated. The day-13 time point marked a period when the ovary was no longer exposed to high circulating levels of the hormone, and OSE morphology resembled the quiescent state. It may therefore be hypothesised that changes to OSE parameters were either the direct result of a reduced availability of estradiol, or due to a decreased sensitivity to estradiol. The latter is most likely for two reasons:

1. Levels of estradiol in ovarian tissue remained significantly elevated at day 13, though circulating levels had reached a nadir. Therefore OSE continued to be exposed to high concentrations of the hormone.
2. A second EV injection dose-equivalent to the first and given on day 14, failed to result in equivalent changes to OSE. This suggests OSE is able to self-regulate its exposure to estrogen.

If the OSE is able to control its own microenvironment, it may, at least in the initial stages of exposure to estrogen, exercise a degree of control over a hyperplastic (and/or hypertrophic) response. As estradiol accumulates in ovarian tissue, this level of control may become compromised. In support of this notion is the insidious increase in OSE depth and total OSE volume observed by day 28, a full 14 days after the second EV injection. Since it was anticipated that the OSE response to estradiol would be most pronounced within 48 hours of giving the hormone, mean OSE volume was not measured at this time point. Consequently the relative contribution of hyperplasia or hypertrophy (or a combination of both processes) to total OSE volume at day 28 cannot be concluded.
The striking elongation of ovaries appears to have resulted from progressive decreases in the amount of surface involutions and deep invaginations in treated ovaries, alongside a significant depletion of corpora luteal volume, resultant from an extended period of anovulation. Accordingly, a large but non-significant increase in total ovarian volume in treated mice by day 2 (P = 0.07) was followed by decreases in volume, and at the conclusion of the experiment ovarian volumes in EV treated mice were smaller than those of control mice, although again not significantly. The combination of decreases in these morphometric parameters over time in treated ovaries had the effect of reducing the surface area of the ovary. Interestingly, a non-significant trend towards increases in ovarian surface area and OSE involutions was apparent by 28, and was accompanied significant increases in OSE depth and total OSE volume. It may therefore be postulated that this trend could continue if estradiol exposure to OSE was further prolonged.

The appearance of OSE at day 13 was, apart from one animal, that of a return to the quiescent state, and may have occurred as a consequence of EMT (1.2.2.2). This would be difficult to assess morphometrically, given that OSE occupies so little of ovarian volume (1/1000) in this state (Wright et al., 2005), thus any alteration to stromal volume would be very small. An increase in the rate of apoptosis of OSE cells is also possible, however a pilot analysis revealed no apoptotic OSE cells on treated or untreated ovaries following 1 month of EV. Moreover, Burdette et al (Burdette et al., 2006) found no evidence of apoptosis involving OSE after gonadotrophin induced ovulation in mice, concluding that murine OSE does not undergo apoptosis in vivo. Therefore, a third possibility and one lending itself to investigation is that of OSE cell depletion by sloughing from the ovarian surface. In order for this to occur, OSE cells would have to lose cell-to-cell contact. Evidence of this event would be a loss of cell polarity preceded by a loss of E-cadherin.

A novel finding in this experiment was the highly significant degree of hypertrophy in OSE cells within 48 hours of EV treatment. Although this has not been reported in the literature, it is not entirely unexpected. Uterine epithelium has long been recognised as undergoing both hypertrophy and hyperplasia in response to estrogen, and myometrial tissue responds to estrogen similarly (Barks and Overholser) (Martin et al., 1973). One
unexpected finding after only 48 hours of estradiol exposure was that of OSE metaplasia. This cellular response is best demonstrated in figure 4.7a, where cilia are clearly seen on OSE facing the bursal space. Previous reports of ciliated ovarian surface epithelium are not limited to, but are well documented in, OSE lining inclusion cysts (Auersperg et al., 2001) (Fleming et al., 2007). The growth of a ciliated epithelium is representative of an oviductal (Müllerian) phenotype and as such is additionally recognized as being precursory to OSE neoplasia (Auersperg et al., 2001).

Over the course of the experiment, other morphological features were seen in treated ovaries. These included the occasional formation of atypical inclusion cysts (Figure 4.5c) that had an epithelial lining several layers thick. The appearance of these inclusion cysts was different to that of hilar inclusion cysts seen in older control animals (Figure 4.4d). The cyst wall did not have a collapsed profile or a thin epithelial rim surrounding the cyst lumen. Furthermore, the cytoplasm of cells lining these cysts did not appear translucent or secretory and the apical membranes of cells facing the cyst lumen were non-ciliated. This finding is in contrast with reports of ciliated luminal OSE cells seen in regular inclusion cysts. The development of atypical inclusion cysts may have been coincidental to estradiol treatment, since they were not common, yet control ovaries did not contain this type of cyst at any timepoint and it is hypothesized they may represent another form of metaplastic process.

Observed increases in vascularity, particularly in the peripheral regions of the ovary underlying the tunica albuginea, but also within papillary excrescences occurred (Figure 4.5f). Additionally there were examples of what appeared to be lipid accumulation in the ovarian interstitium and in oviductal epithelial cells (Figure 4.5g). The reason for an increased blood supply is not clear, but exogenous estradiol has been shown to regulate blood flow and vascular permeability in the uterine tissue of ovariectomized rats (Plowchalk and Teeguarden, 2002). It is plausible that a high dose of estradiol may improve blood flow not only to the ovary, but also within the ovary. Lipid accumulation could relate to the ability of the ovary to synthesise estrogen de novo, although to date it has not been established whether OSE cells can produce steroid hormones from cholesterol in vivo (Rae and Hillier, 2005). Nevertheless, this substrate may be available following exposure to exogenous estrogen.
In summary, results from this chapter have shown that significant alteration to OSE morphology and morphometrics occur within 48 hours of exposure to estradiol in the older mouse ovary. The acute response is one of rapid OSE hypertrophy, metaplasia, and histologic change strongly suggestive of hyperplasia. When the ovary is exposed to the same dose of estradiol within 14 days, this response is severely attenuated. It is hypothesized this muted response is the result of a reduction in the acute sensitivity of OSE to estradiol. Secondly, it is proposed that the rapid OSE growth and metaplastic change seen by day 2 and subsequently substantially subdued by day 16 despite a further dose of estradiol; *a priori* involves alteration to cell-cell adhesion. The following and final experimental chapter of this thesis will address the nature of the observed change in responsiveness to repeat estradiol exposure.
CHAPTER 5: Immunohistochemical analysis of estrogen receptor α, estrogen receptor β, and E-cadherin following exposure to exogenous estradiol

5.1 Introduction

In the previous chapter, it was hypothesized that changes to OSE morphology and morphometric parameters occurred as a consequence of an alteration to the level of sensitivity of OSE to estradiol. Evidence in support of this hypothesis was the observation that both variables returned to baseline levels by day 13 in treated mice. Circulating levels of estradiol had also decreased by day 13, but ovarian tissue levels of the hormone remained significantly elevated, thereby prolonging exposure of the epithelium to estradiol.

When a second estradiol injection was administered dose-equivalent to the first, serum and ovarian tissue estradiol levels reached only 50% of those recorded following a first exposure to the hormone, and there was a significant attenuation of the OSE morphologic response. It was subsequently proposed that in addition to increases in the metabolic clearance of estradiol, mouse OSE exercised a degree of control over its microenvironment. It is therefore possible OSE may be equipped to self-regulate its exposure to estrogen during short periods of elevated tissue estradiol concentration, but could progressively lose this ability when subjected to repeated or prolonged periods of exposure.

One plausible hypothesis is that OSE may regulate its response to estrogen through controlling levels of functional estrogen receptor. Therefore the specific major aims of the experiments outlined in the first half of this chapter were to:

(1) Use immunohistochemistry to localize and compare ERα and ERβ protein expression in the older mouse ovary and in particular the OSE.
(2) Use immunohistochemistry to provide a qualitative comparison of both ER subtypes 48 hours following exposure to a single and a repeat depot estradiol injection (days 2 and 16).

(3) Use confocal microscopy and immunofluorescence to demonstrate dual label for ERα and ERβ on normal (older) OSE, and provide a semi-quantitative analysis of ER on OSE 48 hours following first estradiol exposure.

(4) Demonstrate ER protein expression in oviductal epithelia (also derived from embryonic coelomic epithelium and an example of a Müllerian duct-derived epithelium) and ascertain if ER protein expression patterns differ from that of OSE.

Studies of ER in normal OSE have used a variety of in vitro and in vivo techniques to investigate the localization and expression of both wildtype estrogen receptor subtypes, with the majority of studies using rat ovary. These and many other studies using monkey, human, hamster, mouse, cow and sheep, have localized ERα to OSE at both mRNA and protein levels (refer Chapter 1, table 1.1). In contrast, fewer studies (Saunders et al., 1997) (Saunders et al., 2000) (Wang et al., 2000) (Cardenas et al., 2001) (Symonds et al., 2003) (Hishikawa et al., 2003) (Lindgren et al., 2004) (Juengel et al., 2006) have shown the presence of ERβ protein on OSE in vivo. Of those, patterns of ERβ distribution within OSE cells for the most part show a homogeneous nuclear distribution. However, expression patterns of both ERα and ERβ are known to alter throughout the estrous cycle (Cardenas et al., 2001) (Gaytan et al., 2005). Cyclic regulation of rodent ERα expression and distribution in OSE has been reported using immunohistochemistry, and shows levels of ERα to be highest during metestrus and diestrus, and lowest during estrus (Gaytan et al., 2005). This contrasts with that reported for rodent uterine epithelia, where messenger RNA levels for ERα are reportedly highest during proestrus, while those of ERβ are highest during metestrus and diestrus, and lowest during estrus (Wang et al., 2000). An expression profile for ERβ in OSE has not been described.

ERβ is abundant in the ovary, yet reports of the immunohistochemical expression of this receptor on OSE are highly variable (refer Chapter 1, table 1.1). In vivo studies by
Saunders et al, (Saunders et al., 2000) and Wang et al, (Wang et al., 2000) reported the presence of ERβ on marmoset, human and rat OSE using immunohistochemistry. Saunders and colleagues collected ovaries during the late follicular stage of the ovarian cycle, conceivably when high endogenous levels of estradiol may have significantly reduced ER expression, yet a strong nuclear stain was reported (image not shown for OSE), while Wang’s group did not specify the stage of the estrous cycle animals were in when sacrificed. Thus, not only may there be interspecies variation in the expression of this receptor on OSE, there may be intraspecies variation secondary to the stage of the ovarian cycle, and therefore the relative influence of estradiol on the ovary.

Additionally, the influence of antibody choice and the subsequent application protocol should always be considered as potentially influencing immunohistochemical results. For instance, Serotec (U.K) supplies a monoclonal ERβ1 antibody directed against human ER-beta (clone PPG5/10). This widely used antibody comes in two different strengths, with a 10x concentration (the MCA1974S) supplied to allow for very short primary incubation times. The single strength (MCA1974) is supplied for the purposes of overnight incubation at 4°C. Inadvertent substitution of the 10x concentrate for the single strength antibody, alongside overnight incubation, could markedly increase staining intensity and additionally result in background stain.

To date there has been only one report of the effects of exogenous estradiol on ER expression in normal OSE in vivo. The study, by Bai et al, (Bai et al., 2000) demonstrated the presence of ERα protein on rabbit OSE, and further showed that transcriptional down-regulation of ERα occurred following short-term estradiol treatment of OSE cells in culture. The down-regulation of ERα was subsequently paralleled by a lack of OSE proliferative response. Results from Bai’s study were suggestive of an obligatory role for ERα in the initial proliferative/hyperplastic OSE response. A similar role for ERβ remains to be established.

It has been hypothesized most recently in the literature, that the differential expression of ERα and ERβ in ovarian epithelial tumors may constitute a critical step in ovarian carcinogenesis and hormone unresponsiveness (Wong and Leung, 2007) and increasingly studies cite downregulation of ERβ relative to ERα occurring with
progression to epithelial oncogenesis, including OSE (Roger et al., 2001) (Stabile et al., 2002) (Foley et al., 2000) (Horvath et al., 2001) (Brandenberger et al., 1998). Since estradiol is implicit to the differential regulation of these receptors, the effects of exogenous estradiol on ER expression in vivo requires intensive investigation.

The two preceding chapters in this thesis have presented and discussed the dramatic alteration to OSE morphology and morphometry that occurs within 48 hours of the administration of a depot estradiol treatment. Furthermore, it has previously been established in vitro that ERα is a requirement of the hyperplasia constituting part of this response, a response enhanced by the presence of stroma (Bai et al., 2000). The cell proliferation that is obligatory to the process of culturing sufficient cells for in vitro experimentation has been reported to result in a progressive loss of ERα and disruption to the distinctly nuclear distribution pattern of this receptor (Bai et al., 2000). Hyperplasia and hypertrophy in vivo may also elicit such changes to ER. Concurrent with this may be the disruption to cell-cell interfaces, the consequence of which conceivably involves alteration to cell junctions, intercellular spaces and cell adhesion molecules. The continued expression of proteins critical for the maintenance of OSE integrity may therefore be mutualistically dependent on the presence of ER.

E-cadherin, a major cell adhesion molecule (1.2.1.2), may, along with the catenins, play a key role in the control of cell growth induced by estrogen in vivo. In three-dimensional cell culture, cancer cells that maintain E-cadherin function form cellular aggregates and are growth inhibited by cell-cell contact. Such a mechanism is a natural regulator of cell proliferation in normal epithelia. A mechanism linked to this is the E-cadherin induction of p27, a cyclin-dependent kinase inhibitor. Levels of p27 induced by E-cadherin have been shown to saturate and prevent the activity of E-cdk2, a cyclin-dependent kinase that controls progression through the G1 to the S-phase of the cell cycle. Immediate (within 6 hours) changes to the levels of E-cdk2 in E-cadherin-expressing cellular aggregates, are accompanied by more delayed (within 48 hours) decreases in cyclin D1, further reinforcing the cell cycle arrest effects of increased E-cdk expression (St Croix et al., 1998). E-cadherin mediated suppression of cell growth observed in three-dimensional culture (as evidenced by [3H]-thymidine incorporation) is secondary to decreases in the amount of cell signaling through receptor tyrosine
kinases (soluble growth factor receptors) such as EGFR. This signals a role for E-cadherin in the inhibition of mitogenic activity (St Croix et al., 1998), and raises the possibility that E-cadherin expression may initially increase when levels of estradiol increase, in what could conceivably be a homeostatically controlled mechanism to prevent excessive cell growth. In fact, estradiol has been shown to cause rapid up-regulation of E-cadherin on OSE in vivo (MacCalman et al.; 1994) while simultaneously potentiating EGFR and IGFR-1-mediated OSE cell growth by increasing receptor binding availability and affinity. Accordingly it may be postulated that E-cadherin antagonises the effects of estradiol in vivo by limiting the extent of estradiol-induced hyperplasia and hypertrophy.

Further in vitro support for this hypothesis is found in the estrogen deprivation of normal epithelial cell lines, resulting in a progressive loss of functional E-cadherin. A significant loss of this molecule’s function in response to loss of estrogen may take considerable time however, since the mechanism has been reported as dependent on the induction of apoptosis, which is not known to occur until after 6 days of estrogen deprivation. The eventual down-regulation of E-cadherin is thought to result from O-glycosylation of the newly synthesised E-cadherin, blocking its transport to the cell surface where it assumes its functional capacity (Zhu et al; 2001). It may be proposed therefore, that when serum and ovarian levels of E2 declined during the present study, levels of E-cadherin may also have declined.

In the unstimulated state, mouse OSE is reported as having a ubiquitous expression of E-cadherin (Auersperg et al., 2001). This chapter of the thesis therefore further aims to:

2. Compare expression patterns of E-cadherin on OSE on day 2 and day 16 following a first and subsequent estradiol exposure.
3. Use confocal microscopy to co-localize E-cadherin and ER on OSE and oviduct and compare expression patterns.
5.2 Methods

5.2.1 Tissue retrieval and preparation for immunohistochemistry

A new group of twenty-eight 7-10 month old Swiss Webster mice were required for this set of experiments. Current animal ethics approval was in place (application number #69/07).

