A Study of Inflammatory Signalling in Epithelial Ovarian Cancer and the Normal Human Mesothelium

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Declaration

It is hereby declared that this thesis has been composed by the candidate in its entirety and the work is the candidate's own, or whenever a contribution has been made by a colleague that this has been duly acknowledged.

This work has not been submitted for any other degree or professional qualification.

Kenneth Scott Fegan
March 2009
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Finally I would like to acknowledge the support and encouragement of my wife Claire.
Abstract

Epithelial Ovarian Cancer (EOC) kills more women annually in the United Kingdom than any other gynaecological cancer. Survival rates for women diagnosed with EOC have not improved over the past 30 years, due to the often advanced stage at presentation, where widespread intra-peritoneal dissemination has occurred. The natural history of the disease remains uncertain but the ovarian surface epithelium (OSE) is a strong candidate for the tissue of origin. The OSE undergoes cyclical damage and repair in women of reproductive age following ovulation, which can be considered an acute inflammatory event. Factors that prevent ovulation (pregnancy, breastfeeding and contraceptive pill use) also protect against the development of EOC. Previously published data show that the OSE is able to up-regulate the enzyme 11-beta hydroxysteroid dehydrogenase type 1 (11βHSD1) in response to inflammation, the enzyme responsible for converting inactive cortisone to anti-inflammatory cortisol. This thesis hypothesises that 11βHSD isozymes are deregulated in ovarian cancer; that the peritoneal surface epithelium (PSE) is indistinguishable from the OSE in its response to inflammation and should be considered a potential source of some “ovarian cancers”; and finally that the expression of the tumour suppressor gene OPCML (OPioid binding Cell adhesion Molecule-Like) is altered by inflammation. These hypotheses were examined at three levels. Firstly, primary cultures of EOC were established, and glucocorticoid metabolism and the response to inflammation was compared to normal OSE. Results from these investigations reveal that the 11βHSD1 response to IL-1α stimulation is impaired in EOC compared to normal OSE at the mRNA level but there is no significant difference when 11βHSD1 enzyme activity is measured in these tissues. When basal levels of 11βHSD1, 11βHSD2 and COX2 are compared amongst untreated samples of EOC and OSE, there was a significant correlation between 11βHSD1 and COX2 mRNA expression (P<0.001). 11βHSD2 mRNA expression was significantly higher in the EOC specimens compared to OSE (P<0.05). Secondly the response to inflammation was compared in primary cultures of human peritoneal surface epithelial (PSE) cells and OSE. The data suggest that the mRNA response to inflammation was similar in OSE and PSE, but that the 11βHSD1 enzyme activity was reduced in PSE (P<0.05), which may result in differences in tissue healing. Finally, the effect of inflammation on the expression of the ovarian cancer associated tumour suppressor gene (TSG), OPCML (OPioid binding Cell adhesion Molecule-Like) and the other members of the IgLON family, was examined in OSE. These results suggest that OPCML mRNA expression can be induced by IL-1α, an effect that is inhibited by cortisol.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>11βHSD</td>
<td>11-beta hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>AI</td>
<td>Aromatase inhibitor</td>
</tr>
<tr>
<td>BOT</td>
<td>Borderline tumours of the ovary</td>
</tr>
<tr>
<td>CA125</td>
<td>Cancer Antigen 125</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>COCP</td>
<td>Combined oral contraceptive pill</td>
</tr>
<tr>
<td>COX1</td>
<td>Cyclooxygenase 1</td>
</tr>
<tr>
<td>COX2</td>
<td>Cyclooxygenase 2</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E</td>
<td>Cortisone</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EOC</td>
<td>Epithelial ovarian cancer</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen receptor</td>
</tr>
<tr>
<td>F</td>
<td>Cortisol</td>
</tr>
<tr>
<td>FIGO</td>
<td>International Federation of Gynecology and Obstetrics</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>FSHR</td>
<td>Follicle stimulating hormone receptor</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosyl phosphatidylinositol</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>Gene Response Elements</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>H6PDH</td>
<td>Hexose 6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotrophin</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF1α</td>
<td>Hypoxic inducible factor 1α</td>
</tr>
<tr>
<td>HNT</td>
<td>Neurotrimin</td>
</tr>
<tr>
<td>HOX</td>
<td>Homeobox</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>hTERT</td>
<td>Human telomerase reverse transcriptase</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferonγ</td>
</tr>
</tbody>
</table>
IgLON Immunoglobulin LSAMP OPCML Neurotrimin
IL Interleukin
IL-1 Interleukin-1
IL-1α Interleukin-1α
ILRA Interleukin1 receptor antagonist
kDa Kilodaltons
LH Luteinising hormone
LOH Loss of heterozygosity
LOX Lysyl oxidase
LSAMP Limbic system associated membrane protein
MMP Matrix metalloproteinase
mRNA Messenger RNA
NAD Nicotinamide adenine dinucleotide
NADPH Nicotinamide adenine dinucleotide phosphate
NEGR1 Neuronal Growth Regulator 1
OPCML opioid-binding cell adhesion molecule-like protein
OSE Ovarian Surface Epithelium
PCOS Polycystic ovarian syndrome
PCR Polymerase chain reaction
PPC Primary peritoneal cancer
PR Progesterone receptor
PSE Peritoneal surface epithelium
PTEN Phosphatase and tensin homolog
RNA Ribonucleic acid
RT-PCR Reverse transcription PCR
SIR Standardised incidence ratio
SMAD Sma and Mad related protein
SP-A Surfactant protein-A
STAT Signal transduction and activator of transcription
TIMP-1 Tissue inhibitor of metalloproteinase-1
TNFα Tumour necrosis factor α
TSG Tumour suppressor gene
VEGF Vascular endothelial growth factor
UICC International Union Against Cancer
Chapter 1

General Introduction
1 General Introduction

1.1 Clinical Aspects of Epithelial Ovarian Cancer

More women die each year from epithelial ovarian cancer than any other gynaecological malignancy. Compared with many other cancers, improvements in ovarian cancer survival have been modest and overall survival remains poor with only 32% alive at 5 years (figure 1.1).

![Figure 1.1 Trends in ovarian cancer survival in Scotland from 1980-2004 Ages 15-99. (ISD, 2010)](image)