Mice underwent daily vaginal smears for 3 consecutive estrous cycles to confirm cyclic behavior prior to commencement of the experiment. As observed previously (3.3.1), with other mice this age, aberrant cycles were not uncommon. Using the model and experimental protocol previously outlined (3.2.1-3.2.2), ovaries recovered on day 2 and day 16 were used to study the effects of estradiol on estrogen receptor subtype and E-cadherin expression 48 hours following each EV injection. Mice were randomly assigned to two control and two treatment groups as follows:

**Group A**

*Control*  
\( n = 8 \)  
*EV treated*  
\( n = 6 \) (received 1 dose EV 10\( \mu \)g/g body weight)

**Group B**

*Control*  
\( n = 8 \)  
*EV treated*  
\( n = 6 \) (received 2 doses of EV 10\( \mu \)g/g body weight)

Since hormonal variations within the estrous cycle may have had implications for levels of estrogen receptor and E-cadherin, additional mice were assigned to control groups. This was to increase the likelihood that sufficient numbers of ovaries obtained at sacrifice were representative of metestrus and diestrus stages of the estrous cycle, when ER expression is more likely to be maximal.

Ovaries were removed from the animals and prepared as described previously for morphometry and morphometric analysis in the preceding chapter (4.2.1). An exception was the decision to increase the pH of the paraformaldehyde fixative. A pilot analysis of ovarian tissue derived from 3 animals showed increasing fixative pH from
7.6 to 8.9 resulted in excellent immunohistochemical localisation of ER, in particular the ERβ1 isoform (refer 5.2.4 (a) for further detail). Blocks of processed tissue were trimmed and sectioned using the Leica Jung RM 2025 microtome (Leica Microsystems, Germany) and a disposable knife (Feather, Cell diagnostics, Dublin). A principal aim of these experiments was to develop a protocol to study the OSE expression of ER using both thin and thick sections. The intention was to cut sections to 5μm to qualitatively assess levels of both receptor subtypes on OSE using DAB for visualisation, and to use 20μm sections to localise the receptors using a fluorescent-linked secondary antibody for quantitative analysis in conjunction with confocal imaging. In a pilot study using DAB only, ovaries were serial sectioned at either 5 or 20 μm. Surprisingly, using 5μm sections, ERβ was not detectable, however when section thickness increased to 20μm, this subtype was detected as a particulate reaction product in OSE. Consequently, 20μm sections were used for ERβ, while 5μm sections were used for ERα. A random set of numbers was generated (GraphPad Software) to provide a non-biased sample of sections (n=5) from throughout the ovary for each receptor subtype. Sections were immersed briefly in 30% (v/v) etoh, floated onto a 42°C water bath and collected onto Superfrost® Plus slides (Biolab). Tissue adherence was achieved by placing all slides on a 45°C hotplate for up to 30 minutes for 5μm sections and 1 hour for 20μm sections, followed by incubation overnight in a 37°C incubator. To prevent curling of 20μm sections during repeated sectioning, the tissue block was periodically dismounted from the block holder and placed face down in the hot water bath for up to 15 seconds at a time. Thick sections also required longer in the water bath to achieve optimal expansion prior to being picked up onto the slides.

5.2.2 Estrogen receptor -α and -β immunohistochemistry

The following protocol was developed from two original estrogen receptor protocols designed for the detection of prostate ER (Androgen Research Group, Department of Anatomy and Structural Biology, School of Medical Sciences, University of Otago) and ER from brain frozen sections (Herbison Lab, Centre for Neuroendocrine Research, Department of Physiology, University of Otago). After de-waxing in two changes of xylene and rehydration of tissue through a series of graded alcohols (4.2.2), sections were rinsed in two changes of double distilled water (ddH₂O). Endogenous
peroxidases were blocked with 3% (v/v) hydrogen peroxide in methanol/TBS followed by two 5-minute washes in TBS with Triton (0.5%), and one 5-minute wash in TBS alone. Antigen retrieval required the pre-heating of 0.01 M citrate buffer (pH 6) (Appendix C) for 3 minutes, followed by microwaving sections in the buffer on a rolling boil for 10 minutes using a Sharp Carousel 1000-watt microwave oven. Sections were cooled over a period of 5 minutes with the progressive addition of cold citrate buffer, then allowed to sit for a further 5 minutes prior to a rinse using TBS/Triton (0.3%), followed by three 1 minute washes in double distilled water. Sections were immersed in TBS/Triton (0.3%), placed on a rotating table, and washed for 30 minutes, followed by two rinses in TBS.

Protein block was achieved by incubating sections with TBS/Triton and 0.25 % BSA for 30 minutes in a moist chamber at room temperature. Primary antibody incubation was performed overnight in a moist chamber at 4°C using C1355, rabbit polyclonal, (ERα) (Upstate/Millipore, CA) diluted 1:100, or NCL-ER-beta, mouse monoclonal clone EMRO2 (Novacastra, U.K) diluted 1:50 in antibody dilution buffer. Dilution buffer contained TBS with 0.3% Triton, 0.25% bovine serum albumin (BSA) and 2% normal serum of the species in which the secondary antibody was made (refer to Appendix B). The addition of the 2% normal serum as stated was to control levels of non-specific binding of the secondary antibody.

Following 10-minute washes* (TBS, TBS/Triton (0.3%) and additional TBS), sections were incubated in a humidity chamber for 60 minutes at RT with secondary antibody; biotin-conjugated donkey anti-rabbit IgG (Amersham Bioscience, U.K), ERα, or biotin-conjugated goat anti-mouse IgG (Amersham), ERβ, diluted 1:200 in TBS. Excess antibody was removed from sections with three washes in TBS, TBS/Triton (0.3%) and TBS as above. Signal amplification was achieved with a 30-minute incubation in a 1:100 dilution of streptavidin biotinylated horseradish peroxidase reagent (Sb-HRP, Amersham) at room temperature. After three consecutive 10-minute washes* to remove traces of sb-HRP, visualisation was achieved using diaminobenzadine (Fast DAB, Sigma). Signal development was terminated with two rinses in ddH2O, followed by Tris /HCl for 5 minutes and a two more rinses in ddH2O. Sections were counterstained for 6 seconds in Gills #2 haematoxylin diluted 1:4 in
ddH20. Positive controls included uterus (ERα) and oviduct and skin (ERβ). Negative controls omitted primary antibody in the dilution buffer, or consisted of a non-immune IgG control diluted 1:100 (α) or 1:50 (β) in dilution buffer.

Both primary antibodies exhibited high specificity to the respective receptor isoform, demonstrating no cross reactivity between isoforms. Preliminary trials comparing two other primary antibodies that included NCL-ER-6F11 mouse monoclonal anti-estrogen receptor-α (Novocastra, U.K) and Rabbit anti-estrogen receptor-β polyclonal Z8P (Zymed Laboratories Inc. San Francisco) were also performed. These antibodies gave similar results to the above antibodies, but it was decided that one polyclonal and one monoclonal should be used for the purposes of double label immunohistochemistry. As the NCL-ER-beta monoclonal (Novacastra) and the C1355, rabbit polyclonal, (ERα) (Upstate/Millipore, CA) gave consistently satisfactory results, these were the eventual two antibodies chosen.

NCL-ER-beta is reported to be specific to the wildtype form of the human ERβ protein C-terminus, a region that demonstrates 98.5% sequence homology between human and mouse (Saunders, 1998). There is also greater than 90% sequence homology in the ligand-binding domain between wildtype mouse and human ERβ receptor (ERβ1). The advantage of NCL-ER-beta as an epitope specific to wildtype ERβ1 is that this epitope is not present in a known rodent ERβ variant, the ERβcx or ERβ2 identified by Chu and Fuller in 1997 (Chu and Fuller, 1997) and recorded by Peterson et al, 1998 (Petersen et al., 1998). ERβ2 encodes a distinct ERβ receptor subtype with an 18 amino acid insertion within the ligand-binding domain (Petersen et al., 1998). Accordingly it exhibits altered ligand binding and transcriptional activation properties as yet uncharacterized, and was therefore excluded from any quantitative analysis of ERβ in this study.
5.2.3 E-cadherin immunohistochemistry

E-cadherin immunohistochemistry protocol was adapted from that supplied by the Androgen Research Group (Fleming, JS and Mc Quillan, J) and was performed on 5μm thick paraffin embedded sections.

After de-waxing and re-hydration of tissue through a series of graded alcohols (4.2.2) sections were rinsed in two changes of double distilled water. Antigen unmasking was achieved with 1 mM ethylenediaminetetraacetic acid disodium salt (EDTA), (pH 8, Appendix B). EDTA solution was preheated in the microwave for 3 minutes, slides immediately immersed in the reagent and maintained on a rolling boil for 10 minutes. Sections were cooled in the retrieval buffer as previously described (5.2.1.2). Rinses in distilled water and phosphate buffered saline (PBS) (pH 7.4) preceded the blocking of endogenous peroxidases with 3% (v/v) H2O2 in methanol for 5 minutes. Peroxide solution was removed with three 1 minute rinses in ddH2O and tissue permeability enhanced with two consecutive 30 minute washes in PBS/Triton (0.5%) v/v, followed by two 5 minute rinses in PBS.

Non-specific binding was blocked by incubating slides for 10 minutes at RT with 20% (v/v) normal goat serum, the serum specific to the species in which the secondary antibody was made. Following a brief rinse in PBS sections were incubated with Avidin Block (Avidin/Biotin Blocking Kit; Vector Laboratories, U.K) for 30 minutes at room temperature. A second rinse in PBS was given prior to application of Biotin Block for a further 30 minutes. This blocking procedure ensures all endogenous biotin, biotin receptors, or avidin-binding sites resident within tissues are blocked prior to the addition of the labelled avidin reagent. It therefore additionally reduces non-specific binding. After another brief rinse in PBS, primary antibody incubation was performed overnight in a moist chamber at 4°C using rat anti-mouse E-cadherin monoclonal primary antibody (Clone ECCD-2, Zymed Laboratories) diluted 1:40 with PBS/Triton (0.3%) and BSA 0.25%.

Control sections included omitting primary antibody from the dilution buffer and replacing primary antibody with an equivalent concentration of Ig isotype serum.
5.2.3 E-cadherin immunohistochemistry

E-cadherin immunohistochemistry protocol was adapted from that supplied by the Androgen Research Group (Fleming, JS and Me Quillan, J) and was performed on 5μm thick paraffin embedded sections.

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Non-specific binding was blocked by incubating slides for 10 minutes at RT with 20% (v/v) normal goat serum, the serum specific to the species in which the secondary antibody was made. Following a brief rinse in PBS sections were incubated with Avidin Block (Avidin/Biotin Blocking Kit; Vector Laboratories, U.K) for 30 minutes at room temperature. A second rinse in PBS was given prior to application of Biotin Block for a further 30 minutes. This blocking procedure ensures all endogenous biotin, biotin receptors, or avidin-binding sites resident within tissues are blocked prior to the addition of the labelled avidin reagent. It therefore additionally reduces non-specific binding. After another brief rinse in PBS, primary antibody incubation was performed overnight in a moist chamber at 4°C using rat anti-mouse E-cadherin monoclonal primary antibody (Clone ECCD-2, Zymed Laboratories) diluted 1:40 with PBS/Triton (0.3%) and BSA 0.25%.

Control sections included omitting primary antibody from the dilution buffer and replacing primary antibody with an equivalent concentration of Ig isotype serum.
Primary antibody was removed with two 5-minute washes in PBS, followed by three 10-minute washes in PBS/Triton 0.3%, and three 1-minute rinses in PBS. Application of secondary antibody, biotin-SP-conjugated AffiniPure Goat anti-rat IgG (Jackson Labs) diluted 1:100 in PBS followed with incubation in a moist humidity chamber for 1 hour at RT. Secondary antibody was removed with three consecutive 10-minute washes in PBS, PBS/Triton (0.3%), and PBS. A 1:50 dilution of sb-HRP (Amersham) in PBS was applied for 1 hour at room temperature, with sections in the humidity chamber. After two 5-minute changes in PBS/Triton (0.3%) and two further rinses in PBS, the peroxidase signal was developed with DAB for 10 minutes. The reaction was stopped with 2 changes of ddH2O. Sections were counterstained for 4-6 seconds in Gill's#2 haematoxylin diluted 1:4 in ddH2O. After rinsing in changes of tap water and distilled water, sections were dehydrated through a series of graded ethanol (one change of 70% for 1 minute, one change of 95% for 2 minutes, followed by two changes of 100% for 2 minutes). Sections were cleared in 2 changes of xylene (2 minutes each), and coverslipped with dibutyl phthalate with xylene (DPX) mountant.

5.2.4 Optimization of immunohistochemical protocol

For optimal immunohistochemical detection of ER and E-cadherin on OSE, the above protocols were developed through repeated testing and optimization of existing protocols. Alterations and the rationale for their inclusion are listed.

*Estrogen receptor*

(a) Increasing pH of the fixative

Experiments to localise ER following periods of exposure to exogenous estradiol were designed bearing in mind possible clinical applications. For instance having potential diagnostic or prognostic significance in OSE neoplasia. Progress toward such clinical applications has to date been hampered by well established routine fixation methods that often fail to optimally conserve the structure of both nucleic acids and proteins in tissues. This includes use of formaldehydes, of which paraformaldehyde is a polymerised form that depolymerises in solution to produce formaldehyde. The resultant loss of high quality RNA or protein may lead to spurious results, particularly at the molecular level where the effect of such fixation has been commonly shown to
induce mutations (Srinivasan et al., 2002). For this reason, fresh frozen tissue is presently considered best for molecular studies including in situ hybridization, a technique that was initially considered for use in this thesis alongside immunohistochemical localisation of ER.

Unfortunately, morphology is unduly compromised using frozen sections, and in situ hybridization experiments using ovary have never been successful in detecting ERβ in OSE at mRNA level, while detecting levels of ERα with relative consistency (refer Table 1.1). This indicates that in vivo, ERβ mRNA may be present in lower abundance in OSE than in developing follicles, and also lower than levels of ERα mRNA in OSE. A highly specific localisation of ERβ was desired as a principal outcome for the current set of experiments, therefore in situ studies were not considered further. Research into optimizing fixation for use in in situ hybridization nevertheless yielded valuable information for promoting optimal conservation of nucleic acids. This had potential useful applications in preserving antigenicity of the protein and consisted of modifying the fixation protocol by significantly increasing the pH of the paraformaldehyde fixative (refer 5.2.1).

(b) Permeabilization of tissue

Existing protocol used 0.5% Triton (v/v) in all washes and as part of the antibody dilution buffer. Since estrogen receptor is reported as nuclear, the permeabilization step is a critical inclusion in this protocol. However the 0.5% used in existing protocol was excessive and unnecessary. Its presence within buffers caused rapid drying of sections, in particular the thick sections used for detection of ERβ. Furthermore, the continued presence of a soapy substrate on sections undergoing incubation steps, led to difficulties in keeping reagents on the section, despite the use of hydrophobic pens used to create barriers around sections. For these reasons, the amount of triton used in buffers was reduced from 0.5% to 0.3% in all but the first wash. In addition, each wash in buffer containing triton, was followed by rinses in TBS alone, to rid the sections of excess triton and allow for efficient cording off of the section prior to the application of agents used in incubations.
(c) Moist incubation

It is traditional to perform incubations using immunohistochemical reagents in humidity chambers, thereby protecting sections from drying in room air. ER immunohistochemistry to detect the beta isoform presented a unique challenge due to the thickness of the section having increased tendency to dry out. Therefore in addition to use of the chamber and wet (dH₂O) tissues lining the bottom of the chamber underneath slides, all chambers were double wrapped in ‘Gladwrap’ plastic wrap prior to incubation. This included short (30 minute) bench top incubations at room temperature.

(d) Decreasing the amount of BSA protein in the protein block steps and in the antibody dilution buffer.

A reduction in the amount of protein in the protein blocking solution and in the antibody dilution buffer from 3% to 0.25% significantly reduced drying of sections during incubation times and did not result in increases in non-specific binding if used in conjunction with 2% normal serum from the species in which the secondary antibody was made.

(e) Use of oviduct as a positive control for ERβ1

Recommended positive controls for ER include the use of uterus for ERα and the use of skin for ERβ (Data sheet Novacastra, U.K). Preliminary trials revealed oviduct to be superior to skin as a positive control for murine ERβ.

E-cadherin

The original protocol (refer 5.2.3) for this immunohistochemistry also used 0.5% triton in buffers. This was therefore reduced to 0.3% following the first buffer wash. Extra PBS rinses were given following all incubations in buffer containing triton. Oviduct additionally served as an excellent positive control for E-cadherin.