The poor survival associated with EOC is explained by the fact that most women who develop the disease present at an advanced stage and therefore the disease is less amenable to treatment and cure. Thus survival figures are inversely related to the FIGO stage of the malignancy (see table 1.1).
Table 1.1 FIGO staging of ovarian cancer with associated 5 Year survival rates. Modified from (Benedet, J.L. et al. 2000; Heintz, A.P. et al. 2003).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Definition</th>
<th>5 Year Survival Rate (%)</th>
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<tbody>
<tr>
<td>I</td>
<td>Growth confined to ovaries</td>
<td></td>
</tr>
<tr>
<td>Ia</td>
<td>Growth limited to one ovary; no ascites present containing malignant cells; No tumour on external surface; capsule intact</td>
<td>91.7</td>
</tr>
<tr>
<td>Ib</td>
<td>Growth limited to both ovaries; no ascites present containing malignant cells; No tumour on external surfaces; capsules intact</td>
<td>76.0</td>
</tr>
<tr>
<td>Ic</td>
<td>Tumour either Stage Ia or Ib but with tumour on surface on one or both ovaries or with capsule ruptured or with ascites present containing malignant cells, or with positive peritoneal washings</td>
<td>81.2</td>
</tr>
<tr>
<td>II</td>
<td>Growth involving one or both ovaries with pelvic extension</td>
<td></td>
</tr>
<tr>
<td>IIa</td>
<td>Extension and/or metastases to uterus and/or tubes</td>
<td>78.8</td>
</tr>
<tr>
<td>IIb</td>
<td>Extension to other pelvic tissues</td>
<td>64.0</td>
</tr>
<tr>
<td>IIc</td>
<td>Tumour either Stage IIa or IIb but with tumour on surface on one or both ovaries or with capsule ruptured or with ascites present containing malignant cells, or with positive peritoneal washings</td>
<td>69.5</td>
</tr>
<tr>
<td>III</td>
<td>Tumour involving one or both ovaries with histologically confirmed peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastases. Tumour is limited to the true pelvis, but with histologically proven malignant extension to small bowel or omentum.</td>
<td></td>
</tr>
<tr>
<td>IIIa</td>
<td>Tumour grossly limited to the true pelvis with negative nodes but with histologically confirmed microscopic seeding of peritoneal surfaces, or histologically proven extension to small bowel or mesentery.</td>
<td>57.0</td>
</tr>
<tr>
<td>IIIb</td>
<td>Tumour of one or both ovaries with histologically confirmed implants, peritoneal metastases of abdominal peritoneal surfaces, none exceeding 2cm in diameter; nodes are negative</td>
<td>43.8</td>
</tr>
<tr>
<td>IIIc</td>
<td>Peritoneal metastases beyond the pelvis &gt;2cm in diameter and/or positive retroperitoneal or inguinal lymph nodes</td>
<td>30.2</td>
</tr>
<tr>
<td>IV</td>
<td>Growth involving one or both ovaries with distant metastases. If pleural effusion is present, there must be positive cytology to allot to stage IV. Parenchymal liver metastases.</td>
<td>13.7</td>
</tr>
</tbody>
</table>
The growth of the tumour within the peritoneal cavity is often asymptomatic thus the term “silent killer” is used to describe the disease. There is considerable lack of understanding of the natural history of EOC particularly its mode of onset and mechanisms of dissemination. Once suspected, management of EOC involves excision of the tumour, most often by laparotomy, total abdominal hysterectomy and bilateral salpingo-oophorectomy (TAH and BSO), peritoneal lavage and omentectomy. The purpose of surgery is not only to remove the tumour and be able to make a histological diagnosis but also to assess degree of spread of the tumour within the peritoneal cavity, allowing the FIGO stage to be determined (Table 1.1). The advantage of this international system of staging is that trials can be compared and correlated at a global level, adding value to research. The disadvantages are that the staging is arbitrary, based on anatomical extent of the disease and not its histology, and it does not reflect the fact that the majority of cases are stage IIIc or IV at presentation. In addition the staging presumes a step-wise progression of the disease which may not reflect the natural history of the disease. The uncertainty over EOC’s natural history makes it a difficult disease to screen for and there is no test currently available that fulfils the necessary screening criteria set down by the World Health Organization (Wilson J et al. 1968).

Some studies have shown that the more tumour that is removed the better the outcome, such that patients with residual tumour measuring less than 1cm have improved survival rates (Bristow, R.E. et al. 2002; Bristow, R.E. et al. 2002). However another explanation for these results could be that the biological nature of the tumours is different and the more aggressive tumours (with poorer prognoses) are more difficult to surgically debulk to less than 1cm. Evidence for this is supplied by Berchuck et al who have discovered that there are genetic differences between tumours that are optimally and sub-optimally cytoreducible on microarray analysis (Berchuck, A. et al. 2004). Following histological diagnosis, intravenous chemotherapy with a platinum based agent (carboplatin or cisplatin) is standard practice in the United Kingdom with or without paclitaxel. Recent clinical studies
have suggested that survival can be extended by using intra-peritoneal chemotherapy in combination with intravenous chemotherapy (Armstrong, D.K. et al. 2006) albeit with an increase in abdominal complications. A major criticism of this study however is the adjuvant use of intravenous chemotherapy – to truly assess the efficacy of intra-peritoneal chemotherapy then a randomized control trial of intravenous versus intraperitoneal chemotherapy alone is required. Furthermore the dosing schedule meant that the patients in the intra-peritoneal arm were receiving paclitaxel in two out of every three weeks as opposed to once every three weeks in the intravenous arm.

Preventing the intra-peritoneal spread of EOC is a highly desirable therapeutic target, particularly because it is unusual for the disease to disseminate beyond the coelom. Unlike many cancers e.g. breast cancer which commonly spreads haematogenously, EOC is largely confined to the peritoneal cavity, forming widespread nodules on the surface of the peritoneum or replacing the adipose tissue of the omentum. This is often associated with a rise in serum levels of CA125 (MUC16), a glycoprotein tumour marker which is raised in 80% of EOC cases (Bast, R.C. et al. 1983). Although CA125 is a useful marker of disease progression and response to treatment its source is likely to be the peritoneum (Epiney, M. et al. 2000) and subsequently it is often raised in non-malignant disease making it a poor screening tool for early detection of EOC. When EOC does spread beyond the peritoneum it is usually to abdominal lymph nodes or the pleural cavity: another mesothelium. However it is the effect of intra-abdominal tumour that in many cases ultimately leads to death. Multiple chemoresistant tumour nodules develop on the serosal surface of the bowel and peritoneum which impairs normal motility and function (figure 1.2). The stasis of luminal contents then causes distension of the bowel, leading to pain and vomiting and predisposes to bacterial infection whereby the vomitus can become faeculant. Nutrient, electrolyte and fluid absorption across the bowel is unable to occur normally. Further deterioration of bowel function occurs in the now critically undernourished patient, with localised disruption of vascular perfusion and ischaemia and finally the patient succumbs after a distressing and often prolonged course of
bowel obstruction. If extension of EOC to the bowel could be prevented then prolongation of life for patients with ovarian cancer would follow.

Figure 1.2 Laparoscopic view of liver and peritoneal surface in patient with FIGO Stage IV serous ovarian carcinoma. Picture courtesy of Dr G Walker.

Figure 1.2 shows the extent of tumour burden commonly discovered at initial laparotomy. The omental involvement alone may consist of billions of malignant cells. Given this scenario, the complete removal of the entire malignant component by surgery alone is not possible and even after subsequent chemotherapy it is unlikely that all malignant cells would be entirely destroyed. In the author’s opinion the overall goal of ovarian cancer research should be to prevent EOC ever reaching such an advanced stage.

The development of a preventative strategy requires a thorough understanding of the natural history of ovarian cancer. This includes; identifying the cell type of origin and the necessary oncogenic transforming factors, determining what controls propagation of the transformed cell locally and subsequently how malignant cells are dispersed from the initial locus. From here; identifying what processes are involved in the adhesion of disseminated cells at distant sites within the peritoneum, and how proliferation of the metastatic cell occurs.
1.2 Origins of Epithelial Ovarian Cancer

The source of EOC is widely held in the literature as being the ovarian surface epithelium (OSE) (Auersperg, N. et al. 2001; Murdoch, W.J. et al. 2002). The term epithelium is a misnomer as the OSE is not a true epithelium, arising as it does from mesodermal tissue. This led to the generation of the term mesothelium by Minot in 1890. The evidence for EOC arising from OSE is somewhat circumstantial and indirect: the risk of developing EOC increases with number of lifetime ovulations and ovulation causes repetitive and cyclical injury to the OSE (Espey, L.L. 1994; Fleming, J.S. et al. 2006; Ness, R.B. et al. 2000). Many authors then hypothesise that the regeneration of the OSE following ovulation renders the OSE progenitor cell population vulnerable to genetic mutation and therefore oncogenic transformation.