5.2.5 Single label immunofluorescence

Immunohistochemical localisation of ERα and ERβ and E-cadherin used specific fluorescent markers chosen for their individual excitation and emission spectra.
Fluorochromes were linked to secondary antibodies obtained from Molecular Probes, Inc; Eugene, Oregon, as listed in Table 5.1.

### Table 5.1. Immunohistochemistry using fluorescent probes

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
<th>Fluorochrome</th>
<th>Excitation Wavelength</th>
<th>Emission Wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>C1355 Rabbit polyclonal 1:100</td>
<td>Donkey anti rabbit 1:200</td>
<td>Alexa Fluor 488</td>
<td>488</td>
<td>Green BP 505-530</td>
</tr>
<tr>
<td>ERβ</td>
<td>NCL-ER-beta mouse monoclonal 1:50</td>
<td>Goat anti-mouse 1:200</td>
<td>Alexa Fluor 555</td>
<td>543</td>
<td>Red LP 560 or 560-610</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>E-CCD-2 Rat-anti-mouse monoclonal 1:40</td>
<td>Goat anti-rat biotinylated 1:200</td>
<td>Avidin- Alexa Fluor 488</td>
<td>488</td>
<td>Green 505-530</td>
</tr>
<tr>
<td>Reference nuclear stain (ER)</td>
<td>1:1000 in TBS</td>
<td>TO-PRO-3</td>
<td>633</td>
<td>Blue 650-LP</td>
<td></td>
</tr>
</tbody>
</table>

Immunohistochemical detection of ER using fluorescent probes required minimal alteration to the existing protocol for use with DAB. Since signal was not required to develop with use of streptavidin-biotin link, and visualization was permitted by way of direct conjugation of fluorochromes to the secondary antibody, time from removal of the secondary antibody to mounting of the specimens was significantly reduced. Secondary antibody application was performed in the dark and sections incubated in a black humidity chamber for 2 hours at RT. All precautions were taken to ensure that sections did not dehydrate (5.2.4 c) during this long bench top incubation. Following incubation with the fluorochrome-tagged secondary antibody, all sections were given three 2-minute washes in TBS and the fluorescent nuclear counterstain TO-PRO-3 (T3605 monomeric cyanine nucleic acid stain; Invitrogen, USA) pipetted onto sections at a concentration of 1:1000 in TBS to specifically label cell nuclei.
After two further rinses in TBS, sections remained hydrated and were coverslipped using Vectashield antifade mountant (Vector Laboratories, U.K), protected from light with aluminium foil, and stored at 4°C until study with the Confocal microscope.

E-cadherin secondary antibody and fluorescent probe were kindly gifted by the Herbison lab (Centre for Neuroendocrine Research, University of Otago). The secondary antibody was biotinylated, requiring the probe to contain an avidin link. Incubation time did not alter as a result.

5.2.6 Confocal microscopy

Specimens were visualised using the Zeiss Axioplan upright confocal microscope with the LSM 510 control software (Windows NT) (Fig. 5.1). Confocal microscopes offer several advantages over conventional fluorescent microscopes. The confocal has the ability to entirely eliminate out of focus flare produced by thick specimens labelled with fluorescent probes. This results in a better signal to noise ratio and superior resolution. The microscope utilises a laser as its light source and optically scans biological specimens. Dichroic mirrors in the scan head of the microscope prevent the illumination light (which has a longer wavelength than the fluorescence light emitting from the specimen) from reaching the detectors. Therefore the image produced corresponds only to the fluorescence emitted by the object of interest at a specified level or plane. With the alignment of a pinhole, the diameter of which is determined by the software in line with the desired optical resolution of the specimen, the thickness (optical slice) of the specimen can be controlled producing optical slices less than 2 μm. Consecutive scans through the Z axis may be stacked by the software to produce a ‘Z series’ or 3-dimensional representation of the structure of interest.
Figure 5.1 Zeiss Upright Confocal Microscope imaging
Image courtesy of Mr. A. McNaughton, Otago Centre for Confocal Microscopy.
5.2.7 Semi-quantitative analysis of ERβ expression using confocal imaging and immunofluorescence

Twenty micrometre thick sections obtained from treated and untreated ovaries were stained for immunohistochemical detection of ERβ using goat anti-mouse secondary antibody linked to Alexa Fluor 555, and visualised using the Zeiss upright confocal microscope (5.2.1.6). Short durations of transmitted light at a reduced luminance and the 4x objective lens were used to locate the section on the slide. The specimen was viewed at higher magnification with the 63x objective lens and oil immersion. At higher magnification, the light source was changed to the helium laser (excitation wavelength 543). For each section of ovary (n=4, control, n=5, treated), 3 areas of OSE were selected at random. Once selected, the area was twice further magnified with use of the zoom function of the microscope software. At this point immunostaining was easily identifiable in control animals, but the settings for the microscope required additional calibration to produce the best signal to noise ratio using a pinhole (optical slice thickness) of less than 2μm.

The point at which visualization of the receptor became optimal (best signal to noise ratio) was achieved by adjusting the detector gain setting of the software. The minimum possible laser intensity was used to obtain images and laser intensity held constant throughout all scanning. If laser intensity increases during image acquisition it causes faster bleaching of fluorochromes, and would make comparisons between fluorescent profiles invalid.

Configurations were recorded for each section viewed, and detector gain settings averaged over all sections examined in a single scanning session to provide a baseline or reference setting in untreated ovaries. These pre-configured settings produced a fluorescence intensity profile for OSE from control animals, that could be used for comparison with images of OSE obtained from EV treated ovaries examined during the same scanning session. Each scanning session included OSE profiles from sections taken first from control animals and then from EV treated animals. Ovary sections which had the primary antibody omitted or consisted of an IgG isotype control were
included with each batch of immunohistochemical slides to act as negative controls, while adjacent oviduct served as an internal positive control for the presence of ERβ. Negative control sections were scanned following control animal sections. Established baseline confocal settings obtained from control ovaries were then used to semi-quantitatively determine fluorescence emission from negative control specimens relative to untreated (control) and EV-treated OSE.

An arbitrary OSE length of 50μm was chosen to measure fluorescence. Using Image J image profile software analysis, a line was routinely drawn through the midline of the length of OSE so as to follow a consistent pattern of measurement. As the line intersected fluorescent signal emitted from estrogen receptor, the signal was transduced and given a numerical value (represented on the y-axis), while length of OSE was projected along the x-axis (Fig 5.1). The software additionally provided a print out of all values. Entry of these values into Excel software allowed for a semi-quantitative analysis of ERβ using Mann Whitney statistical analysis, which does not assume Gaussian distribution of data (GraphPad Prism 4, version 4.0c statistical software, GraphPad Prism Inc; San Diego, California). Confidence interval was 95%. P< 0.05 was considered statistically significant.

5.2.8 Dual and triple label immunofluorescence: ERα and ERβ

Confocal microscopy is a powerful tool for use in determining the colocalization of up to three different antibodies linked to selected fluorochromes, because it is able to detect the different specific wavelengths at which each fluorochrome emits signal (emission spectra, Table 5.1). Therefore two distinct epitopes colocalized to the same cell may be detected concurrently. The above protocols (5.2.2-5.2.3) were modified to colocalize both receptor subtypes in OSE and oviduct.

Double labelling of ERα and ERβ was achieved by incubating sections with the primary and secondary fluorochrome-tagged antibodies described (5.2.2, 5.2.5). Primary antibodies were diluted in antibody dilution buffer in separate eppendorfs. Antibody concentration was adjusted so that when combined with each other in a single eppendorf, the final concentration of antibodies matched the optimized
concentration used when single label was applied (Table 5.1). Likewise, secondary antibodies were diluted 1:100 in dilution buffer in separate eppendorfs and equal volumes of each combined in a single eppendorf, rendering a final dilution of 1:200. Primary and secondary antibodies were mixed by gentle inversion and brief vortexing prior to application onto sections. Use of this method negated the requirement for two consecutive 2-hour incubations at room temperature, and prevented drying of sections. Triple label of structures required the addition of a 10-minute incubation step with the nuclear label TO-PRO-3 diluted 1:1000 in TBS (refer 5.2.5). This incubation followed concurrent application of both secondary antibodies. The remainder of the immunohistochemical protocol was unaltered from that used in single label technique.

5.2.9. Dual label immunofluorescence: ERβ and E-cadherin

Achieving double labeling of E-cadherin and ER-beta on OSE presented the following challenges when developing the protocol:

1. Antigen retrieval required two separate retrieval buffers; since estrogen receptor epitope unmasking was best when 0.01 mol 1⁻¹ citrate buffer was used, while E-cadherin epitope retrieval required 0.1 mol 1⁻¹ EDTA.
2. Phosphate buffered saline (PBS) 0.1 mol 1⁻¹ (pH 7.4) was the recommended buffer for E-cadherin immunohistochemistry, while TBS was recommended for estrogen receptor.
3. E-cadherin immunodetection used a tertiary fluorochrome (Alexa Fluor 488) linked to avidin. Although this carried the advantage of signal amplification, it required the E-cadherin protocol to include a biotinylated secondary antibody (goat anti-rat biotinylated IgG, Jackson Labs). This was not required for estrogen receptor.

To overcome these challenges, the two protocols were run separately until the blocking step for non-specific binding, when double label sections were exposed to 20% normal goat serum for 10 minutes, followed by incubation for a further 30 minutes with TBS/Triton and 0.25 % BSA. Although TBS was used in the protein block for ERβ, PBS was used thereafter in all antibody dilution buffers. Protein block was performed
in a moist chamber at room temperature, after which sections were briefly rinsed in PBS prior to incubation with avidin block for 30 minutes at RT. A further rinse in PBS was followed by application of Biotin blocking solution (5.2.3). For all other wash steps, the ER wash sequence (TBS for 10 minutes followed by TBS/Triton (0.3%) for 10 minutes and TBS alone for 10 minutes) was adhered to, with the exception that TBS was replaced with PBS from application of the primary antibody onward. After avidin/biotin block, sections were isolated with use of a hydrophobic pen and ERβ primary antibody (NCL-ER-beta, mouse monoclonal clone EMRO2 (Novacastra, U.K) and E-cadherin, rat anti-mouse E-cadherin monoclonal primary antibody (Clone ECCD-2, Zymed Laboratories) applied. Optimal dilution for ERβ was previously shown to be 1:50 in antibody dilution buffer, while that of E-cadherin was 1:40. Consequently for dual label, ERβ was diluted 1:25 and E-cadherin 1:20. Both antibodies were then combined in equal volumes to give the desired final concentration. Antibodies were gently inverted and briefly vortexed to ensure adequate mixing. Sections were incubated overnight in a moist humidity chamber at 4°C.

A wash sequence to remove traces of primary antibodies was followed by application of (E-cadherin associated) secondary antibody, biotin-SP-conjugated AffiniPure Goat anti-rat IgG (Jackson Labs) diluted 1:100 in PBS and incubation of sections reduced to 40 minutes at RT. This secondary antibody was removed with three consecutive 10-minute washes (above). Both the secondary antibody for ERβ linked to Alexa Fluor 555, and tertiary reagent avidin-Alexa Fluor 488 had optimal final dilutions of 1:200, therefore, each was prepared at a 1:100 dilution and equal volumes mixed as previously described. Incubation was performed in the dark at room temperature for 2 hours with all precautions taken to avoid desiccation of tissue sections (5.2.4 c). Following incubation sections were given three 2-minute washes in PBS and coverslipped using Vectashield anti-fade mountant (Vector Laboratories, U.K). Protection from light was with aluminium foil, and storage of sections was at 4°C until examined under the confocal microscope.

Control sections for dual label immunohistochemistry had primary antibody omitted from the dilution buffer or primary antibody replaced with equivalent concentration of IgG isotope serum.
5.3 Results

5.3.1 ERα and ERβ protein: immunohistochemical localization and expression

1. Control mice (the older normal mouse)

Results are representative of mice sacrificed in diestrus.

**ERα localization and expression in the reproductive tract of control mice**

Strong nuclear and weaker cytoplasmic expression of ERα was localised to uterine epithelial cells. Nuclei of uterine stromal cells underlying epithelia and surrounding uterine glands additionally showed moderate to intense staining for ERα (Fig. 5.2A). An intense nuclear stain was also seen in oviductal luminal epithelial cells (Fig. 5.3A). In the ovary (Fig. 5.4A), strong expression was noted in nuclei of stromal fibroblast and interstitial cells, while some staining was also observed in all follicle types. Weak to moderate expression of ERα occurred in theca. Inclusion cysts did not stain for ERα, however interstitial cells surrounding the cysts did. OSE showed high expression of the receptor, with staining strongest in epithelial cells lining ovarian surface invaginations and near areas of OSE-mesothelial transition. Likewise, the mesothelial lining of ovarian bursa stained intensely adjacent to the ovary where it adjoined OSE.

**ERβ localization and expression in the reproductive tract of control mice**

When 5μm sections were examined under the light microscope, it appeared that ERβ expression was restricted to follicles in the ovary and oviduct. Once section thickness increased, a discrete particulate staining pattern for ERβ was noted in other parts of the ovary. Weak to moderate expression was observed in uterine epithelial and stromal cells (Fig. 5.2C). Oviduct epithelia appeared to have an intense uniformly nuclear staining pattern for ERβ (Fig. 5.3C 1). On high magnification (Fig 5.3C 2) it could be recognised that this was the result of a high receptor density in these cells compounding the particulate distribution of ERβ, and giving the appearance of a uniformly nuclear stain. In the ovary, ERβ expression was highest in granulosa cells of developing follicles, but was present in all follicle types. Bursal membrane, corpora lutea and OSE also showed ERβ expression. There was an absence of ERβ in theca (Fig. 5.5). Unfortunately, sections viewed (at random) in control ovaries contained no
cysts. Therefore no comparison of ERβ in cysts was possible between treated and untreated ovaries.
**Figure 5.2 ERα and ERβ expression in control mouse uterus**

*(DAB/Haematoxylin)*

(A) Uterine epithelia lining the lumen of the uterus (marked with an asterisk), and uterine glands (both arrowed) show strong nuclear (N) and weaker cytoplasmic (C) stain for ERα. Nuclei of stromal cells also express the receptor (arrowheads).

(B) Negative control where primary antibody was omitted from the dilution buffer showing no reactivity for ERα.

(C) Section of uterus showing weak nuclear stain for ERβ in luminal and glandular epithelium (both arrowed), and stroma (arrowheads). Uterine lumen is marked with an asterisk.

(D) Negative control. Primary antibody replaced with an IgG isotype serum showing no immunoreactivity for ERβ, but some evidence of endogenous peroxidase activity despite pre-treatment with H2O2 and methanol.

Scale bars = 50μm Section thickness = 5μm for ERα and 20μm for ERβ
Figure 5.3 ERα and ERβ expression in control mouse oviduct

(DAB/Haematoxylin)

(A) Oviduct epithelial cells showing nuclear staining for ERα (arrows).
Scale bar = 50μm

(B) Negative control. Primary antibody has been omitted from the dilution buffer and resulted in no staining for ERα. Scale bar = 50μm

(C1) A 5μm thick longitudinal section of oviduct where staining for ERβ is intense and appears uniformly nuclear in luminal epithelial cells (arrows). Scale bar = 50μm

(C2) A 20μm thick cross section of oviduct at higher magnification reveals the particulate nature of the ERβ receptor on oviduct epithelial cells. Scale bar = 25μm

(D) Negative controls for C1 and C2, showing no ERβ expression, where in D1 primary antibody was replaced with IgG isotype serum and in D2 primary antibody was omitted from the antibody dilution buffer. Scale bar = 50μm
Figure 5.4 ERα expression in control mouse ovary

(DAB/Haematoxylin)

(A) Control mouse ovary showing strong expression for ERα in OSE near the point of OSE (O)/mesothelial(M) transition. Stromal interstitial cells in close proximity to small atretic and primary follicles (arrows) also showed high expression of the receptor, while weak to moderate expression was seen in growing follicles (GF), theca (T) and corpora lutea (CL).