![Photomicrograph of section of ovarian cortex demonstrating the presence of an inclusion cyst (ic) and proximity to the ovarian surface epithelium (ose). Inset shows tubal metaplasia occurring within the inclusion cyst. Modified from Auersperg et al. (2001).]
Leading on from “the OSE as cancer source” hypothesis is the suggestion that the OSE is subjected to an oncogenic environment within small cysts in the ovarian cortex referred to as “inclusion cysts” (Auersperg, N. et al. 2001). The name arises from the assumption that they form following injury to the ovary by ovulation and its subsequent repair, resulting in entrapment and inclusion of OSE cells within the ovarian cortex. Interestingly, occasional ciliated cells can often be identified within these cysts (Okamura, H. et al. 2001) suggesting either that metaplasia occurs or that cells shed by the Fallopian tube can also accumulate within them. Although there is some evidence linking their presence to cancer development, again this is mostly circumstantial, as inclusion cysts are more numerous in patients with family histories of ovarian cancer and in normal ovaries contralateral to ovaries affected by ovarian cancer (Mittal, K.R. et al. 1993; Werness, B.A. et al. 1999). Brewer et al have presented evidence that nuclear abnormalities can be detected in women with a higher risk of developing EOC or in the ovaries of women with ovarian cancer (Brewer, M.A. et al. 2004). However, nuclear abnormalities alone do not equate with malignancy and there have been no studies showing that patients with nuclear abnormalities go on to develop EOC. So, unlike other epithelial tumours e.g. cervical or colonic cancer, in EOC there is no clear stepwise progression from a pre-neoplastic lesion to an in-situ tumour to widespread malignancy. There are case reports in the literature of malignant change appearing within inclusion cysts but these are rare and anecdotal (Aoki, Y. et al. 2000). Some authors believe, controversially, that the lack of a defined pre-cursor lesion is simply due to not looking hard enough (Scully, R.E. 1995). Another possibility is not that pre-cursor lesions in the OSE are being missed but that the precursor lesions exist in a different tissue altogether: the Fallopian tube or peritoneum for example. To explain the absence of a precursor lesion, it is possible that once the disease develops it rapidly progresses beyond the inclusion cyst. Alternatively some cases of EOC may not arise within an inclusion cyst. However, one of the attractions of the inclusion cyst hypothesis is that it explains how tumours can appear at FIGO Stage IA, without breaching the capsular surface of the ovary. If EOC developed from OSE from the exterior surface of the ovary in every case then early stage cancer would not be seen,
as malignant cells would be more likely to shed and disseminate within the pelvis than invade into the ovarian stroma.

The literature regarding the genesis of inclusion cysts is controversial. Evidence that their formation is linked with ovulation was presented by Radisavljevic, however this is a histological study of thousands of ovaries and a purely descriptive study (Radisavljevic, S.V. 1977). The author does describe the presence of the inversion of the ovarian surface that occurs at sites where ovulation has recently occurred (as shown by the presence of an underlying corpus albicans). In addition there is a description of inclusion cysts that form following the enclosure of the fimbrial end of the fallopian tube, which the author suggests is evidence of “auto-transplantation”. A more dynamic study of inclusion cyst generation was undertaken using a mouse model, where ovaries from incessantly ovulating mice were compared with those from breeding mice and fetal ovaries. It appeared that inclusion cyst development was more closely related to age than number of lifetime ovulations (Clow, O.L. et al. 2002). A similar study from Australia, which also used a murine model discovered that inclusion cyst number was greater in the incessantly ovulating group (Tan, O.L. et al. 2005). In the human, a study examining incidence of inclusion cysts in women undergoing oophorectomy for benign gynaecological conditions demonstrated that the association between number of ovulations and presence of inclusion cysts was weak and the use of the combined oral contraceptive pill (COCP) and pregnancy was not associated with a statistically significant reduction in number of inclusion cysts (Heller, D.S. et al. 2005). From several epidemiological studies it is apparent that the risk of developing EOC is proportional to the number of lifetime ovulations (Fathalla, M.F. 1971; Ness, R.B. et al. 2000; Ness, R.B. et al. 2001; Purdie, D.M. et al. 1999; Purdie, D.M. et al. 2003). Therefore if inclusion cyst formation was necessary for ovarian cancer development, it would be expected that inhibition of ovulation by the oral contraceptive would also reduce the incidence of ovarian inclusion cysts. In summary the evidence for inclusion cysts being a pre-cursor for EOC is weak and other possible sources need to be considered.
1.3 Alternative Tissue Origins of EOC

Returning to the origins of ovarian cancer some authors have challenged the dogma that EOC arises from the OSE (Dubeau, L. 1999; Piek, J.M. et al. 2004). The authors point out deficiencies in the OSE theory but they fail to provide substantive evidence for any other theory of origin of EOC, although one candidate tissue they discuss is the secondary Mullerian system. This tissue includes the rete ovarii and paratubal cysts but the author acknowledges that little is known about their origin or indeed how they could be the source of ovarian cancer. However one point that is raised is that tumours that are designated primary peritoneal carcinomas are indistinguishable from serous epithelial ovarian cancers. Dubeau cites this as evidence that the OSE cannot be the cell of origin of EOC whereas what this finding contributes is the possibility that the same factors which cause the development of EOC are equally able to cause oncogenic change in other cell types including Fallopian tube epithelium and the mesothelial surface or peritoneal surface epithelium (which will be abbreviated to PSE to acknowledge the close relationship between the peritoneal mesothelial cell and the OSE).

A further explanation is that in ovarian tumours not arising in inclusion cysts the transformed OSE cell is shed from the surface of the ovary at an early stage and the fertile milieu of the peritoneum allows attachment and further proliferation. That cells from ectopic tissue can attach to the peritoneum has been demonstrated in models of endometriosis where endometrial epithelium and shed menstrual epithelial cells are able to attach in vitro to human peritoneal tissue (Witz, C.A. et al. 2002; Witz, C.A. et al. 2001). It is therefore conceivable that transformed EOC cells could attach to the peritoneum once they have disaggregated from the initial tumour growth.
In many epithelial cell types when epithelial cells are removed from their home environment they undergo apoptosis; a process known as anoikis (Liotta, L.A. et al. 2004). Moreover this phenomenon appears to be absent in epithelial ovarian cancer cells which, once freed from their origin, appear to be able to survive not only in the distant peritoneum but also anchorage-free, suspended in ascitic fluid. Another area of potential targeted therapy therefore is the possible reconstitution of anoikis induced apoptosis in disseminated EOC cells. It is presumptive however to assume that peritoneal metastases arise from disseminated cells from a precursor. In a similar hypothesis to the metaplastic theory of endometriosis, it might be the case that it is not the transformed OSE cells themselves that disseminate to cause widespread disease but that there are oncogenic proliferative factors released into the coelomic cavity which stimulate metaplasia, and ultimately dysplasia, in the peritoneum.

1.3.1 Peritoneal Biology and Pathophysiology

The peritoneum plays an important role in the progression of EOC and indeed EOC could be considered to be a peritoneal disease rather than a disease of the ovary. However it is remarkable how little work has been undertaken to look at the role of the tumour/peritoneal interface in EOC. The importance of such research is clear as the peritoneum is a potential target organ in which dissemination of EOC could be treated or even prevented. Yet most of the knowledge of peritoneal function comes from studies concerned with peritoneal dialysis and its complications.

The evolution of the coelomic cavity occurred some 550 million years ago and enabled organisms to develop an internalised organ system, permitting development of the lungs and gastrointestinal tract. The initial benefit may have been to allow segmentation and burrowing abilities of the common ancestor which was likely to have been a worm-like organism, however the persistence of the coelom in evolution emphasises its importance. Indeed, morphological studies of several mammalian
species have shown that the mesothelium is essentially the same in most mammals. The role of the cells lining the coelom is that of a separator such that when opposing sides of the coelom come into contact, there is no adhesion between the surfaces. Thus the lining of the cavity must be non-adhesive and frictionless. In the human the peritoneum covering the abdominal wall is termed the parietal peritoneum and that covering the organs the visceral peritoneum. The link between structure and function of the peritoneum was first documented in the early 18\textsuperscript{th} century by Douglas (Douglas, J. 1730) who described it as;

\begin{quote}
“\textit{everywhere smooth and even and lubricated by a fluid in order to preserve it from those inconveniences which otherwise have followed its continual attrition with other viscera}”.
\end{quote}