(B) Negative control. Primary antibody replaced with IgG isotype serum showing no staining. Scale bars = 50μm
**Figure 5.5 ERβ expression in control mouse ovary**

*(DAB/Haematoxylin) Section thickness 20\(\mu \text{m})*

(A) Control mouse showing strong nuclear expression of ERβ in granulosa cells of growing follicles (arrows). Staining of OSE forming a small involution can also be seen at this magnification (arrowheads). Higher magnification of OSE, refer Fig.5.7. Scale bar = 50\(\mu \text{m})*

(B) Stromal interstitial cells (arrows) in close proximity to both degenerating atretic (asterisk) and growing follicles (arrows) also stained strongly for ERβ. Scale bar = 50\(\mu \text{m})*

(C) Cells of corpora lutea expressing ERβ shown at 100x mag. At this magnification, the particulate pattern of stain for ERβ can clearly be seen. Scale bar = 12.5\(\mu \text{m})*

(D) Negative control. Primary antibody replaced with IgG isotype serum showing a small degree of non-specific stain present in cells of corpora lutea at all times. Scale bar = 50\(\mu \text{m})*


**EV treated mice**

*ERα and ERβ localization and expression in the reproductive tract of older mice after a single estradiol injection*

Two days after estradiol treatment there was strong uterine epithelial expression of ERα but markedly decreased levels of ERβ. Expression in oviductal epithelia was similar to controls for ERβ but reduced for ERα. In the ovary, ERα varied from moderate to strong expression in ovarian follicles, but was decreased in stroma underlying OSE, and in bursa compared to controls. ERβ expression was similar to control mice in ovarian stroma but decreased relative to controls in ovarian bursa. Although estradiol had some effect in down-regulating ERβ in follicular granulosa cells, staining for the receptor was clearly visible. On OSE, expression of both estrogen receptors was markedly reduced compared to control ovaries. The reduction in ERα expression occurred uniformly across OSE cell shape and loci. In contrast loss of ERβ expression was most in squamous and cuboidal OSE cells. Pockets of strong ERβ expression remained in areas of OSE invagination, OSE abutting points of OSE/mesothelial transition, and in areas of OSE showing a tendency toward metaplasia (columnar and layered epithelium).

Table 5.2(a) summarises the localization and expression patterns of both estrogen receptor subtypes on day 2; in ovary, oviduct, and uterus from control mice sacrificed at diestrus. Table 5.2(b) gives the same summary for estradiol-treated mice. As the surface epithelium of the ovary was the focus of this thesis, images depicting the regulation of estrogen receptor subtype by exogenous estradiol were restricted to OSE and are shown in Figure 5.6.

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Table 5.2 ER protein expression in older mouse ovary day 2

Table 5.2(a) Control

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<thead>
<tr>
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<td>All follicle types</td>
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<tr>
<td>Corpora lutea</td>
<td>+</td>
</tr>
<tr>
<td>Stroma</td>
<td>++</td>
</tr>
<tr>
<td>OSE</td>
<td>++/#</td>
</tr>
<tr>
<td>Bursa</td>
<td>++</td>
</tr>
<tr>
<td>Oviduct</td>
<td>++</td>
</tr>
<tr>
<td>Cysts</td>
<td>0</td>
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Table 5.2(b) EV treated

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<tr>
<td>Stroma</td>
<td>0-+</td>
</tr>
<tr>
<td>OSE</td>
<td>+</td>
</tr>
<tr>
<td>Bursa</td>
<td>+</td>
</tr>
<tr>
<td>Oviduct</td>
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</tr>
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<td>Cysts</td>
<td>0</td>
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<tr>
<td>Uterus (epithelium)</td>
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<td>++</td>
</tr>
<tr>
<td>Cysts</td>
<td>0-+</td>
</tr>
<tr>
<td>Uterus (epithelium)</td>
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</tr>
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</table>

0: None  
+: Weak to moderate  
++: Intense  
#: Stain intensity greater near OSE/mesothelial transition, in invaginations and involutions, areas of cell layering and columnar OSE
5.3.1.1 Comparison of ERα and ERβ protein expression in treated OSE following a single and a repeat estradiol injection (day 2 and day 16)

Figure 5.6 shows typical ER staining/expression patterns in OSE from EV treated mice sacrificed on day 2 and day 16. Light microscopic images shown represent results obtained from 4 repeated immunohistochemical experiments.

**ERα expression**

ERα protein expression in treated mice (Fig. 5.6B) was reduced compared to controls (Fig. 5.6A) in OSE, and in stromal fibroblasts and interstitial cells underlying OSE by day 2, following a first exposure to EV. On day 16 after a second estradiol exposure, treated OSE showed high ERα expression with >95% of cells positive for the receptor (Fig. 5.6C). In contrast, ovarian stromal cells showed progressive loss of ERα. ERα was not detected in cyst epithelium of treated groups.

**ERβ expression**

The particulate staining pattern for ERβ seen in cells of corpora lutea, follicles and oviduct of control mice (Fig. 5.5 (A-C), Fig. 5.3 (C1 and C2), was also evident in these structures in treated ovaries at both time points. In addition, cysts seen on day 2 stained positive for ERβ near OSE/mesothelial boundaries.

Compared to controls (Fig. 5.7A), low, often non-detectable levels of ERβ were seen in OSE overlying corpora lutea and pre-ovulatory follicles, where cells were simple squamous or cuboidal (Fig. 5.7B). Some high expression remained, but was mostly limited to OSE near the OSE/mesothelial boundary, and in areas of cell layering and invagination where OSE cells were columnar in shape. Overall, a down-regulation of ERβ occurred in OSE within 48 hours of treatment. At day 16, following second estradiol exposure, ERβ expression remained reduced in OSE compared to controls (Fig. 5.7C).
Figure 5.6 Comparison of day 2 and day 16 ERα protein expression in OSE of estradiol treated mice (Gills Haematoxylin/DAB)

(A) Control mouse ovary showing high expression of ERα in OSE. Note strong expression in stromal cells (arrows). Scale bar = 25μm

(B) Day 2 EV treated mouse ovary showing reduced expression of ERα in both stroma and OSE. Scale bar = 25μm

(C) Day 16 EV treated mouse ovary showing a return of ERα expression in OSE but not stroma. Scale bar = 25μm

(D) Negative control. Primary antibody replaced with IgG isotype serum showing no immunoreactivity. Scale bar =18μm

(E) Positive control (uterus of estrus mouse) staining for ERα in luminal epithelium Scale bar = 50μm
Figure 5.6 (A-E)
Figure 5.7 Comparison of day 2 and day 16 ERβ protein expression in OSE of estradiol treated mice (Gills Haematoxylin/DAB) Section thickness 20μm.

(A) Control mouse ovary showing the characteristic particulate distribution of ERβ in OSE cells. Scale bar = 25μm

(B) Day 2 EV treated mouse ovary showing a marked decrease in ERβ receptor expression in OSE cells. Scale bar = 25μm

(C) Day 16 EV treated ovary with reduction of ERβ in OSE in comparison to untreated controls. Scale bar = 25μm

(D) Negative control. Primary antibody replaced with IgG isotype serum showing no immunoreactivity. Scale bar = 12.5μm

(E) Positive control. Oviduct of diestrus mouse showing extensive ERβ immunoreactivity Scale bar = 25μm
Figure 5.7 (A-E)
5.3.2 E-cadherin

*Expression in ovary of control mice*

E-cadherin immunohistochemistry was repeated to obtain three sets of results from which observations of expression and patterns of distribution were made for 3 mice per group. OSE of control mice did not show a ubiquitous expression of E-cadherin. Simple squamous to cuboidal OSE cells consistently showed no to low levels of staining (Fig. 5.8 A-B). In contrast, columnar-shaped cells, and areas of OSE invagination and cell layering showed high levels of E-cadherin expression (Fig. 5.8C).

*Expression in ovary of EV treated mice on day 2 and day 16*

OSE of treated mice responded to first estradiol exposure with high levels of E-cadherin expression involving all cell shapes (day 2), (Fig. 5.8D). However at day 16, following a second exposure to EV, E-cadherin expression was markedly reduced in all OSE cell shapes (Fig. 5.8E).
Figure 5.8 E-cadherin expression in OSE

(A-C) Control mouse ovary with zero to low E-cadherin expression in squamous (A) and cuboidal (B), and strong expression in columnar (C) OSE cells. Scale bar = 20μm

(D) Day 2 EV treated mouse ovary showing high levels of E-cadherin expression in cuboidal OSE cells, typical of all cell shapes at this time. Scale bar = 25μm

(E) Day 16 EV treated mouse ovary showing a marked decrease in E-cadherin expression. Scale bar = 25μm

(F) Negative control with primary antibody omitted. Scale bar = 25μm
5.3.3 Single, dual and triple label immunofluorescence to assess receptor loci of ERα and ERβ in normal OSE

Single, dual, and triple immunofluorescent label (refer Table 5.1) was used to confirm and further define light microscopy results for expression and localization of both ERα and ERβ on OSE. The confocal images in this chapter are representative of 2 of the 4 animals studied per group. Images for the remaining 2 animals may be found in CD format in the back pocket of this thesis (Appendix G).

Single immunofluorescent label for ERα showed this receptor to be abundantly expressed on OSE of control mice. Figures 5.9(A) and 5.10(A) show ERα to have a diffuse staining pattern that initially appeared to have a predominately nuclear locus. Large vacuolated areas that were devoid of any immunofluorescent label, refer Fig. 5.9(E), were best observed amongst ERα stain. Single immunofluorescent label for ERβ (Fig.5.9B and Fig. 5.10B) indicated this receptor formed discrete clusters, accounting for the particulate staining pattern observed by light microscopy. The definition of ERβ relative to ERα using dual label immunofluorescence (Fig 5.9C and Fig, 5.10C) found that the two estrogen receptors predominantly localized adjacent to, but not exactly with, each other in OSE cells. To confirm the location of ERα and ERβ relative to the cell nucleus, the nuclear stain TO-PRO-3 (refer Table 5.1) was used (Fig. 5.9E and Fig. 5.10E). With this nuclear counterstain, ERα was seen to localize mostly to nucleus but also to cytoplasm, and colocalization of ERα with ERβ appeared to occur in the cytoplasm. ERβ formed both small and larger clusters inside and outside the nucleus. Nuclear aggregations of ERβ did not necessarily correspond to heterochromatin aggregation.
Figure 5.9 Single, dual, and triple immunofluorescent label for ERα and ERβ in cuboidal OSE of control mice using confocal microscopy

(A) Single label immunofluorescence for ERα expression in OSE using Alexa Fluor 488 (green). ERα receptor has a diffuse expression that is predominantly nuclear in this image. Isolated bright specs of fluorescence for ERα can be seen amongst more diffuse stain. Areas devoid of stain (black) are seen separating sites of receptor expression.

(B) Single immunofluorescent label for ERβ in mouse OSE using Alexa Fluor 555 (red) shows that ERβ forms variable sized clusters.

(C) Dual label for ERα (green) and ERβ (red). ERα is the dominant receptor expressed in mouse OSE. Expression of ERβ relative to ERα varies from cell to cell. Areas where the receptors colocalize appear as yellow and can be seen in 2 out of the 7 cells shown.

(D) Nuclei of mouse OSE cells are labeled with TO-PRO-3 (blue).

(E) Triple immunofluorescent label. Nuclei (blue), ERα (green), and ERβ (red). ERα localizes predominantly to nuclei green/aqua. ERβ localizes to nuclei (pink) and there is some evidence for presence in the cytoplasm (red). Areas of nuclear chromatin aggregation do not always correspond to areas of expression of ERα or ERβ. Areas devoid of all stain are evident in the nucleus.
Figure 5.9 (A-E)
Figure 5.10 Single, dual and triple immunofluorescent label for ERα and ERβ in columnar OSE of control mice

(A) Columnar OSE cells show a more diffuse pattern of ERα expression (green) compared to cuboidal cells. Areas devoid of stain are once again seen within OSE cells expressing ERα. Small isolated bright specs of green immunofluorescence are also present.

(B) In columnar OSE, ERβ (red) shows the characteristic particulate staining pattern seen in cuboidal cells, with the exception that large clusters of ERβ expression within OSE cells appear more numerous.

(C) Dual label for ERα and ERβ show ERβ to occupy some of the non-staining areas previously identified with single label fluorescence for ERα (A). Colocalization of ERα with ERβ is infrequent in columnar OSE compared to cuboidal OSE.

(D) Nuclei stained with TO-PRO-3.

(E) Triple label immunofluorescence in columnar OSE shows ERα to almost exclusively be expressed in the nucleus of the cell (green/aqua) although isolated areas can be identified where ERα localizes to the cytoplasm producing a wispy appearance at the superior nuclear boundaries. ERβ localizes predominantly to the nucleus (pink), but is also seen in small clusters in the cytoplasm (red).

(F) Negative control. Primary antibody omitted, showing no staining for ER.
Figure 5.10 (A-D)
Figure 5.10 (E-F)
5.3.4 ERβ expression profile in control mouse OSE using confocal microscopy and single label immunofluorescence: a method for semi-quantitative analysis of ERβ

Figures 5.11(A and B) show the distribution of ERβ in 10 OSE cells of 2 control mice. A single line, used as a probe to detect the intensity of immunofluorescence emitted by the receptor, was drawn centrally through OSE cells for a length of 50μm producing profiles for each scan. Clusters of ERβ in OSE cells from control mice frequently produced fluorescence spikes of between 200-250 arbitrary units. All sections scanned for semi-quantitative analysis were labelled only for ERβ and only with the Alexa Fluor 555 fluorochrome. Figure 5.12(A-B) shows the OSE profile where replacement of primary antibody with an IgG isotype serum (A) and omission of the primary antibody (B) resulted in no detectable fluorescent signal using pre-determined baseline settings for the LSM software (5.2.1.7), while Fig. 5.13 shows high expression of ERβ in oviduct and provides a positive control.
**Figure 5.11 (A and B)** Immunofluorescence profiling for semi-quantitative analysis of ERβ protein expression in control mouse OSE

The fluorescence emitted by discrete collections of the receptor in 10 OSE cells from 2 mice (A and B) has been captured using the 63x oil objective lens and the 3x zoom function of the LSM software on the confocal microscope. The resulting fluorescence profile is seen below each image and has been plotted using Image J software. Scale bars = 5µm.
Figure 5.11 (A-B) Immunofluorescence profile from 2 control mice.
Figure 5.12 Negative controls used for immunofluorescence profiling of ERβ protein expression. Images captured using (pre-optimized) LSM software settings demonstrate no immunoreactivity.

(A) Negative control 1 Primary antibody replaced with IgG isotype serum
(B) Negative control 2 Primary antibody omitted from the antibody dilution buffer
Scale bars = 5μm
Figure 5.13 Oviduct: positive control for ERβ

Positive control for ERβ: control mouse oviduct showing intense fluorescence in clusters of luminal epithelial cells.
5.3.5 ERβ expression profile in OSE of EV treated mice day 2

Figure 5.14 (A -B) illustrates the marked reduction in fluorescence emitted by ERβ in the OSE of 2 EV treated mice following 48 hours of estradiol exposure. The fluorescence profiles shown are representative of that most frequently observed. Where OSE nearer the OSE-mesothelial juncture or within surface invaginations showed the highest degree of immunofluorescence, OSE overlying larger structures within the ovary was at times completely devoid of fluorescent signal. Figure 5.14B (see scale) illustrates the high degree of sensitivity associated with immunofluorescence profiling.
Figure 5.14 ERβ expression profile in OSE of EV treated mice day 2

When data had been collected from all mice the average fluorescence intensity emitted by ERβ was calculated for each animal group and revealed that an 11-fold reduction in functional ERβ expression had occurred in response to a first exposure to estradiol (Figure 5.15).
Figure 5.14 (A-B) Immunofluorescence profile from two day 2 EV treated mice.
Figure 5.15 Semi-quantitative analysis of ERβ protein expression in OSE of control and EV treated mice (day 2)

The bar graph represents mean fluorescence intensity produced by ERβ expression in ovaries of 4 control mice sacrificed at diestrus and 5 EV treated mice. A total of 5 sections were taken from each ovary and for each section, 3 randomly selected areas of OSE were scanned using the LSM software, and the pre-calibrated optimized settings (5.2.7) on the confocal microscope. All scans of OSE were for 50μm and included 10-13 OSE cells, depending on whether the cells were cuboidal or columnar in shape. Using this method, it was calculated that by day 2, a highly significant decrease in functional ERβ protein expression had occurred in response to a first exposure to estradiol (P<0.0001).
5.3.6 Localization of ERβ relative to ERα in OSE cells using dual label immunofluorescence

To demonstrate the location of ERβ relative to ERα, a dual fluorescence profile was constructed for 3 loci per ovary section where the two receptors were seen in the same plane relative to the Z-axis. In total, five sections were taken at random through the ovary of control mice sacrificed at diestrus (n=4). Representative immunofluorescence profiles for 2 of the 4 mice are shown in figure 5.16 and 5.17 (E) and demonstrate that the point of maximal fluorescence emission for ERβ predominantly originates from a locus nearby ERα. Since the optical slice (pinhole) was less than 2 μm, the distance separating the two receptors from each other in the Z-axis can be estimated to be less than 2μm. However in every profile a degree of fluorescence emittance overlap was seen, suggesting that ERβ colocalizes with ERα in some instances. Representative images from the remaining two mice can be accessed from the CD in the back pocket of this thesis.
Figure 5.16 Dual label immunofluorescence showing the relative location of both estrogen receptor subtypes on OSE of control mice

Mouse 1
Fluorescent label is shown for ERα (green reaction product, Alexa Fluor 488), and ERβ (red reaction product, Alexa Fluor 555)

(A) Characteristic stain of ERα in OSE cells
(B) Particulate distribution of ERβ in OSE cells

(C) Merged fluorescence image of ERα and ERβ shows the close proximity of the two estrogen receptors

(D) Merged fluorescence image shown in (C) where a line (probe), drawn through a selection of 4 OSE cells, gives rise to 5 intercepts (arrows) where ERα and ERβ lie in the same plane relative to the Z-axis. Profiles 1, 2 and 3 show varying degrees of yellow fluorescence, indicating a shared receptor locus. Profiles 4 and 5 show a more distinct red fluorescence for ERβ and a green fluorescence for ERα, indicating a close, but not a shared, receptor locus. Two cells are circled to better illustrate the yellow fluorescence.

(E) Red/green immunofluorescence profile produced by the probe shows fluorescence emission for ERα and ERβ in probed OSE cells. Maximum fluorescence intensity is seen to occur in the same locus in profiles 1, 2 and to some extent in profile 3. Profiles 4 and 5 show a larger gap in peak fluorescence intensity and suggest a more defined level of separation may exist between the two receptors.
Figure 5.16 Dual label immunofluorescence ERα and ERβ
Figure 5.17 Dual label immunofluorescence showing the relative location of both estrogen receptor subtypes on OSE of control mice

Mouse 2
(A-C) Fluorescent label is shown for ERα (green reaction product, Alexa Fluor 488), and ERβ (red reaction product, Alexa Fluor 555) as for Fig 5.10.

(D) Merged image shown in (C) where a line, drawn through a selection of 3 OSE cells (arrows), gives rise to multiple smaller intercepts where ERα and ERβ lie in the same plane relative to the Z-axis. Profiles from the second OSE cell show areas of yellow fluorescence, indicating a shared receptor locus. Cells 1, 2 and 3 also show areas of distinct red fluorescence for ERβ and green fluorescence for ERα, again indicating a close, but not a shared, receptor locus.

(E) Red/green immunofluorescence profile produced by the probe shows fluorescence emission for ERα and ERβ in probed OSE cells. Maximum fluorescence intensity is seen to occur in the same locus in 3 out of 5 profiles from cell 2 and indicates a shared locus for ER in those profiles. Profiles from cells 1 and 3 show separations in peak fluorescence, indicating that in these cells, a distance between receptors occurred and the extent of colocalization of ERα and ERβ was less.
5.3.7 Single, dual, and triple immunofluorescent label to assess receptor locus of ERα and ERβ in normal oviduct for comparison with OSE

As OSE and oviductal epithelia share common embryonic origins, and both express estrogen receptor, a comparative qualitative analysis of ER loci in OSE and oviduct of the same control mice sacrificed in diestrus (n=4), was performed to assess whether the location of ER α and ERβ differed between these tissues.

In oviduct, both ERα and ERβ were expressed in the cytoplasm and nuclei of luminal epithelial cells (Fig.5.18 (E)). This pattern of expression was also evident for ER of uterine luminal epithelium (not shown). The spatial relationship between ER subtype in oviduct appeared to be similar to that of OSE except that expression of ERβ relative to ERα was greater. Furthermore, ERβ appeared to colocalize less with ERα in the cytoplasm of oviductal epithelia when compared to OSE.
Figure 5.18
Single, dual, and triple fluorescent label for ERα and ERβ in the oviduct of 2 control mice using the Alexa Fluor fluorochromes described in Fig. 5.9

Mouse 1
(A) Single label immunofluorescence for ERα in luminal epithelial oviduct cells mouse showing strong ERα expression (green).

(B) Single label immunofluorescence in luminal epithelial oviduct cells showing high expression of ERβ (red).

(C) Dual label for both estrogen receptor subtypes in oviduct show that the abundance of ERα relative to ERβ varies from cell to cell. Evidence for colocalization of both ER (yellow fluorescence) is scant.

(D) Nuclei stained with TO-PRO-3

(E) Triple immunofluorescent label shows ERβ is expressed predominantly in the nucleus (pink), but small clusters also localize to the cytoplasm (red). ERα is expressed in both the nucleus (green/aqua) and cytoplasm (faint green seen peripheral to nuclei). Triple label confirms the sparse degree of colocalization of ERα and ERβ (almost complete absence of yellow immunofluorescent stain). Unstained areas of the nucleus (black) are evident, unoccupied by either ER subtype or chromatin.
Figure 5.18 (A-C) Mouse 1
Figure 5.18 (D-E) Mouse 1
Mouse 2

(A) Single label immunofluorescence for ERα showing a moderate to high expression in luminal epithelial cells of the oviduct.

(B) Single label immunofluorescence for ERβ again showing high expression in oviduct.

(C) Dual label immunofluorescence for both ER showing almost no detectable colocalization (yellow immunofluorescence is negligible).

(D) Nuclei stained with TO-PRO-3.

(E) Triple label showing almost exclusively nuclear (green/aqua) expression of ERα. ERβ localizes predominantly to the nucleus (pink), but large clusters of the receptor are also present outside the nucleus also (red). Lack of colocalization seen with dual label is confirmed with triple label.

(F) Negative control where primary body has been replaced with IgG isotype serum showing no detectable stain for ER.
Figure 5.18 (A-C) Mouse 2
Figure 5.18 (D-F) Mouse 2
5.3.8 Single and dual immunofluorescent label to assess ERβ expression patterns in OSE relative to the cell adhesion molecule E-cadherin in normal OSE

High magnification and the use of E-cadherin to delineate the location of the plasma membrane of both OSE and oviductal epithelia was initially considered a useful method to assess the relationship of ERβ to the cell membrane in both tissues. E-cadherin immunohistochemical expression was previously shown using DAB to be absent or weak in squamous and cuboidal epithelial cells of OSE however, (Fig 5.8.A-B) and sections chosen at random for the present analysis predominantly consisted of cuboidal OSE. Low abundance of E-cadherin therefore required LSM gain settings to be increased, enhancing the likelihood of artefact and making analysis more problematic. For this reason, the following results in three control mice should be viewed with a degree of caution.

E-cadherin expression (green fluorescence) was evident in basal membranes, with stronger expression in lateral and also some apical membranes (Fig.5.19A). ERβ expression was evident from particulate immunofluorescent label (red) and was chiefly confined to inside the cell (Fig. 5.19B). Colocalization of ERβ with E-cadherin in the cytoplasm was additionally suggested when dual label was used (Fig.5.19C). A nuclear label was not used as it was considered a further variable when combining the ERβ and E-cadherin protocols to obtain double label.
Figure 5.19 Single and dual immunofluorescent label for E-cadherin and ERβ in OSE of control mouse

(A) E-cadherin (Alexa Fluor 488, green) was present in low abundance in cuboidal OSE cells. Increasing the detector gain setting on the LSM software allowed signal to be detected on the basal and lateral membranes.

(B) ERβ (Alexa Fluor 555, red) is detected as a particulate product inside OSE cells, with some possible localization to the cytoplasm (circles).

(C) Dual labeling of E-cadherin and ERβ (yellow fluorescence) also suggests that some ERβ co-localizes with E-cadherin in the cytoplasm (circles).

(D) Negative control where primary antibody was omitted from the antibody dilution buffer and which demonstrates artefact in apical cell membranes due to increased noise relative to signal. Scale bars = 5 μm.
5.3.9 Single and dual immunofluorescent label to show ERβ and E-cadherin expression in oviduct epithelia of control mice

E-cadherin was strongly expressed at cell boundaries of oviductal epithelia in comparison to OSE. It was not expressed on the surface of cells facing the lumen of the oviduct, and was at times also absent from epithelial cell-cell boundaries within oviductal tissue (Fig. 5.20A). At high magnification, it was evident that ERβ expression was confined entirely within the nucleus/cytoplasm of oviductal epithelial cells (delineated with E-cadherin, Fig. 5.20C) with no evidence of an association with E-cadherin location.
Figure 5.20 Single and dual immunofluorescence labeling of E-cadherin (Alexa Fluor 488, green) and ERβ (Alexa Fluor 555, red) in control mouse oviduct

(A) Strong immunofluorescent label for E-cadherin was seen delineating the plasma membrane at points of cell-cell adhesion in oviduct (red arrows). It was absent from some cell-cell boundaries between isolated groups of neighbouring cells. E-cadherin was not present on the apical surface of luminal epithelial cells (yellow arrows). L marks the oviduct lumen.

(B) Strong ERβ immunoreactivity was observed in the oviduct epithelial cells.

(C) Dual label of ERβ and E-cadherin shows ERβ does not co-localize with E-cadherin at the cell membrane or in the cytoplasm of oviducal epithelia.

(E) Negative control where primary antibody was omitted showing no immunofluorescence. Scale bars = 10μm
5.4 Discussion

This chapter of the thesis has presented the results of experiments conducted to address the following questions:

1. What is the normal pattern of ERα and ERβ expression in older mouse reproductive tract, the ovary, and specifically the OSE?
2. What happens to normal expression when a single and a repeat dose of estradiol are administered?
3. If there are changes to the level of ER in OSE, can these be quantified using confocal microscopy and immunofluorescence?
4. Does the distribution of ERα and ERβ in OSE cells suggest a degree of receptor shuttling may occur?
5. What is the normal distribution of the cell adhesion molecule E-cadherin in older mouse OSE?
6. Is there any evidence for ER localizing to the cell membrane in OSE and could this be demonstrated using immunofluorescence?
7. Are there any differences between ER expression in OSE and oviduct, an example of Müllerian duct-derived epithelium?
8. Are there any differences between E-cadherin expression in OSE and oviduct?

The main findings of these experiments reveal that in the older mouse reproductive tract, both receptors are abundantly expressed. High levels of ERα expression were found in uterus, oviduct, ovarian stroma and OSE, with variable expression in all follicle types and corpora lutea. No expression was observed in any epithelial inclusion cysts examined. ERβ expression was highest in oviduct (a reliable positive control) and in the granulosa cells of growing ovarian follicles, however all follicle types showed some degree of stain for ERβ. The use of thick sections allowed for a much better appreciation of the normal expression patterns of ERβ, and older mouse OSE, bursae, and stromal interstitial cells surrounding follicles, also expressed high levels of this receptor. Variable ERβ expression was seen in corpora lutea of the ovary and in the uterus. Confocal microscopy and the use of dual and triple immunofluorescent label provided for excellent resolution of ER, considerably
adding to knowledge of normal expression patterns in OSE gained by immunohistochemistry using light microscopy. Subsequently, both ER were found to be expressed in the nucleus and cytoplasm of OSE cells. ERα had a diffuse but uniform staining pattern. In contrast, ERβ formed clusters, giving this receptor a particulate staining pattern. Triple label immunofluorescence defined nuclear boundaries and revealed areas of the nucleus that were black holes, expressing neither estrogen receptor and showing no chromatin aggregation. Dual and triple label immunofluorescence demonstrated the relationship between ERα and ERβ, and showed that although the two receptors colocalize in the cytoplasm of OSE cells, they additionally often appear to be situated very close to each other. Although not quantified, it appeared colocalization of ERβ occurred more frequently in cuboidal OSE compared to columnar OSE, and that in columnar OSE there was relatively more ERβ expression in the nuclei of OSE cells. ERβ relative to ERα expression on individual OSE cells was in addition, variable. Luminal epithelial cells of the oviduct also showed both nuclear and cytoplasmic expression of ER. The relative expression of ERα and ERβ varied from cell to cell as it did in OSE, however the epithelia of the oviduct appeared to have a higher level of ERβ expression than OSE, and like columnar OSE much of this expression was nuclear. Colocalization of ERα and ERβ was uncommon in oviductal OSE.

E-cadherin expression in untreated mouse OSE varied widely according to location when viewed by light microscopy. Squamous and cuboidal shaped cells showed little to no stain. In contrast, cells that were columnar in shape, invested cell surface invaginations of the ovary, or were located near points of OSE-mesothelial transition stained strongly for E-cadherin. When estradiol was first administered all OSE cells responded with a high level of E-cadherin expression. This was not seen following a second dose of the hormone, when a marked decrease in E-cadherin expression was seen in all OSE cells. Dual label immunofluorescence for E-cadherin and ERβ was performed to investigate if a relationship existed between the two in OSE. The results from this experiment should be viewed with caution because of the possibility that adjustment of the LSM settings on the confocal microscope made to optimize viewing of E-cadherin on OSE may have introduced a level of artefact. This was not evident in the negative controls, however, and so it
may be tentatively proposed that very small clusters of ERβ may colocalize with E-cadherin. However, this was not observed in the cell membrane of OSE cells, rather in their cytoplasm.

Two days following first estradiol treatment, there was a large decrease in the expression of both ER subtypes on OSE and ERα in stroma underlying OSE. This was seen using light microscopy to be accompanied by a marked increase in E-cadherin expression OSE. Semi-quantitative analysis of the mean immunofluorescence levels generated by ERβ in OSE of control ovaries and estradiol treated ovaries on day 2 showed that a highly significant downregulation of ERβ had occurred in the OSE of treated mice after first estradiol exposure. On day 16, following a second estradiol injection, levels of ERα on OSE were comparable to controls, while those of ERα in stroma and ERβ in OSE were low. E-cadherin expression was reduced markedly also.

Previous chapters in this thesis have established that within 48 hours of exposure to elevated blood and ovarian tissue levels of estradiol, morphological changes occur in OSE that include epithelial cell stratification, metaplasia and hypertrophy. Experiments from the present chapter additionally show that concurrent with these changes, dramatic decreases in ERα expression occur in OSE and the stroma underlying it, and this is accompanied by a highly significant decrease in expression of ERβ in OSE at this time. In contrast, levels of E-cadherin dramatically increase and this cell adhesion molecule becomes expressed in all OSE cells. The morphological changes seen in OSE, generated by a single exposure to estradiol in the present study, have been documented in the literature as representing early protective responses to factors challenging the structural or genomic integrity of cells within reproductive and other epithelial tissues (Rubin et al., 2007).

A requirement for ERα in OSE proliferation has previously been mooted (Bai et al., 2000), however experiments presented in this chapter reveal that when levels of ERβ expression on OSE are reduced relative to ERα (day 16), the epithelium does not duplicate the rapid hypertrophic, hyperplastic and metaplastic response seen after first exposure to the hormone. This indicates that the acute responsiveness of
the ovarian surface epithelium to estradiol may be reliant on OSE expressing both receptor subtypes in sufficient amounts. In normal murine mammary gland and in ovary, epithelial cells that are actively proliferating lose ERα expression (Zeps et al., 1999) (Bai et al., 2000). It has been reported that in the epithelial cells of mammary gland, ERβ facilitates the return of ERα to the nucleus of cells exposed to estradiol, and mediates their responsiveness to subsequent estrogen exposure (Cheng et al., 2004).