Anatomically the OSE is intimately related to the peritoneal surface epithelium (PSE). Both OSE and PSE are mesothelial cuboidal single cell layers which meet at the ovarian hilum. While the OSE has been extensively characterized, much less is known about the PSE. It covers the entire peritoneal cavity, an area of some 1.8m\textsuperscript{2} (Michailova, K.N. \textit{et al.} 2006) almost the same surface area as the skin. Effluent recovered from the peritoneum following peritoneal dialysis was noted to contain significant amounts of a surface active material and subsequent animal research showed that the mesothelium was able to produce large quantities of phosphatidylcholine, the major constituent of pulmonary surfactant (Dobbie, J.W. \textit{et al.} 1988; Grahame, G.R. \textit{et al.} 1985; Ziegler, C. \textit{et al.} 1989). Electron microscopy of the mesothelial cell has revealed two populations of cells: flattened squamous cells and less abundant cuboidal cells (Mutsaers, S.E. 2002). The significant difference in ultrastructure between these subtypes is that the cuboidal cells have more numerous mitochondria as well as more prominent endoplasmic reticulum and Golgi apparatus, consistent with increased metabolic activity. Dobbie and Lloyd described the presence of lamellar bodies within the mesothelial cell vacuoles, similar to those seen in type II pneumocytes (Dobbie, J.W. \textit{et al.} 1989). Transport across the mesothelium is an active process.
It would appear that the similarity between pneumocytes and mesothelial cells does not end with the morphological similarity. Surfactant protein A (SP-A) is the most abundant of the surfactant proteins and plays an important role in the alveolus where it regulates phospholipid homeostasis thus helping to lower surface tension. It has also been described in the mesothelium, where it is likely to aid lubrication in a similar manner (Dobbie, J.W. 1996). However another role of SP-A is in the innate immune response. SP-A is related to the collectin family of lectins, of which complement C1q is a member. By binding to carbohydrate molecules on the surface of microorganisms SP-A acts as an opsonin and can activate macrophages and other phagocytic cells (Crouch, E.C. 1998). So the PSE may play an important role in host defence as well as acting as a simple lubricating surface. Subsequently the work in this thesis directly examines the ability of the PSE to respond to inflammatory stimuli.

The structural similarities between PSE and OSE are striking as described by Blaustein (Blaustein, A. 1984). Just as Mutsaers has described two types of mesothelial cell, Gillett et al have described two populations of OSE cells (designated A and B cells) (see figure 1.5).
Figure 1.4. Electron microscopy of OSE and PSE showing a) two distinct cell populations on the ovarian surface; A cells being cuboidal and B cells squamous (Gillett, W.R. et al. 1991) b) fan shaped mesothelial cells (large arrows) 24 hours following injury (bar represents 20μm) (Mutsaers, S.E. et al. 2002).
The similarities between OSE and PSE are perhaps not surprising given their similar embryological origins. The intraembryonic coelomic cavity arises from the lateral plate mesoderm at the end of week three in the human. Between weeks five and seven the coelom undergoes septation to generate a future pericardial cavity, two pleural cavities and a peritoneal cavity. By the end of week seven the peritoneal cavity is separate from the other body cavities.

Figure 1.5. Embryology of the peritoneal cavity (kindly reproduced by Ted Pinner). Origin of the lateral plate mesoderm (top). Lower figure indicates fetal ovary at Carnegie stage 18 (44-49 days) demonstrating the similar origins, and juxtaposition of OSE and PSE.
However there are some significant differences between OSE and PSE. Microvilli dominate the surface of the PSE. The function of these is not clear, but they increase the mesothelial surface area and therefore are likely to influence absorption or secretion across the cell surface. They may also play a role in the trapping of secreted proteins or glycoproteins thus acting as a monitor of the peritoneal environment. Alternatively the large surface area simply provides protection of mesothelial integrity. The ability to transport non-coaguable fluid across the peritoneum has been put to clinical use as a method of dialysis for patients with renal failure.

Since Hertzler’s observation in 1919, it has been known that different sizes of peritoneal wounds take the same time to heal. Thus it is unlikely that the mesothelium regenerates from the wound edge alone. The hypothesis that peritoneal wounds are repopulated by free-floating mesothelial cells was tested by Foley-Comer et al using a model of intraperitoneal injury to rat testes (which are in communication with the peritoneal cavity through the tunica vaginalis) (Foley-Comer, A.J. et al. 2002). Labelled mesothelial cells were injected intraperitoneally into rats following testicular injury and the wound site examined at 5 and 8 days post injury. The wounds were completely healed by the labelled cells at 8 days. Furthermore, there were no labelled cells on the wound site if fibroblasts were substituted for the mesothelial cells and there was no binding of labelled cells on non-injured surfaces. This provides strong evidence for a progenitor cell population of mesothelial cells circulating within the peritoneum.

These findings raise further questions: where is the stem cell niche that these progenitors arise from? What signals allow progenitor cells to preferentially adhere to the wounded surface? How is the proliferation of these progenitors switched off? Are the progenitor cells able to differentiate into other mesodermal or epithelial
phenotypes? Does the ovary heal in a similar fashion to this model in the human? The answers to such questions are important clinically. The intraperitoneal spread of EOC could potentially be prevented if the adhesion of malignant cells to the peritoneum could be inhibited. Indeed in vitro work on mesothelial cell cultures has suggested that secreted hyaluron prevents attachment of tumour cells to the peritoneal surface (Jones, L.M.H. et al. 1995). Endometriosis could be prevented if the local peritoneal environment could be manipulated to become hostile to endometrial cells. Finally peritoneal scarring which causes significant morbidity through infertility and abdominal pain could be combated by preferential laying down of mesothelial cells rather than scar tissue formation. There may be a future role for autologous transfusion of mesothelial cells in these conditions.

The regeneration of a mesothelial layer is one end result of peritoneal injury. A more serious consequence is the development of intraperitoneal adhesions. While there is little doubt that the mesothelial cell is able to mount an inflammatory response to injury (Topley, N. et al. 1994), it is likely that there is also some anti-inflammatory activity to prevent adhesion formation. The deposition of fibrin may be the critical factor in determining extent of healing. Normally fibrin is excluded from the peritoneal environment. However in mesothelial injury if blood vessels are injured the procoagulant pathway is preferred and if fibrin is not timeously removed, fibroblast invasion can occur thus paving the way for adhesion formation (Holmdahl, L. 1997). This allows development of a matrix containing fibroblasts, and over time smooth muscle cells and nerve fibres which may explain the chronic symptoms experience by patients with severe endometriosis or intra-abdominal adhesions (Sulaiman, H. et al. 2001).

The close links between the OSE and PSE are also present in their pathological states. Women who have an hereditary predisposition to EOC are also at increased risk of developing primary peritoneal cancer (PPC)(Finch, A. et al. 2006). Patients with PPC present with similar symptoms to EOC (Fromm, G.L. et al. 1990), they carry the same risk factors and there is also increased risk in BRCA1/BRCA2
mutation carriers. At a histological level PPC and EOC are indistinguishable leading to a probable underdiagnosis of PPC of around 10% (Chu, C.S. et al. 1999) (Cormio, G. et al. 2000). The tumours respond to the same chemotherapeutic regimens with similar survival outcomes (Chu, C.S. et al. 1999; Jaaback, K.S. et al. 2006). From many angles, PPC and EOC could be considered the same disease. Particularly as PPC can arise in women without adnexae, the PSE should also be considered a candidate for the tissue of origin of peritoneal EOC. However for this to happen there must be some loss of mesothelial phenotype as otherwise the resulting tumour would be malignant mesothelioma.

The mesothelium itself appears to behave differently in patients with EOC. Looking at peritoneal biopsies in patients with EOC, Zhang et al noticed that rather than the single layer of cells seen in non-EOC patients there was a trend towards a discontinuous mesothelium which would contain more than one layer of cells (Zhang, X.Y. et al. 1999). They assumed this was because the mesothelial cell layer reacted to the presence of disease by shedding mesothelial cells. It is highly likely that an increase in mesothelial proliferation is a consequence of EOC, however it is not yet clear what stimulates such a response. One criticism of this study is that is not apparent whether the peritoneal biopsies were taken from similar anatomical areas in the EOC and non-EOC patients. Also it is possible that the discontinuous mesothelial layer in the EOC patients could have been a result of surgical handling as the timing of the biopsy in relation to the surgery is not defined. Nevertheless the mesothelium is likely to play an important role in the response to EOC dissemination.