Experiments conducted as part of this PhD were not designed to address the specific timeframe involved in the OSE response to estradiol. Instead they were designed to align the response of the surface epithelium to estimated maximal and minimal serum estradiol levels. For this reason, it cannot be said exactly how quickly the OSE responded to the estrogen, although the rate of cellular hyperplasia was thought to exceed BrdU label available to OSE within 24 hours of subcutaneous estradiol injection (4.4). Equally, it is not possible to state precisely when a significant down-regulation of both ER subtypes occurred in response to initial estradiol exposure, yet this was clearly demonstrated 48 hours after EV using conventional immunohistochemistry and light microscopy; and in the case of ERβ, was quantified using immunofluorescence and confocal microscopy. Furthermore, ER downregulation has been shown in this thesis to coincide not only with markedly elevated estradiol levels in the blood, but also in the ovary. Exogenous estradiol has previously been reported to rapidly suppress ERα mRNA expression both in vitro (Nawaz et al., 1999, Bai et al., 2000) and in vivo (Hatsumi and Yamamuro, 2006,) and in the latter case occurs within 12 hours of hormone administration, with the receptor population failing to return to pre-treatment levels within 48 hours. In both cases, downregulation of the receptor in response to estradiol may occur as a consequence of some or all the following:

1. E2-induced rapid division of OSE cells may result in less ER as the cell number outstrips the availability of substrate required for the synthesis of new receptors
2. E2 may induce ER on OSE to undergo proteasome degradation
3. E2 may induce ER on OSE to control its own transcription via a short negative feedback loop
4. E2 may differentially regulate ER in OSE
5. Stromal ER may act synergistically with ER on OSE to control its expression
6. ER subtypes may regulate each other

These mechanisms are not mutually exclusive and at any one time, all may be acting on OSE. Alternatively, one or more mechanisms may prove to be homeostatic, while the prevalence of another may signal pathogenesis. Whatever the mechanism(s), the effects of estradiol on ER in this series of experiments appeared to be more enduring for ERβ than ERα, since ERβ expression remained downregulated following a second estradiol exposure, whereas that of ERα returned to pre-treatment levels. For this reason, and in consideration of time constraints, a semi-quantitative analysis of ER expression to establish the extent of receptor downregulation using confocal microscopy was performed only on ERβ and only for day 2 following initial estradiol exposure. The resulting analysis of immunofluorescent profiles produced by ERβ on OSE of treated mice, revealed the extent of downregulation of ERβ to be highly significant (P=<0.0001). Optimization of both the immunofluorescent protocol and the methodology used to obtain ER fluorescence profiles were paramount to the validity of these results, and possibly provide the first application of such a technique for use in quantifying receptor protein levels in vivo.

Interestingly, when LSM gain settings on the confocal microscope were increased maximally above pre-determined baseline settings obtained from control ovaries, the receptor could be seen, but the signal to noise ratio became sub-optimal. Nevertheless, the continued presence of ERβ on OSE suggests that the mechanism for the observed disappearance of this receptor from OSE 48 hours after a single estradiol exposure, involved downregulation and not a destruction of the receptor.

Furthermore, results of experiments presented in this chapter using conventional immunohistochemistry with DAB and light microscopy, have indicated that E2 may differentially regulate both receptor subtypes depending on the duration of estradiol exposure. Evidence to support this hypothesis, is that levels of ER expression in OSE varied between the two receptor subtypes only following a second estradiol
exposure, yet the amount of estradiol administered was dose-equivalent to the first. Moreover, estradiol has been shown in this study to differentially regulate ER subtype spatially, since ERα expression patterns in OSE and stroma were altered independently of each other regardless of the same degree of estradiol exposure.

ERα expression in ovarian stroma was downregulated after initial estradiol treatment and expression decreased further following a second depot injection of EV. In contrast ERα expression on OSE reverted to pre-treatment levels. Whether a relationship exists between stromal expression of ERα and patterns of ERβ in OSE remains to be determined, but a role for stroma in OSE proliferation has been suggested. Bai and colleagues (2000) reported that when rabbit OSE cells were co-cultured with ovarian stromal cells in the presence of estradiol, the amount of OSE cell growth was initially significantly more than when OSE cells were cultured with estradiol in isolation (Bai et al., 2000). However when OSE cells were expanded in cell culture medium over a 1-week period with or without ovarian stromal cells, they progressively lost ERα expression and ER transcriptional activity became undetectable (Bai et al., 2000). The authors state that this finding negates the possibility that the initial enhanced growth response of OSE cells to estradiol was due to the effect of ovarian stromal cells on OSE ERα expression. The in vivo independent regulation of stromal and OSE ERα expression presented in this chapter support Bai’s findings.

The use of confocal microscopy with dual and triple label immunofluorescence in this study, has allowed for the definitive localization of ERα and ERβ in normal OSE cells of older mice. Traditionally, both receptors have been thought to be nuclear (2.2.2), however the present study has shown that they also reside outside the nucleus. A small proportion of the ERβ1 subtype may additionally colocalize with E-cadherin in the cytoplasm of OSE cells, however this preliminary finding requires further support from more experiments. Since the optical slice taken to image the two estrogen receptors using dual fluorescent label was less than 2 microns when ERα and ERβ were oriented in the same plane, it is proposed that in many OSE cells, ERα and ERβ lie within a very short distance of less than 2μm from each other, and appear to colocalize in the cytoplasm. Taken together, the
observed concurrent nuclear and cytoplasmic expression of these receptors in any OSE cell, and their variable expression from cell to cell, suggests ER may continuously shuttle between nucleus and cytoplasm and that some OSE cells may bind estradiol and other ligands more than others, depending on their individual expression of receptor subtype. A similar spatial relationship exists between ERα and ERβ in the oviduct, where both receptors again localize predominantly to the nucleus, but also to the cytoplasm of luminal oviductal cells, suggesting that shuttling between the nucleus and cytoplasm also occurs in oviduct. Observed qualitative differences in ER expression between the two sites were the greater abundance of ERβ in oviduct epithelia compared to OSE, and the almost nonexistent colocalization of ER in oviductal epithelia compared to OSE. The pattern of ER subtype distribution in oviduct epithelia appeared to resemble more that seen in columnar epithelium, where the expression of ERβ was predominately nuclear. The differential expression of estrogen receptor subtypes in nucleus and in cytoplasm has been documented in other reproductive tissues including breast (Shaaban et al., 2008) (Lazennec et al., 2001) and placenta (Brodowska et al., 2007) and may be of important diagnostic and prognostic significance. For instance the over expression of ERβ-1 in tissues has been shown to have antiproliferative and pro-apoptotic effects on breast cancer cells in vitro (Lazennec et al., 2001) (Paruthiyil et al., 2004). Furthermore, Shabaan et al (2008) have recently shown that whereas cytoplasmic expression of ERβ-1 is associated with better overall survival from breast cancer, nuclear expression of ERβ-1 is not (Shaaban et al., 2008).

E-cadherin dual label immunofluorescence was able to confirm that in the oviduct, ERβ did not localize to the cell membrane, nor colocalize with E-cadherin in the cytoplasm. Due to the low level of E-cadherin expression in cuboidal and low columnar OSE obtained for analysis through random sampling, noise signal was produced when attempting to optimize LSM software settings for viewing ERβ and E-cadherin simultaneously, This introduced a degree of artefact that made interpretation of the findings more challenging. Nevertheless, results did show ERβ was located within the cell boundaries of OSE cells, and occasional small clusters of ER reaction product were seen to colocalize with E-cadherin in the cytoplasm of some OSE cells (Fig 5.19C). Further investigation is required to confirm this
finding, however cytoplasmic expression of E-cadherin is reported in oral squamous cell carcinoma, and is associated with loss of heterozygosity in the adenomatous polyposis coli (APC) gene associated with progression to tumor formation in colorectal cancer (Gao et al., 2005).

In OSE studied using immunohistochemistry and light microscopy, the finding that within 48 hours of first exposure to estradiol, E-cadherin expression was enhanced, supports similar findings of MacCalman (1994). This group reported that in vivo, administration of estradiol to mice rapidly induced E-cadherin levels in OSE (MacCalman et al., 1994). Furthermore, as previously referred to in the introduction to this chapter, E-cadherin has been shown in vitro to induce cell cycle arrest by interference with tyrosine kinase signalling and the control of soluble growth factors that can activate ER such as EGF (Nelson et al., 1991) (Ignar-Trowbridge et al., 1992). It may therefore be postulated that estradiol, working through functional ER stimulates OSE hypertrophy, metaplasia and hyperplasia, and up-regulates levels of E-cadherin on OSE with first exposure to the hormone. In turn, the upregulation of E-cadherin in vivo may act to prevent further estradiol-induced growth and migration of OSE (St Croix et al., 1998).

It is interesting that a second exposure to estradiol did not elicit the same morphologic and morphometric response from OSE as the first. ER responded differentially to a second exposure also, with ERβ but not ERα downregulated in OSE, although ERβ expression appeared increased in OSE when compared to controls, and that seen after first estradiol injection. Levels of E-cadherin expression decreased substantially in OSE in response to a second estradiol exposure. This may indicate that repeat exposure to high levels of estradiol downregulates ERβ relative to ERα on OSE, and that decreased expression of ERβ may over time negatively regulate levels of E-cadherin.
CHAPTER 6: General discussion

6.1 General discussion

This PhD thesis has presented a series of integrative *in vivo* experiments to support an overriding hypothesis relating some early events in the development of sporadic ovarian epithelial cancer to prolonged and repeated estradiol exposure. The hypothesis is based on the observation that the ovarian surface epithelium responds to estrogen with a morphologic, morphometric, and immunohistochemical signature, that in many ways mimics that seen during transition to ovarian epithelial neoplasia. Moreover, changes seen in older OSE in response to depot estradiol treatment in these experiments may represent this epithelium’s earliest attempts to maintain homeostasis by dynamically altering its microenvirons.

During embryologic development, pregnancy, and throughout ovulatory cycles, the ovarian surface epithelium is exposed to high endogenous levels of estradiol from sources that include placenta, pre-ovulatory follicles, corpora lutea and ovarian stroma. Despite long exposure to estradiol, OSE at these times appears to retain the capacity to revert from a proliferative, metaplastic epithelium to a quiescent simple epithelium when endogenous estrogen levels wane (as seen in the neonate following withdrawal of placental estrogen), or possibly be non-responsive to the mitogenic effects of estradiol (as in pregnancy when OSE is reported to be a flat cuboidal epithelium) (Papadaki and Beilby, 1971) (Gaytan et al., 2005).

There may be several reasons not only for the OSE proliferative response to estradiol at such times, but also its refractory potential. For much of embryogenesis and fetogenesis, OSE is in contact with adjacent stroma because the presumptive basement membrane normally segregating epithelium from stroma is incomplete. Once these membranes are formed in late gestation, OSE may be less influenced by mitogenic stromal factors that include members of the TGFβ superfamily, in particular the activins, and estrogen produced by stromal interstitial cells (1.2.2.1). Additionally, α-fetoprotein (AFP) present in the developing yolk sac and made by the fetal liver, may inhibit the proliferative and metaplastic response of OSE *in vivo*, since it is a potent
antagonizer of the actions of estradiol (Mizejewski, 2004). Nagata et al., 2006 (Nagata et al., 2006) established from a longitudinal study of 600 pregnant women that estradiol levels in the cord blood of infants delivered at term (40 weeks gestation in humans), ranged between 554 - 41,300 pg/mL (mean = 5907), while maternal serum estradiol at term ranged between 3,960 – 67,300 pg/mL (mean = 31,419), P = 0.001. In fact, serum levels of estradiol were shown throughout pregnancy to be markedly elevated when compared to the non-pregnant state (70-220 pg/mL) (Nagata et al., 2006) (Strauss, 2004), and this is interesting, as traditionally estriol is considered to be the significant estrogen of pregnancy, probably because unconjugated estriol is produced almost exclusively by the fetoplacental unit and therefore is a reliable indicator of fetal wellbeing.

Estriol is present in extremely high quantities during pregnancy, and although having only 1% of the potency of estradiol and decreased affinity for ER, prolonged exposure to this hormone has nevertheless been shown to result in potent estrogenic effects through receptor binding to uterine ER in rats injected with estriol (Clark et al., 1977). Nagata’s study revealed that significant increases in maternal serum estriol and estradiol concentrations during pregnancy were matched by increases in serum AFP in both maternal blood and infant cord blood taken at delivery, with maternal levels ranging from between 47.8 - 954 ng/mL (mean = 185.0) and those of the infant from between 22,300 - 327,000 ng/mL (mean = 96,503), normal <20 ng/mL (Nagata et al., 2006). It is not known whether normal adult OSE expresses AFP, however oviduct does (Mizejewski, 2004). In adult life, elevated levels of AFP have traditionally been associated with oncogenesis involving liver, testis and ovary, and AFP is a routine measure of residual or returning carcinoma in germ cell tumors of the gonad (Mizejewski, 2004).

Aside from the putative influence of AFP, markedly elevated levels of progesterone throughout gestation most likely offset the effects of estrogen on OSE. Progesterone is synthesized by the placenta from cholesterol and concentrations in maternal blood 10-20 fold higher than the luteal phase of the ovulatory cycle, are maintained from 10 weeks gestation in what has been termed a ‘progesterone storm’ (Strauss and Lessey, 2004). Progesterone facilitates marked apoptosis in OSE by Fas/Fas ligand mediated
activation of caspases, and additionally enhances the anti-proliferative effects of TGFβ (2.2.2). It has also been shown to inhibit estrogen-induced mitogenesis in uterine epithelia by blocking both the nuclear translocation and the activation of cyclin dependent kinases and their respective cyclin partners (Tong and Pollard, 1999).

Throughout successive ovulations during reproductive years, the corpus luteum produces progesterone in abundance during the luteal phase of the cycle. Thus, a similar mechanism to that above may protect OSE from excessive hyperplastic activity when micromolar concentrations of estradiol are exuded onto OSE at the time of ovulation in vivo (Belin et al., 2000) (Gougeon, 2004). Progesterone may also be offsetting any oncogenic influences of inflammatory mediators such as TNFα and the leukotrienes and prostaglandins, by virtue of limiting oxidative damage to OSE cells (Murdoch et al., 2001).

The perimenopausal period is a time when the neuroendocrine axis controlling reproductive cycles is in a state of decline. It is now well documented that the hormonal mechanisms underlying the transition to the menopause and the responses of the gonad are fundamentally similar in rodents and humans (Wise, 1982) (Wise et al., 2002). Less well accepted at this time is the notion that the transition to the menopause represents a time of disregulated control of estradiol synthesis in the ovary. Such disregulation, conceivably the direct consequence of neuroendocrine decline affecting the reproductive hormonal axis, may result in isolated bursts of estradiol production in the ovary, and in part explain the paradox of hyperestrogenism reported in older premenopausal women (Wise et al., 2002) (Prior, 2005) (Santoro et al., 1996). Although the combined results of control mice in these PhD experiments have not shown evidence of increased estradiol concentrations in blood, two mice (6%) sacrificed during proestrus did exhibit markedly elevated estradiol levels in ovarian tissue, and may suggest that older mouse ovary also produces excess amounts of estradiol at this time. To confirm this finding, a larger cohort of identically aged-matched older mice would be required, with all mice sacrificed in proestrus.

That hyperestrogenism may occur during the transition to the menopause is a phenomenon not at odds with perimenopausal symptomology reported in older
women. For instance estrogen-induced alterations to vascular dynamics and body fluid regulation such as vasomotor hot flushes and bloating are well documented in the literature. Excessive intermittent production of estrogen by the gonad may potentially account for some of these commonly recognized symptoms. It is also not disputed that polyovulatory cycles resulting in multiple births occur more frequently with age (Beemsterboer et al., 2006). The maturation of more than one large preovulatory follicle in response to neuroendocrine dysregulated FSH surges (Prior, 2005) would theoretically expose OSE in vivo to increased perturbation through higher levels of estradiol during ovulation.

The mouse model used for the present analyses could be criticized for the fact that this animal is naturally polyovulatory, and a pilot study conducted did not assess if changes to the numbers of follicles ovulated per cycle occurred as a consequence of age. Polyovulatory cycles represent the norm for this species however, and as previously alluded to, numbers of ovulatory follicles per cycle are regulated when neuroendocrine control of ovulation is optimal (2.1.1). Interestingly a decrease in the number of large preovulatory follicles does not occur with a decline in neuroendocrine function in middle-aged mice, despite an overall decline in the follicle population (Ishikawa and Endo, 1996) (Gosden et al., 1983). Therefore the ovaries of middle-aged mice sampled in proestrus may also have contained high endogenous levels of estradiol secondary to a coincidently greater number of large preovulatory follicles in a single estrous cycle.

As cycles become progressively more erratic leading up to the menopause in women, the amount of anovulatory cycles also increase (Prior, 1998) and the possibility exists that an ovary that has been hyperstimulated by intermittent high levels of FSH secretion may contain unruptured luteinized follicles as well as greater numbers of large pre-ovulatory follicles. Unruptured luteinized follicles have been reported in women undergoing superovulation for infertility using clomiphene citrate (Randall and Templeton, 1991) as well as significantly larger dominant follicles and higher serum estradiol concentrations (Haritha and Rajagopalan, 2003). Large corpora luteal-like structures were identified in older estrogen-treated mouse ovaries in the present study, and were queried as being unruptured luteinized follicles (4.3.1.2). They appeared to bear no relationship however, to OSE stratification, invagination or metaplasia.
Murdoch and colleagues have also previously reported that unruptured luteinized follicles in sheep had no apparent deliterious effects on overlying OSE (Murdoch, 1994).

It could be hypothesized that once the menopause occurs, signs of intermittent estrogen stimulation on OSE should abate, and the epithelium return to the quiescent state. Indeed, this was what was observed in the current study in estrogen-treated animals when E2 levels in the blood were lowest. A reserved ability of OSE to return to the quiescent state following each ovulation prior to the menopause, and the maintenance of this state after menopause, may prove crucial to OSE in the long term, because once ovulations have ceased, the protective influence of progesterone produced by corpora lutea is no longer available. The years after the menopause therefore, mark the only time when progesterone cannot be synthesized in sufficient quantities to antagonize the mitogenic effects of estradiol.

Estradiol assays conducted as part of this thesis have demonstrated that exposure to a large dose of E2 results in an accumulation of the hormone; first in ovarian tissue, but over time and with repeated exposure, also in the blood. These findings are not unique to exogenous administration of estrogen or indeed to ovary, since estradiol has been shown to accumulate in normal prostatic stromal tissue with advancing age (Krieg et al., 1993) and in the present study, estradiol was observed to produce cumulative effects on uterus as evidenced by time and dose-dependent increases in uterine weight. Serum levels of E2 are also known to increase over time in women taking HRT (Zimmerman et al., 2000). Furthermore, estradiol synthesized via aromatization from androgens, accumulates in adipose tissue in the form of fatty acid esters that are lipophilic. When serum estradiol levels are low, such as in the postmenopausal state, the accumulation of esterified estradiol is maximal in adipose tissue and it is proposed that the accumulation of the estradiol esters acts as an estradiol reservoir (Badeau et al., 2007). It is therefore intriguing that increased adiposity has recently been shown to correlate to increased risk of ovarian cancer (Leitzmann et al., 2009). An incidental but perhaps important observation in the present study was that in middle age, increased adiposity (particularly abdominal) is a feature common to female mice and humans.
According to the free hormone hypothesis (Mencel, 1989), only the unbound fraction of the hormone may exert its effects by specifically binding to receptors found on the target tissues. Thus for estradiol to exert its actions on OSE, three criteria must be filled. First this member of the estrogen family must be able to be synthesized through to its end product (estradiol). For this to occur, OSE itself must house the enzymes to synthesize estradiol from cholesterol de novo, or nearby stroma may synthesize a precursory substrate. The substrate can complete conversion through to estradiol if OSE contains the enzymes that would allow this. Secondly, OSE must express the specific functional receptors for estradiol, so that transcription may occur and may result in the downstream activation of estrogen target genes encoding proteins that include growth factors. Thirdly, estradiol coupled to binding proteins such as albumin, or sex steroid binding globulin (not produced by rodent liver) must become unbound and potential sources of estradiol in OSE or nearby stroma; sulphated estrones and androgens, converted to estradiol. Esterification and conjugation of estradiol to sulphur groups may thus provide a mechanism for restricting the potentially carcinogenic activity of cumulative estradiol in a multitude of tissues that include OSE. It follows that in order to block estradiol’s effects in vivo at the protein level, conjugation reactions using sulphur transferases alongside interruption of enzymatic pathways specific to OSE synthesis of estradiol (for example aromatase), and the blocking or manipulation of OSE receptors for estradiol is required.

The literature presented in this thesis has revealed that OSE possesses not only the enzymes required for de novo synthesis of estradiol, but also those necessary for interconversions between estrone and estradiol and androgens and estradiol (2.2.1). Added to this, OSE and stroma both contain the receptors for androgens and estrogens alongside growth factors that include EGF, IGF, KGF, and PDGF. The cognate receptors for these growth factors, whose activation all enhance the mitogenic OSE response to estradiol have either been found in stroma or in OSE itself. A recent study has additionally shown in vivo immunohistochemical evidence of steroid sulphatase (STS); which hydrolyzes (effectively deconjugates) biologically inactive estrogen sulphates to active estrogens in both normal and tumorigenic OSE. There is also confirmation that the primary ovarian cancer cell line SKOV-3 responds to inflammatory cytokines with increased levels of STS mRNA (Harlow et al., 2008).
contrast, older mouse OSE is reported as having decreased levels of EST, the enzyme responsible for conjugation of estrone to cleaved sulphur groups, preventing its conversion to active estradiol (Zimon et al., 2006). Taken together, it is possible that in older ovaries, STS alongside reduced levels of EST may increase levels of estradiol in OSE. Thus older OSE has the capability to synthesize estradiol from available substrate in situ, but may be unable to exert control over its cumulative effects by increasing the rates of sulphonation.

Normal older ovaries observed in experiments conducted as part of this thesis displayed a convoluted surface and areas of OSE invagination and cysts where OSE became columnar in shape and stratified. Cell layering additionally occurred close to invaginated areas of OSE, but not generally over large structures such as corpora lutea and preovulatory follicles. The appearances of estradiol-treated ovaries resembled an exaggeration of this phenotype and the following were observed: areas of marked cell stratification, large epithelial papillary excrescences, deep cortical invaginations leading to blind-ended clefts, inclusion cysts, hypervascularization of the ovary, possible lipid droplet formation, and the appearance of corpora luteal-like structures which may have represented unruptured luteinized follicles. Morphologic alteration to OSE caused by estradiol largely occurred within 48 hours of initial exposure, but may have occurred well within 24 hours of estradiol treatment as suggested by the failure of BrdU pulse label to match the rate of entry of OSE cells into the S-phase of the cell cycle. A notable consequence of such proliferation appeared to be the significant increase in OSE invagination, enfolding the OSE and surrounding it almost entirely with stroma, while in other areas of the ovary, OSE proliferated directly outwards toward the ovarian bursa and contributed to the formation of large papilloma (4.3.1.2). OSE cells also hypertrophied significantly.

Such a response is hypothesized to be a protective mechanism in the first instance, and demonstrates the highly plastic nature of OSE in response to mitogenic stimulus. However, this response alters the structural relationships that normally exist between the ovarian stroma and OSE, and increases the surface area of OSE in contact with stroma. In the absence of continued stimulation by estradiol, it appears OSE has the capacity to revert to the quiescent state. However present experiments have shown that
a repeat estradiol exposure alters the initial OSE response. It is possible OSE may not deal as efficiently with successive exposures to estradiol over time and may progressively lose the ability to quiesce if levels of ERβ are not optimally restored. Conceivably this could result in aberrant OSE hyperplasia and metaplasia, and predispose OSE to oncogenesis.

The idea that the ovarian surface epithelium possesses phenotypic plasticity is not new, and was first proposed by Auersperg’s group in 1984 (Auersperg et al., 1984) who suggested this was the result of OSE being an uncommitted phenotype (1.2.2.3). Such plasticity has been highlighted in terms of the OSE’s ability to undergo EMT during the process of ovulation, and that an inability to do so may result in the trapping of the epithelium in cortical stroma, possibly forming inclusion cysts and predisposing it to oncogenesis in this manner. The predisposition is thought to be secondary to specific stromal-epithelial interactions that include not only the paracrine activity of growth factors and cytokines, but also that of estrogen found in cyst fluid (Wong and Auersperg, 2003). In effect, zones of epithelial cell inclusion and OSE lining deep cortical invaginations become surrounded by stroma expressing mitogenic growth factors such as EGF, TGFα and others (2.2.3.1). The establishment of a morphological phenotype that may create such a microenvironment occurs very quickly in vivo in response to exogenous estradiol, as the present study has shown, and appears independent of ovulation.

The role of ovulation as the obligatory factor in the etiology of OSE perturbation leading to epithelial ovarian cancer has been challenged, since proliferation occurs in OSE that is not involved in the ovulatory event (Gaytan et al., 2005). Moreover mice lacking FSH receptor, FORKO mice, exhibit high levels of FSH and LH but do not ovulate, and show morphological and immunohistochemical evidence of OSE proliferation, epithelial inclusion cyst formation, and the formation of tumors in the ovary that resemble human serous papillary adenomas (Chen et al., 2007). Interestingly, FORKO mice, like postmenopausal women, have low circulating levels of estrogen, but very high levels of androgens, which nevertheless may convert to estradiol to bring about the observed effects.
The present study has shown ERα and ERβ reside both within the nucleus and the cytoplasm of OSE cells, supporting the concept of nuclear-cytoplasmic shuttling of these receptors previously reported in cultured mouse cells (Dauvois et al., 1993). ERα was abundantly expressed not only in OSE but also in neighboring stroma in the basal (non-estrogen-stimulated state). However, when estrogen was administered, levels of ERα in stroma and OSE quickly become downregulated. Results also suggest that ERα in stromal tissue may be more acutely responsive to E2 than ERα localized to OSE, since downregulation of this receptor was still apparent in stroma following a second estradiol injection, while a recovery of ERα to basal levels was seen in the adjacent OSE.

The period immediately preceding ovulation represents a time of greatly elevated estradiol production in the pre-ovulatory follicle, and conceivably could produce similar effects on ERα expression in OSE and stroma, to that seen with exogenous administration of the hormone. Therefore in vivo EMT may be opposed by the actions of estrogen through downregulation of ERα expression. Thus a requirement for EMT may be the positive regulation of ERα. Indeed Park et al (2008) (Park et al., 2008), proposed ERα to be the ‘critical mechanistic link’ in mediating the promotion of EMT in BG-1 ovarian adenocarcinoma cell lines following 48 hours incubation with estradiol in vitro. In Park’s study, EMT was correlated to cells assuming increased motility, becoming fibroblast-like and showing decreased E-cadherin expression. When BG-1 cells were cocultured with estradiol for 48 hours, E-cadherin expression was significantly suppressed coincident with the induction of EMT transcription factors Snail and Slug (Park et al., 2008). Selective knockout of ERα significantly reversed these changes. If the response of normal OSE cells to estradiol in vivo were to be the same as that observed by Park and researchers after 48 hours of E2 treatment, there would likely be a downregulation of OSE expression of E-cadherin to accompany the loss of ERα from OSE and stroma. This was not the case for the in vivo experiments reported herein using normal OSE, a result supported by those of MacCalman et al, using mouse OSE (MacCalman et al., 1994). The differences in these findings may relate to the use of in vitro cell culture methods (absence of stromal influences) in the former experiment, or to the different species used. However it may
also be indicative of the contrary response of established cancerous cells exposed to estradiol, when compared to normal OSE cells first exposed to this hormone.

*In vivo*, estrogen enhances EGF expression and that of its receptor EGFR in uterine and vaginal epithelia (Mukku and Stancel, 1985) (Gardner et al., 1989) (Lingham et al., 1988). Epithelia of oviduct, uterus, the superior portion of vagina, and OSE, all derive from embryonic coelom, and EGF is expressed on OSE and stroma both *in vitro* and *in vivo* (Rodriguez et al., 1991). Its receptor is a member of the erbB-HER receptor tyrosine kinase family involved in the activation of a plethora of signaling pathways that include Ras-Raf-MAPK, Rac-JNK-p38 MAPK, PI3K and PLCγ-1 cascades (Wong and Leung, 2007) as well as the JAK/STAT pathway (Henson and Gibson, 2006). This growth factor is therefore recognized as a potent regulator of estradiol-induced cell growth within Müllerian-derived tissues, and its pro-survival/anti-apoptotic influence is evident in many other tissues (Henson and Gibson, 2006). EGF induces estrogen-responsive proteins and promotes rapid and significant DNA synthesis in reproductive tract epithelia, independent of gonadotrophins (Nelson et al., 1991) (Ignar-Trowbridge et al., 1992) suggesting that considerable cross talk exists between EGF, E2, and other cell survival and apoptotic signaling pathways (Henson and Gibson, 2006). Furthermore, EGF has been reported to induce EMT of human cultured OSE cells in association with hydrocortisone (Ahmed et al., 2006), and in association with HGF in the presence of an extracellular matrix (2.2.3.2), supporting the possibility that EMT may require stromal interactions with OSE.

It is therefore possible the mitogenic activities of EGF *in vivo* may be transiently enhanced by estradiol administration, but EMT-related activity decreased by a significant reduction in stromal ERα expression. A decline in EMT produced by exposure to a large dose of estradiol may explain the initial upregulation of E-cadherin on OSE concurrent with OSE hyperplasia, metaplasia and hypertrophy *in vivo*. Further experiments directed toward establishing a temporal relationship between estradiol administration, EGF/EGFR induction and E-cadherin synthesis would be critical to establishing if this mechanism exists.
A concurrent downregulation of ERβ occurred on OSE within 48 hours of estradiol in experiments conducted as part of this thesis. This receptor subtype was not strongly expressed in stroma in the mouse ovary, and evidence for its existence in human ovarian stroma remains inconclusive. In contrast both ER subtypes were highly expressed in mouse oviduct during diestrus. In the non-estrogen-stimulated state, there was a healthy population of ERβ on OSE in the present study; but with initial estradiol exposure strong expression was evident only at points of OSE invagination and metaplasia, areas of OSE cell stratification, and near the OSE/mesothelial portions of the ovary. Most of the ovarian surface appeared devoid of this receptor subtype after a single depot estradiol injection. Interestingly, this pattern of expression paralleled that observed for E-cadherin in control mouse ovary (where expression has previously been reported as constitutive) (Auersperg et al., 2001). Following a second estradiol exposure, levels of ERβ were greater than that observed after initial estradiol exposure, but still appeared downregulated when compared to those of control mice.

In contrast, ERα levels on OSE appeared refractory to the continued influence of elevated estradiol because receptor expression recovered to high levels despite a repeat estradiol exposure. Coincidental to the partial downregulation of ERβ following the second estradiol injection, E-cadherin expression was suppressed in all OSE cells and the morphological response to E2 attenuated. ERα expression in ovarian stroma (as stated) and uterus continued to be downregulated in response to repeat E2. Taken together, if the presence of functional ERα in ovarian stroma is required for EMT, the propensity for stroma to induce EMT of OSE cells that may have previously migrated into it, likely remains reduced with repeat and extended exposure to estradiol in vivo.

Additionally, ERβ may have a role to play in a return of OSE to homeostasis following ERα-mediated OSE mitogenesis. This could conceivably occur via a return of ERβ in OSE facilitating the relocation of ERα to OSE nuclei, since the attenuated OSE response to estradiol at day 16 corresponded to higher levels of ERβ expression than those observed after the first estradiol injection, and a strong return of ERα in OSE. E-cadherin expression in OSE was reduced in response to these same conditions following repeat estradiol, again reflecting the commencement of a return to OSE homeostasis. Therefore, it may be hypothesized that over time, successive rounds of
estrogen-induced OSE proliferation, metaplasia, hypertrophy and quiescence, may cause small numbers of OSE cells to periodically enter the stromal compartment where they may become unable to undergo EMT due to reduced levels of stromal ERα, and instead form aggregates and possibly cysts. Although it is not surprising that ERα expression throughout the ovary decreases with time from the onset of the menopause (Brodowska et al., 2007), the consequences for EMT may be reliant more on the expression of levels of ERα relative to ERβ in stroma and neighbouring OSE, than on levels of ERα alone.

In conclusion, estradiol exposure in older ovaries results in an accumulation of this mitogenic hormone both in the ovary and the blood. Estradiol causes a disregulation of ER in OSE and ovarian stroma, and may decrease the ability of OSE to undergo epithelial mesenchymal transition in vivo via disruption of ERα and E-cadherin expression, and possibly ERβ. It is proposed that the morphologic and morphometric responses of OSE observed in the present study after a first exposure to exogenous estradiol represent a hyper responsive homeostatic control mechanism. The normal function of such a mechanism may underlie regulatory changes to ER and E-cadherin expression by virtue of controlling the levels of stromal growth factors and paracrine influences that could include inflammatory mediators. Further studies to clarify the role of growth and paracrine factors in conjunction with regulation of ER by estradiol are required. Changes to OSE morphology brought about by estradiol alter the structural association that exists between OSE and stroma, conceivably exposing proportionately more surface epithelial cells to the paracrine influence of stromal-derived factors, and potentially disrupting homeostatic control of OSE. Over time, repeated exposures to high levels of estradiol may therefore predispose the older ovary to the development of an ovarian epithelial carcinoma.
6.2 Limitations of this work and future directions

Experiments presented in this PhD thesis have been designed in an effort to investigate possible links between estradiol, and the development *in vivo* of morphologic, morphometric, and immunohistochemical features that have been reported to occur during progression of OSE to the cancerous state. Due to the integrative aspirations of this work, there are limitations that require acknowledgement. In the first instance, it would be beneficial to know the exact time course for the proliferative and metaplastic processes that occurred on OSE, and therefore the speed at which disregulation of estrogen receptor expression occurred in OSE and stroma. If this were known, much more information could be obtained relating to the temporal disruption of ER on E-cadherin levels. Since the acute response of OSE to estradiol was shown to happen within 48 hours but was possibly well established inside 24 hours, a cumulative BrdU labeling strategy such as that used by Burdette et al (Burdette et al., 2006), and the sampling of animals beginning two hours after administration of the first estradiol injection and thereafter continuing at 2 hourly intervals for the first 48 hours, would help address this problem.