As alluded to above, an understanding of the interaction between tumour and PSE is an essential requisite to be able to prevent peritoneal spread of cancer. Once the cancer has grown large enough to be able to shed tumour cells from its surface, the free-floating metastasis must be able to attach to the peritoneal surface for further progression of the cancer to occur. These metastatic cells are often aided in their transport by an increase in intraperitoneal fluid, ascites. The mechanism by which
malignant ascites is produced is not clear. In contrast to non-malignant conditions where reduced plasma oncotic pressure or raised hepatic portal vein pressure leads to an accumulation of a transudate, malignant ascites has a high protein concentration (similar to serum) implying active transport of protein across the peritoneal membrane. The stimulus for the generation of ascites is not known but there are potentially many compounds that are involved. In a study of ascites from various primary tumours vascular endothelial growth factor (VEGF) was markedly increased in all cancers compared to non-malignant ascites (Zebrowski, B.K. et al. 1999). Furthermore, ovarian cancer ascites had significantly higher VEGF levels compared to ascites from colon and gastric cancer patients. Mesothelial cells and cancer cells themselves have been implicated in the production of VEGF (Stadlmann, S. et al. 2005). These findings provided an indication for trialling the monoclonal VEGF antibody bevacizumab in ovarian cancer. While the response rate has varied from 8-100% in phase II trials, there has also been a reported rate of bowel perforation of over 5% as a serious side-effect (Han, E.S. et al. 2007). VEGF can be regulated through up-regulation of hypoxia inducible factor-1α (HIF1α) so the low oxygen tension environment of tense ascites may have a role to play in the up-regulation of VEGF.

However as a consequence of these studies the manipulation of the peritoneal environment does appear to be a successful strategy for some EOC patients. Other studies looking at the composition of ascitic fluid in patients with EOC have demonstrated high levels of tumour necrosis factor α (TNFα), IL-6, IL-10, IL-12, and neopterin compared to serum (Melichar, B. et al. 2006; Moradi, M.M. et al. 1993; Zeimet, A.G. et al. 1998). Neopterin is a pteridine compound produced following breakdown of guanosine triphosphate (GTP) by GTP cyclohydrolase I, an enzyme up-regulated in macrophages by interferon-γ (IFNγ) (Huber, C. et al. 1984).

The peritoneum plays an important role in immune surveillance. The description of small whitish spots on the serosal surface of the greater omentum led to further investigation as to their nature. These “milky spots” are tiny accumulations of
lymphoid tissue and are a component of the lymphoreticular system. The mesothelial layer is disrupted at these milky spots and the lack of a basement membrane allows migration of lymphoreticular cells such as macrophages and lymphocytes. Thus an immune response can be initiated rapidly to any antigenic challenge from within the peritoneum.

Despite the close relationship the peritoneum has with the immune system, most EOCs evade a significant immune response until they become quite advanced. A major component of the immune presenting cells in the peritoneum is the monocyte/macrophage population. The cell component of the milky spots consists of 50% macrophages. The literature regarding the immune response to ovarian cancer is rather contradictory with some studies suggesting that the response to the tumour is suppressed while others suggest an up-regulation. This may simply reflect the considerable heterogeneity that exists not only amongst cancers but also amongst patients’ immune systems. Comparing monocyte/macrophage populations in chemo-naïve patients with EOC and healthy subjects, Gordon provided evidence that these cells have impaired function (Gordon, I.O. et al. 2006). Using assays of phagocytosis and antibody-dependent cytotoxicity, they demonstrated that both these mechanisms of cell death were impaired in EOC. Furthermore the macrophage cultures from patients with EOC produced IL-6, IL-8, IL-10 and TNFα. So these data would suggest that impaired monocyte function could explain tumour progression of EOC. There was some evidence of an anti-tumour effect in this study however as co-culture with macrophages (from EOC patients) inhibited growth of an EOC cell line. The authors postulated that this growth suppression was due to TNFα.

While the innate immune system may affect tumour progression there is a greater potential for therapeutic intervention by enhancing adaptive immunity. Although tumours present antigens that can be targeted by the cellular immune mechanisms, they are rarely removed altogether. The failings of the anti-tumour response are also exemplified by the inability of tumour vaccines to resolve malignancy as well as the phenomenon of immune tolerance. Ascites of patients with EOC has a significant
number of T regulatory cells ($T_{REG}$) compared to their blood or ascitic fluid form patients with non-malignant ascites (Curiel, T.J. et al. 2004). These $T_{REG}$ are also more abundant in EOC tumours than in normal ovaries. In vitro $T_{REG}$ caused suppression of T cell proliferation. Finally $T_{REG}$ content of the tumours were correlated with poor survival even after controlling for stage and surgical debulking. There is therefore reasonable optimism that inhibiting $T_{REG}$ function in EOC tumours is a realistic strategy for therapy in the future.

The peritoneum’s ability to heal has further clinical relevance in the condition endometriosis. Here, areas of endometrial glandular tissue are ectopically sited within the peritoneal cavity. Depending on the extent of the disease the clinical sequelae may include pain, infertility and adhesion formation. However there is a paradox between symptoms and extent of disease. Some studies have shown evidence of endometriosis at sterilization in women without symptoms of up to 22% (Moen, M.H. et al. 1991), leading to some authors to suggest that it is so common that it should not be considered a disease (Evers, J.L.H. 1994). Indeed in one study that examined outcomes in 39 women with endometriosis diagnosed incidentally at laparoscopic sterilization there was a higher rate of pelvic pain in the matched control group (Moen, M.H. et al. 2002). However the experience of pain is complex and asymptomatic women with endometriosis may have different coping strategies or reasons not to report pain as a symptom, making the correlation between symptoms and histology difficult. Endometriosis requires further consideration as it has many features of a pre-malignant disease, is associated with EOC and causes damage to the peritoneal surface epithelium.

1.3.2 EOC and Endometriosis

The origins of endometriosis have not been conclusively defined but Sampson’s theory of endometrial implantation (Sampson, J. 1927) appears to carry more weight than the alternative hypothesis first suggested in 1898 that endometriosis arises from metaplasia of the peritoneum (Iwanoff, N. 1898). However there are enough differences between endometriosis and endometrium to suggest that the endometrium
may not be the source of endometriosis (Redwine, D.B. 2002). Multiple studies have demonstrated an increase in pro-inflammatory mediators in patients with endometriosis. In keeping with the theme of this thesis where inflammation is postulated to predispose to EOC, it is perhaps not surprising that these patients are at increased risk of developing EOC.

Several studies have suggested an increased relative risk or odds ratio for EOC in patients with endometriosis and these are critically reviewed by Vigano (Vigano, 2007). In an extensive study based in Sweden, women with a diagnosis of endometriosis had an increased risk of developing EOC at >10 years with a standardised incidence ratio (SIR) of 2.5 (Brinton, L.A. et al. 1997). Further analysis revealed that the site of endometriosis was important as women with ovarian endometriomata had a SIR of >3.0. However the cases in this study were women who had been hospitalized as a result of their endometriosis. If it is accepted that the majority of cases of endometriosis are asymptomatic then the inpatient cohort are perhaps an inaccurate representation of disease incidence. Moreover if these asymptomatic women were included in the analysis it is probable that the prevalence of EOC would drop. Thus the selection of inpatients as cases may bias the outcome and a more extensive study involving ever diagnosed endometriosis would give a more accurate assessment of causality in EOC.

Figure 1.6. Studies demonstrating evidence of an association between endometriosis and endometrioid/clear cell EOC (Vigano, 2007). The horizontal axis represents odds ratio and horizontal lines the confidence interval, the size of the box is proportional to the sample size.

A consequence of the doubling in risk of EOC with endometriosis is a debate over how individual patients with endometriosis should be counselled. The overall lifetime risk of developing EOC remains small and the risk of surgical complications is of a similar order. It would seem unwise currently to recommend prophylactic BSO in women with endometriosis, however a large prospective trial may be helpful in deciding whether such an intervention would be indicated in these women. If endometriosis had a significant role in overall EOC risk then Beral’s Million Women Study should have uncovered a preponderance of endometrioid/clear cell EOCs in women on oestrogen only HRT in a manner similar to development of endometrial adenocarcinoma (Beral, 2007). However the only subtype that appeared to be increased with HRT use was serous, although the association with endometriosis was not specifically looked for. In Ness’ study of 767 cases of EOC, 66 (8.4%) had a history of endometriosis, which was higher than the matched controls (85 out of
This gave an odds ratio of 1.7 (CI 1.2-2.4) for endometriosis as a risk factor for EOC. In addition Ness and co-workers demonstrated a risk reduction with tubal ligation and hysterectomy, which, if reflux menstruation causes endometriosis, would reduce incidence of endometriosis. So although some of the cancers may have been attributable to previously undiagnosed endometriosis, the overall contribution of endometriosis to EOC development is likely to be less than 10%. The association with inflammation lends some biological plausibility to endometriosis being a risk factor for EOC. The other potential mechanism is through sex steroid receptor signalling as ER and PR are both present in endometriosis although levels are not synchronous with those in the endometrium which alter with the menstrual cycle (Metzger, D.A. et al. 1988).

Purdie’s study of Australian women demonstrated that it was protection from the ovulatory events in a woman’s 20s that had the most significant effect on reducing risk of EOC in later life (Purdie, D.M. et al. 2003). By inference this would suggest that ovulation between the ages of 20-29 carries the highest risk of causing disease. Yet EOC is principally a disease of older women with the majority of cases occurring after the menopause. One explanation for this anomaly is that the first “hit” of genetic damage is caused in the OSE during the reproductive years and there is a second hit that occurs after the menopause. It is possible that it is the stem cell component of the OSE that is damaged during the reproductive years but which only begins to replicate following the hormonal changes at the menopause. The obvious endocrine protagonists following the menopause are the gonadotrophins which become significantly and persistently raised (Cramer, D.W. et al. 1983). Yet in vitro work has suggested that follicle stimulating hormone (FSH) and luteinising hormone (LH) do not cause any increase in OSE proliferation (Ivarsson, K. et al. 2001).

Rather than inhibition of ovulation being the protective mechanism of the COCP, an alternative hypothesis is that it is the steroid component of the pill that has a more dominant effect against EOC. There is overwhelming evidence for the protective effect of the COCP on EOC but the mechanism for this is unknown (Collaborative
Group on Epidemiological Studies of Ovarian, C.; Greer, J.B. et al. 2005; Gwinn, M.L. et al. 1990; Siskind, V. et al. 2000). The most likely beneficial component of the COCP is the progestin moiety. The reasons for suggesting this are firstly that some other protective factors for EOC also have prolonged raised progesterone levels e.g. pregnancy. In addition twin pregnancy, where the maternal progesterone is raised more than in singletons (Haning, R.V. et al. 1985), carries a higher protective effect than a singleton pregnancy (Whiteman, D.C. et al. 2000). Secondly, progesterone has demonstrable anti-inflammatory effects in OSE, where it inhibits the rise in COX-2 following IL-1α treatment (Rae, M.T. et al. 2004). Thirdly, COCP preparations with higher efficacy progestins have a greater protective effect on EOC risk than those with low potency progestins (Schildkraut, J.M. et al. 2002). Finally, the other component of the COCP, synthetic oestrogen, appears to be mitogenic in OSE and therefore unlikely to protect against EOC (Gotfredson, G.S. et al. 2007; Syed, V. et al. 2001).

While sex steroids may protect against EOC if taken pre-menopausally, they appear to increase the risk of developing EOC when taken post-menopausally in hormone replacement therapy (HRT) (Beral, V. 2007; Lacey, J.V., Jr. et al. 2002; Riman, T. et al. 2002; Rodriguez, C. et al. 2001) although this is disputed by some (Purdie, D.M. et al. 1999; Sit, A.S. et al. 2002).

<table>
<thead>
<tr>
<th>Last reported HRT use</th>
<th>Years of HRT use*</th>
<th>Cases/ population (1000s)</th>
<th>Relative risk (95% CI)</th>
</tr>
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<tbody>
<tr>
<td>Never users</td>
<td>..</td>
<td>1342/4747</td>
<td>1.00</td>
</tr>
<tr>
<td>Past users</td>
<td>33</td>
<td>391/868</td>
<td>0.98 (0.88-1.11)</td>
</tr>
<tr>
<td>All current users</td>
<td>77</td>
<td>1240/1871</td>
<td>1.20 (1.05-1.32)</td>
</tr>
<tr>
<td>Oestrogen-only</td>
<td>92</td>
<td>242/859</td>
<td>1.34 (1.13-1.60)</td>
</tr>
<tr>
<td>Oestrogen+ progestagen</td>
<td>69</td>
<td>414/669</td>
<td>1.14 (1.01-1.28)</td>
</tr>
<tr>
<td>Other</td>
<td>70</td>
<td>84/519</td>
<td>1.22 (0.98-1.53)</td>
</tr>
</tbody>
</table>
Using data from the Million Women Study group, which was set up to investigate links between HRT use and breast cancer, Beral found that risk of developing EOC was increased amongst users of HRT for longer than 5 years (Beral, V. 2007). There were a significantly greater number of serous tumours in the HRT users group but no difference in other histological subtypes. Interestingly there was also a higher rate of serous borderline tumours in the HRT users. The study also demonstrated that unlike COCP use, where the protective effects appear to last many years after cessation of use, previous HRT use appeared to have no residual effect on disease risk. In Beral’s analysis users of HRT were also more likely to have been COCP users, suggesting that the protective effects of the COCP are abrogated by HRT use.

The mechanism by which HRT use increases EOC risk is unclear. Both HRT and COCP involve administration of exogenous oestrogens and progestins, but they act in quite different endocrine environments. Ethinyl-estradiol in the COCP inhibits gonadotrophin induced production of estradiol from the ovary, while HRT users have high gonadotrophin levels but minimal ovarian production of estradiol. It may therefore be the dynamics of estradiol production that predispose to cellular transformation rather than absolute dose. So the COCP may be protecting the ovary from rapid swings in potentially harmful estradiol levels.

A glaring question from the Million Women Study is not why are HRT users at increased risk of developing EOC but what stops the majority of HRT users from developing EOC? The incidence of the disease is still small in users compared to non-users (2.6 per 1000 women versus 2.2 per 1000 women) so if exogenous oestrogen is the cause of EOC there must be protective mechanisms in place to offset its effects. An interesting study yet to be undertaken should measure serum estradiol and correlate this with risk of developing EOC. If HRT is causative for EOC then a
dose response effect should be seen, with higher estradiol levels being associated with greater risk of developing EOC.

The potential for oestrogen to cause EOC should prompt a search for other mechanisms by which estradiol could impact on ovarian tissue. While in the younger woman ovarian production of estradiol is predominantly via 17β-hydroxysteroid dehydrogenase, in the post-menopausal state the cytochrome P450 enzyme aromatase synthesizes predominantly estrone from androstenedione. Inhibitors of this pathway are widely used in breast cancer treatment (ATAC Trialists' Group 2005). One study described aromatase in the OSE and on measuring activity demonstrated that levels were higher in normal tissue than in EOC tumours (Cunat, S. et al. 2005). Furthermore data from Phase II trials have indicated that patients with EOC and ER-positive tumours might benefit from aromatase inhibitor (AI) therapy (Bowman, A. et al. 2002). Indeed this was then confirmed by Smyth et al who described a disease stalling effect of the AI letrozole in a phase II trial of women with ER-positive EOC(Smyth, J.F. et al. 2007). One study has demonstrated that estradiol and estrone cause down-regulation of the progesterone receptor (PR) in both OSE and EOC cancer cell lines (Mukherjee, K. et al. 2005). Patients positive for PR appear to have a better prognosis than those negative for PR (Hempling, R.E. et al. 1998; Lee, P. et al. 2005; Sevelda, P. et al. 1990). Therefore ER and PR typing may assist patient care by their use in treatment decision algorithms.