It may be argued that the estrogen-treated mice in the present study would respond to estradiol with an LH surge that could result in the ovulation of follicles and contribute to the morphological and morphometric changes seen. Although this possibility cannot be discounted, constituent volumes of both large follicles and corpora lutea were not greater in treated ovaries compared to controls 48 hours following the first E2 injection, suggesting that ovulation was not a significant contributory factor to these results. Engelhardt et al, (1989) were able to demonstrate that administration of estradiol valerate to cows, although resulting in a premature LH surge, failed to cause ovulation of preovulatory follicles, instead promoting their atresia (Engelhardt et al., 1989). However to completely address the possibility that ovulation may occur spontaneously in older mice exposed to EV, the addition of a GnRH analogue or antagonist could be used in future studies to prevent ovulation prior to administration of estradiol.
A third consideration relates to time. Experiments in this thesis were confined to a time period of 1 month and just two depot injections of estradiol. A pilot experiment exposed virgin Swiss Webster mice of six weeks of age up to ten months of age to two months of estradiol, and included a total of five depot estradiol injections. Images obtained from the older (7-10 month old) mice demonstrated possibly advanced ovary changes that included disruption of the basement membrane, OSE cell aggregation, and retrograde migration of OSE aggregates into stroma. These changes were not seen in any of the younger mice. Although it was this pilot study that inspired the present work, the main experiments for the PhD thesis did not extend to 2 months because some mice treated for this length of time during the pilot study developed abscesses on their necks from repeat depot injections, and all had vaginal infections from the estrogen by the time of sacrifice. If these complications could be avoided, it would be very desirable to repeat the experiment over an extended time frame and compare these results to a large cohort of younger virgin mice. Injections were the chosen delivery method over estradiol pellets because they were generally better tolerated in these small animals, and were considered to be best achieve the depot effect.

An additional comment is one relating to inclusion of mRNA studies as part of this work. Although this was achievable in theory, laser capture microdissection (LCM) was considered to be the only method that could be used to isolate OSE from the ovaries for in vivo analysis. OSE represented less than 1% of the (2-3mm diameter) ovary in control mice. Furthermore, OSE isolation for Northern blot using fetal porcine ovary appeared not without problems (Ryan et al., 1996). LCM was therefore considered impractical to isolate OSE from stroma to investigate the individual contribution of each compartment in such small animals. It is conceded, however, that LCM to obtain OSE mRNA for quantitative PCR and also receptor proteins of interest such as ER, EGFR, and particularly levels of STS relative to EST for Western blot, would not only be very desirable but highly achievable using a larger species. Harlow and colleagues have recently confirmed the presence of STS in normal human OSE in vitro. Furthermore they reported increases in STS mRNA in the SKOV ovarian cancer cell line concurrent with high levels of ERα and ‘barely detectable’ levels of ERβ following exposure to inflammatory cytokines (Harlow et al., 2008).
A fulfilling endpoint of this work would be the application of the underlying hypothesis to experiments using human tissue from perimenopausal women and women ever exposed to high exogenous sources of estradiol. It would not be difficult to find women fitting the latter description. For example women in the 1960s and 70s who were prescribed first generation oral contraceptives in which the estradiol content was excessive would in 2009 be in their 60s, a time coinciding with the highest incidence of ovarian cancer. Additionally, the use of high dose estrogen therapy for emergency contraception (common in the 1960s and 1970s) remains to this day in parts of Europe (Haspels, 1994). Moreover, even though there has been some attention given to educating women about the risk of ovarian and breast cancers associated with the use of HRT, many women throughout the world continue to be prescribed this hormonal supplement for the management of perimenopausal symptoms.

Notwithstanding the curiosity that underpins this body of work, a parting immunohistochemical experiment was performed ‘just to see’ if ERα and ERβ immunohistochemical expression on human OSE resembled that observed in the mouse model. The resulting double label immunohistochemistry used archived human paraffin embedded ovarian tissue (Otago Ethics Committee, number 99/12/113). Imaging was by confocal microscopy and is shown in figure 6.1. Although the archived ovarian tissue was three years old and no doubt had lost a large degree of antigenicity, results were encouraging.
Figure 6.1
Dual fluorescent label shown for human ERα (bright green reaction product, Alexa Fluor 488), and ERβ (bright red reaction product, Alexa Fluor 555)
REFERENCES


BARKS, O. L. & OVERHOLSER, M. D. Estrone effects on uterine musculature. *The Anatomical Record, 70*, 401-411.


235


ESPEY, L. L. (1967) Ultrastructure of the apex of the rabbit graafian follicle during the ovulatory process. Endocrinology, 81, 267-76.


EVANS, H. M. & SWEAZY, O. (1932) Ovogenesis and the normal follicular cycle in adult mammalia. CAI Western Medicine, 36, 60.


GRECO, T. L., DUELLO, T. M. & GORSKI, J. (1993) Estrogen receptors, estradiol, and diethylstilbestrol in early development: the mouse as a model for the study


fimbria of the fallopian tube in a BRCA1 carrier undergoing prophylactic

cancer development: epithelial-cell-stromal-cell interactions and steroid
hormone action in normal and cancerous mammary gland. Breast Cancer Res,
5, 208-15.


expression by exogenous 17beta-estradiol in the mammary glands of lactating

receptors from divergent species with a polymerase chain reaction technique:
complete cDNA sequence of the mouse androgen receptor and isolation of
androgen receptor cDNA probes from dog, guinea pig and clawed frog. Biochem Biophys Res Commun, 171, 697-704.

HENSON, E. S. & GIBSON, S. B. (2006) Surviving cell death through epidermal
growth factor (EGF) signal transduction pathways: implications for cancer


cyclic changes in the primate oviduct and endometrium. IN NEILL, J. D. (Ed.)

ovarian surface epithelial cell mitosis or apoptosis depending on the presence
or absence of an extracellular matrix. Endocrinology, 140, 2908-16.

Expression of oestrogen receptor alpha and beta in cultured human ovarian
surface epithelial cells. Mol Hum Reprod, 4, 811-5.

in rats during the embryonic period and the first three weeks postpartum. Biol Reprod, 53, 1208-21.


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251


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APPENDIX A

Fixatives and staining protocols:

4% (w/v) paraformaldehyde (100mL)
Dissolve 4g paraformaldehyde in 50 mL ddH₂O.
Heat while stirring to 60°C.
Add 7 drops 1 M NaOH.
Turn heat down and continue stirring until solution clears.
Filter through wet strength filter paper and make up to 100mL with 2x PBS.
For all protocol except that for estrogen receptor check pH is approx. 7.4.
For ER protocol pH to 8.9 with NaOH.

Periodic Acid Schiff (PAS) staining of paraffin wax sections with Methyl Blue
Picric Acid counterstain

1. De-paraffinize sections and bring sections to water.
2. Oxidize sections for 10 minutes in 1% Periodic acid.
3. Rinse in 3 changes of distilled water.
4. Place in Schiff’s solution for 20 minutes.
5. Wash in running water for 20-30 minutes.
6. Differentiate in 70% alcohol for 5-10 minutes or longer if necessary.
7. Wash in running water.
8. Stain nuclei with Weigert’s iron haematoxylin for 5-10 minutes.
9. Wash in running water.
10. Counterstain in methyl blue picric acid mixture for 30 seconds while agitating.
11. Brief rinse in water and dehydrate through 70%, 95%, and absolute alcohols.
13. Mount with DPX.

Schiff’s solution
Basic fuchsin 2.5g
Potassium metabisulphite 5.0g
1 N Hydrochloric acid 25mL
Distilled water 500mL

Use plastic forceps only in Schiff’s solution
**Methyl Blue Picric Acid**

Methyl blue 40mg  
Sat. aq. Picric acid 100mL

**Weigert's iron haematoxylin**

**Solution A**

Haematoxlin 1g  
95% alcohol 100mL  

**Solution B**

29% Ferric chloride in water 4mL  
Distilled water 95mL  
Concentrated Hydrochloric acid 1mL

Mix equal parts of stock solution A and B

**Gills Haematoxylin**

Distilled water 730mL  
Ethylene glycol 230mL  
Haematoxylin 2g  
Sodium iodate 0.2g  
Aluminium sulphate 17.6g  
Glacial acetic acid 20mL
APPENDIX B

Paraffin embedding schedule

Tissues were removed from fixative and immersed in 70% alcohol prior to wax embedding. An automated Shandon Hypercentre XP was used for embedding and programmed as follows:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Temperature</th>
<th>Vacuum</th>
<th>Immersion time</th>
<th>Drain time</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% etoh</td>
<td>Ambient</td>
<td>No</td>
<td>3 hours</td>
<td>15 mins</td>
</tr>
<tr>
<td>95% etoh</td>
<td>°C</td>
<td>No</td>
<td>1 hr</td>
<td></td>
</tr>
<tr>
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<td>°C</td>
<td>No</td>
<td>1 hr</td>
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<tr>
<td>wax</td>
<td>60°C</td>
<td>Yes</td>
<td>1 hr</td>
<td></td>
</tr>
</tbody>
</table>

Tissue was removed and placed in paraffin blocks.
APPENDIX C

Buffers used for immunohistochemistry

1. BrdU

0.01 M Phosphate buffer (PB) (2L)
Na₂HPO₄·2H₂O 2.42g
NaH₂PO₄·2H₂O 0.812g

Dissolve in 1L ddH₂O and pH to 7.2. Make up to 2L with ddH₂O.

0.01 M Phosphate buffered saline (PBS) (2L)
NaCl 16g
KCL 0.4g
Na₂HPO₄·2H₂O 2.8g
KH₂PO₄ 0.4g

Dissolve in 1L ddH₂O and pH to 7.4. Make up to 2L with ddH₂O.

Proteinase K buffer (100mL)
0.1 M Tris 1.21g
5 mM EDTA 0.19g

Dissolve in ddH₂O. Adjust to pH 7.4 with HCl

Antibody dilution buffer
0.01 M PB 10mL
NaCl 90mg
Tween 20 10μL
BSA 100mg

2. Estrogen receptor

Citrate buffer for antigen retrieval
Citric acid 2.1g
MilliQ water 1L

pH to 6.0 with strong NaOH.

Stock solution A (Tris Base 0.2 M) (1L)
Tris Base 24.2g
ddH₂O 1L
Stock solution B (HCl 0.2 M) (1L)
Concentrated HCl 17mL
ddH₂O 900mL

Make up to 1L with ddH₂O

Tris/HCl working solution (1L)
Stock solution A 125mL
Stock solution B 96mL
ddH₂O 500mL

Adjust pH to 7.6 with solution A or B. Make up to 1L with ddH₂O

Tris/HCl/NaCl (TBS) (1L)
NaCl 8.7g
Tris/HCl 1L

Tris/HCl/NaCl/Triton (0.3% and 0.5%)
Tris/HCl/NaCl 100mL
Triton 0.3/0.5mL

Antibody dilution buffer
Tris/HCl/NaCl/Triton (0.25%) 100mL
BSA 0.25g

3. E-cadherin

EDTA buffer for antigen retrieval
EDTA 0.29g
MilliQ water 500mL

Gently heat EDTA in MilliQ water to dissolve. Cool, pH to 8.0 and make up to 1L with milliQ water.

Antibody dilution buffer
PBS 100mL
Triton 0.5%
BSA 0.25%
APPENDIX D

Estradiol extraction for tissue estradiol RIA

Tissue extraction
All extractions must be performed in glass LP4 tubes.
Weigh all tissue and record the weight. Add 50mL 70% methanol per 1 g tissue and cut/homogenize into very small pieces. Leave (covered) overnight at 4°C. Centrifuge at 1500x g for 30 minutes and tip off supernatant into glass LP4 tubes. Aquavap and freeze any remaining sample. When reconstituting sample, match the volume of zero standard used for reconstitution to that of the methanol used for extraction.

Assay procedure using the DSL 39-100 estradiol assay kit

1. All reagents to reach RT prior to commencement of the assay, with gentle inversion several times to mix reagents thoroughly before use.
2. Assay standards and controls in triplicate and unknown samples in duplicate.
3. Label all tubes and arrange: total counts, non-specific binding (NSB), standards, controls and unknowns (sample).
4. Pipette 200µL of the supplied standards (0, 1.5, 5.0, 50.0 and 150.0 pg/mL) and controls (prescribed high and low) to the bottom of the labeled glass tubes. The volume of the unknowns is pre-calculated according to the requirements of the standard curve. Add 300µL of zero (0pg/mL) standard to NSB tubes.
5. Add 100µL of the 3rd Generation Estradiol antiserum to all tubes except the total count and NSB tubes.
6. Vortex all tubes immediately.
7. Incubate all tubes for 4 hours at 2-8°C.
8. Add 100µL of the 3rd Generation Estradiol [I-125] reagent to all tubes and vortex immediately.
9. Cover tubes with foil and incubate for 20-24 hours at 2-8°C
10. Add 1 mL of the precipitating reagent to all except total count tubes and vortex all tubes immediately.
11. Incubate for 20-30 minutes at RT.
12. Centrifuge all except total count tubes (1500 Xg) in a refrigerated centrifuge at 4°C.
13. Decant all tubes (except total counts) into a radioactive waste sink by inverting tubes simultaneously using a sponge rack. Allow tubes to drain for 1-2 minutes and blot excess drops adhering to tube rims before returning them to the upright position.
14. Count all tubes for 1 minute in a gamma counter.
APPENDIX E

Veterinary Prescription for estradiol valerate and ethics consents

VETERINARY PRESCRIPTION

Date: 7.5.04

To supply Primogen Depot (estradiol valerate)

2 boxes 3 x 10mg ampoules/box, total of 6 ampoules

For the purpose biomedical research in compliance with Animal Ethics Committee ABC protocol #35/02.

Supplied to Ms L Gulliver, working in the research laboratory of Dr Peter Hurst, Department of Anatomy and Structural Biology, University of Otago.

Labelled: For Animal Treatment Only

Dose administered to laboratory mice: 10μg/mg body weight by subcutaneous injection, repeated every 2wks for a total of five injections.

Dr John C Schofield, BVSc, MRCVS, DACLAM
Director of Animal Welfare
University of Otago
Date: 22/4/02

Dr. P. Howat
Pharmacy

Dear P. Howat,

Thank you for your reply to the provisos requested for protocol 10/02. These were considered at the most recent meeting of the Otago University Animal Ethics Committee and I am pleased to advise that your protocol has now been approved in full.

Yours sincerely,

Barbara Lee
Secretary
Otago University Animal Ethics Committee
Dr P Hurst  
Anatomy & Structural Biology  
Otago School of Medical Sciences

Dear Dr Hurst

Re: Application 69/07

This is to acknowledge receipt of your application for the use of live animals in the programme/project entitled: Does regulation of estrogen receptor (ER) occur in older mouse ovarian surface epithelium in response to initial and a repeat exposure to exogenous estradiol?

Meetings of the Committee are held the second Wednesday of every month (except January). Your application will be considered for approval at the next meeting.

Yours sincerely

[Signature]

PP Barbara Lee  
Secretary  
Otago University Animal Ethics Committee
APPENDIX F

Conferences attended and conference presentations during the course of this thesis.

Attended
Familial Cancer Research and Practice (Combined meeting of kConFab and AOCS & Family Cancer Clinics of Australia and New Zealand) 18-21 August (2004), Couran Cove, Queensland.

Conference presentations


Future publications and presentations

Invited publication: Ovarian Cancer review article for the Pharmacy Journal of New Zealand (NZJP) due October 2009.

21st meeting of the European Association for Cancer Research, Oslo Norway 26-29 June 2010: Abstract submission is pending.