It is unlikely that the natural history of EOC will be definitively documented in the human as such a study allowing confirmed early malignant disease to be observed without treatment would be unethical. However with increasing use of ultrasound as an investigative tool in gynaecology it has become apparent that not only are ovarian cysts a common finding in postmenopausal women, but they also have a low risk of becoming malignant (Bailey, C.L. et al. 1998; Castillo, G. et al. 2004; Kroon, E. et al. 1995; Modesitt, S.C. et al. 2003; Valentin, L. et al. 2003). What protects some patients from developing ovarian cancer while others remain susceptible? The answer to this is undoubtedly complex and multifactorial however genetic
susceptibility may play a role and this will be explored in more detail later in this chapter.

1.3.3 The Fallopian Tube

An alternative hypothesis to the argument that the OSE is capable of transforming into other epithelial cell types is that these separate histologies have different cells of origin. Thus the mucinous subtype has a gastro-intestinal source, clear cell and endometrioid tumours arise from explanted endometrial tissue and serous from the Fallopian tube. Furthermore, there is increasing evidence that the Fallopian tube may have a role in the development of ovarian cancer (Crum, C.P. et al. 2007; Kindelberger, D.W. et al. 2007; Lee, Y. et al. 2007). Firstly, in women with BRCA1 or BRCA2 mutations undergoing prophylactic salpingo-oophorectomy, there is an increased risk of having an occult Fallopian tube carcinoma (Finch, A. et al. 2006). Secondly, women with BRCA1 or BRCA2 mutations whose ovaries alone are removed have an increased risk of developing primary peritoneal carcinoma than those undergoing complete salpingo-oophorectomy (Paley, P.J. et al. 2001). Thirdly, there are histological similarities between the Fallopian tube luminal epithelium and ovarian papillary serous adenocarcinomas. The proximity of the fimbrial ends of the Fallopian tubes to the ovarian surface also provides a biological explanation for the discovery of ciliated tubal-like cells within ovarian inclusion cysts. The extension of a Fallopian tube carcinoma to involve the adjacent ovary could be an early step in the progression of such a malignancy and by the time of surgery the origin of the primary tumour may be difficult to ascertain. Indeed this situation would explain why a step-wise progression of ovarian cancer is not seen in the OSE.

1.4 Histological Subtyping of EOC

The prognosis for patients diagnosed with EOC is heavily dependent on stage at initial presentation (table 1.1). However tumour grade and histological subtype also influence prognosis and response to treatment. While it is standard practice to use
the FIGO staging system in the United Kingdom to describe the clinical stage of the disease, TNM staging is also used internationally, and is directly comparable to the FIGO system (table 1.2)

<table>
<thead>
<tr>
<th>FIGO</th>
<th>UICC</th>
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Table 1.2 Comparison of FIGO and International Union Against Cancer (UICC) staging systems for EOC.

A consequence of the uniform cuboidal appearance of the OSE is that some authors assume that this means the OSE is in an undifferentiated state (Auersperg, N. et al. 2001). The transition from a simple cuboidal phenotype to a more mesenchymal phenotype is well documented, and this ability to undergo metaplasia gives support to the possibility that the OSE can differentiate into cell types resembling those from the reproductive tract. Furthermore during the transformation into cancerous tissue the histological subtypes found in cancers reflect the epithelial components found throughout the genito-urinary tract, such that serous tumours represent the Fallopian tube, endometrioid tumours the endometrium, clear cell histology the transitional epithelium of the bladder, and mucinous tumours the endocervix (figure 1.9). In most series of advanced ovarian malignancy, such as that described by the Gynecologic Oncology Group (Omura, G.A. et al. 1991), the most common form is serous (60%) then endometrioid (16%), and more rarely mucinous (4.5%) and clear
cell (3.7%). Mixed histologies can also arise (6%) as well as undifferentiated forms (4.1%).

Figure 1.8. Examples of the most common histological subtypes of EOC (haematoxylin and eosin, magnification A-C X10, D X40). A) Serous carcinoma B) Mucinous carcinoma C) Endometrioid carcinoma D) Clear cell carcinoma. Pictures courtesy of Dr A Williams.

While this is an appealing and tidy model, there are several arguments suggesting that this view is too simplistic. Firstly, although mesenchymal transformation of cultured OSE cells can be demonstrated there have been no studies demonstrating metaplasia of OSE to endometrial, endocervical, or tubal epithelial cell types. Secondly, the OSE does not arise from the Mullerian duct. Thirdly, there is growing evidence that rather than EOC being a disease arising from one particular cell type it is more likely that it is a heterogeneous disease with different risk factors for different histological subtypes. For some time it has been apparent that many
mucinous tumours are derived from gastro-intestinal primary cancers, in particular appendiceal adenocarcinoma (Fujii, S. et al. 1986; Seidman, J.D. et al. 1993; Skaane, P. et al. 1986; Sumithran, E. et al. 1992; Thorsen, P. et al. 1991; Young, R.H. et al. 1991). Furthermore, mucinous tumours appear to differ from non-mucinous tumours in their epidemiological risk factors, with oral contraceptive pill use being less protective for the mucinous group and ovulation being a much greater risk factor in non-mucinous tumours, while family history of EOC increases risk for non-mucinous but not the mucinous subtypes (Purdie, D.M. et al. 2003). Similar discrepancies in risk factors between different subtypes can also be seen for other histologies. In a study examining development of EOC and previous gynaecological surgery, hysterectomy and tubal ligation were found to be protective for EOC, but only for endometrioid and clear cell carcinomas (Rosenblatt, K.A. et al. 1996). One weakness of this study is that although the odds ratios suggested a protective effect, the confidence intervals crossed unity. Whether the risk factors differ for individual histological types of EOC is controversial and there are almost equal numbers of studies for and against. In one of the largest, involving 767 women with EOC and 1367 age-matched controls, there were no significant differences in reproductive risk factors amongst the different histologies, however once these subtypes were analysed, the number of cases (52 mucinous cases for example) may have been too small to be able to demonstrate a true difference; an accusation denied by the authors in their discussion (Modugno, F. et al. 2001). A study looking for loss of heterozygosity (LOH) in only stage I ovarian cancers demonstrated similar allelic loss, resulting in grouping of endometrioid, clear cell and grade 3 tumours (Wang, V. et al. 2005). This appeared to suggest that there is considerable heterogeneity amongst ovarian cancers. A final unexplained observation is why the incidence of the histological subtypes is skewed in favour of serous adenocarcinomas. Presumably if the OSE is truly capable of differentiation during malignant transformation then the type of tumour should be equally prevalent.

The means by which different EOCs could arise are likely to be multiple but one common pathway may be through disruption of homeobox (HOX) genes (Cheng,
W. et al. 2005). The genes *Hoxa9*, *Hoxa10* and *Hoxa11* are known to control differentiation of the Mullerian ducts into Fallopian tubes, uterus and cervix. Cheng et al. demonstrated firstly that although the HOX proteins are differentially distributed throughout the Mullerian tract in the human, there are none detectable by immunohistochemistry on the human OSE. Secondly, on examining a panel of ovarian cancers, HOXA9 protein was found in serous, endometrioid and mucinous EOCs, HOXA10 in endometrioid and mucinous but not serous and HOXA11 only in mucinous tumours. After transfecting a mouse OSE cell line (MOSEC) with these individual HOX genes, they created a mouse model of ovarian cancer by intraperitoneal seeding of the transfected cells. On histological assessment of the tumours those expressing *Hoxa9* developed tumours resembling papillary serous EOC, those expressing *Hox10* developed glandular tumours resembling endometrioid carcinoma and those expressing *Hoxa11* had tumours with cells containing clear cytoplasm much like mucinous EOC. Inoculation of mice with MOSEC cells transfected with *Hoxa7*, which appeared to be important for determining degree of differentiation rather than tissue type of differentiation, also resulted in low-grade tumours. While this study still does not provide direct evidence for the OSE being the tissue of origin for EOC, it does provide an explanation for the multiple histologies seen in this disease.

The existence of borderline tumours of the ovary (BOT) (Schaner, M.E. et al.) throws up some difficult questions as to their role in the genesis of EOC. Thus they behave clinically like benign tumours and although they can recur the prognosis for patients with borderline tumours is often excellent. The histological characteristic of the tumour is a lack of invasion beyond the basement membrane of the epithelial tissue. The majority of these tumours are serous and share many features of the Fallopian tube epithelium but a mucinous subtype may also be reported (see figure 1.10). They are staged in an identical fashion to EOC and can have peritoneal implants, which if they become invasive result in poorer outcomes (Prat, J. et al. 2002). Molecular analyses of serous BOTs (SBOTs) do not show the same genetic profile as malignant tumours: there is no characteristic *TP53* mutation in SBOTs.
Gene expression profiling shows that serous and mucinous BOTs are distinct entities and that there is considerable heterogeneity amongst mucinous tumours, possibly implying different oncogenic events (Wamunyokoli, F.W. et al. 2006). Whilst ras mutations are common in both mucinous BOT and mucinous EOC, they occur in serous BOT but not carcinomas. These observations suggest that while mucinous EOC could arise from a mucinous BOT it is very unlikely that such a process occurs in serous EOC. Looking at loss of heterozygositis (LOH) and K-RAS mutations in a series of SBOTs, Sieben et al have provided evidence that the implants in SBOT are from clonal expansion of the disease rather than the generation of multiple tumours (Sieben, N.L. et al. 2006). Further investigation of K-RAS and BRAF mutations in SBOTs by microarray showed a high rate of mutations in these genes but none or low rates in serous carcinomas of the ovary (Sieben, N.L.G. et al. 2005). The high rate of these mutations in SBOTs implicated the RAS-RAF-MEK-ERK-MAP kinase signalling pathway and further analysis of the genes involved in this pathway showed a significant difference between EOC and SBOTs. In these borderline tumours there appears to be an uncoupling of the proliferative and invasive components of malignancy. Prime suspects for tissue invasion are the matrix metalloproteinases (MMPs). Although Sieben et al showed a significant difference in extracellular matrix (ECM) associated gene expression between SBOTs and EOC, the difference was a down regulation of all genes in SBOTs apart from tissue inhibitor of metalloproteinase-1 (TIMP-1). However follow-up quantitative real-time PCR demonstrated only a 1.6-fold up-regulation of this gene and zymography showed MMP-9 activity in the cancers, but the published zymogram is not convincing and pro-MMP-9 is present in the SBOTs. Nevertheless further research into molecular mechanisms of control of ECM degradation is indicated to explain the differences between SBOT and EOC. This has considerable potential for gene therapy where aberrant EOC cells could be made non-invasive by switching off their MMP activities and could return the tumour to a SBOT-like state.
Another way of distinguishing different types of EOC is by tumour grade; G1, low grade being well differentiated, G2, moderately differentiated, and G3 high grade and poorly differentiated. It has been proposed that low grade tumours are anteceded by borderline tumours given the similarity of genetic alterations in low grade and BOTs whilst high grade tumours have less genetic mutations identified apart from p53 mutations that are found in high grade serous and carcinosarcomas (Shih Ie, 2004). This scheme of characterization moves away from the traditional surgical methods of disease staging and considers the disease in terms of genetic alterations that influence the cellular biology. This emphasises the observation that what is currently termed “ovarian cancer” is more likely to be a collection of different diseases arising from different sources and different genetic mutations rather than
one disease. By moving towards this way of thinking it is probable that the methods
of treating the disease in the future will be more tailored to individual patients based
on genetic analysis of their tumour, rather than administration of uniform treatments.

1.5 OSE in ovarian biology

In the female of reproductive age the single layer of mesothelial cells that cover the
surface of the human ovary is necessarily disrupted on a monthly basis by the release
of an oocyte from the ovarian cortex. This event results in significant trauma to the
ovarian surface which must be repaired quickly in time for the next ovulation. While
it is clear that the endocrine signalling responsible for the timing of ovulation is
precisely controlled there is also evidence that the OSE has its own intracrine
mechanisms to aid this reparative process. However the exact sequence of events
that occur at the time of ovulation remain vague. During enlargement of the
dominant follicle in the first half of the menstrual cycle, hydrostatic pressure
increases the convexity of the ovarian surface. While some authors propose that the
OSE undergoes apoptosis prior to ovulation (Murdoch, W.J. et al. 2002), it is far
from clear how this is regulated. There may be a physical stimulus from increasing
tension between OSE cells as the cortex enlarges, or there may be paracrine signals
arising from the follicle itself. For ovulation to occur there has to be proteolytic
breakdown of the ovarian surface by matrix metalloproteinases. While there is
evidence that the OSE is capable of synthesising such enzymes it is not clear what
the relative contributions of OSE, ovarian stroma and oocyte complex are in
collagenase/proteinase production. Recent work in OSE demonstrates that this tissue
is capable of synthesising both MMP-9 and Lysyl oxidase (LOX), an enzyme
necessary for tissue remodelling and collagen cross-linking (Rae, M.T. et al. 2008).
Furthermore the synthesis of these compounds is influenced by interleukin-1α and
the potent anti-inflammatory steroid cortisol (Kendall’s compound F). Therefore the
local degradative effect of these enzymes causes thinning of the ovarian cortex and stigma formation from where the oocyte complex can breach the ovarian surface. It is clear from direct visualization of this process in the human at laparoscopy that localised inflammation is considerable (see figure 1.11).

![Figure 1.10. Inflammation of the ovarian surface following ovulation (picture courtesy of Prof SG Hillier).](image)

However the pro-inflammatory cascade caused at ovulation must also be quickly resolved in order to repair the ovarian surface and prepare the ovary for its next ovulatory episode. Examination of the effect of IL-1α in primary cultures of human OSE allowed Yong et al to conclude that there are also anti-inflammatory mechanisms initiated at the time of ovulation that limits local tissue disruption and allows resolution of inflammation (Yong, P.Y. et al. 2002). They measured levels of 11-beta hydroxysteroid dehydrogenase (11βHSD1) mRNA and enzyme activity in cultures of human OSE in the presence and absence of IL-1α to simulate inflammation in vitro. The principal action of this protein in the human is the conversion of inactive cortisone to the potent anti-inflammatory steroid cortisol by reduction of the 11-keto group. Although it is part of the alcohol dehydrogenase superfamily, 11βHSD1’s behaviour in vivo appears to be mainly as a reductase. The
The addition of cytokine caused a significant rise in 11βHSD1 mRNA in a dose-dependent fashion and could be abrogated by the addition of the IL-1 receptor antagonist (IL-RA). The mRNA rise was replicated in the 11-oxoreductase activity of these cells, suggesting that the OSE is able to increase local levels of cortisol following an inflammatory insult and hence aid in the resolution of tissue damage.
Further work on the anti-inflammatory signals in the OSE by Rae et al confirmed the rise in 11βHSD1 mRNA with IL-1α treatment but when cortisol was also added concomitantly there was a further rise in 11βHSD1 mRNA expression (Rae, M.T. et al. 2004). Assuming this is reflected in the level of the enzyme, it would appear that there is a positive feedback mechanism in place whereby in a background of inflammation cortisol is able to enhance its own production. There was no change in levels of the inactivating enzyme 11βHSD2 upon treatment in these experiments so any alteration in glucocorticoid metabolism was likely to have arisen from the effects on 11βHSD1. By examining cyclo-oxygenase-2 (COX-2) expression in their OSE samples the substantial anti-inflammatory effect of cortisol could be confirmed. COX-2 mRNA levels rose with IL-1α but were returned to near control levels by the addition of cortisol. Interestingly, progesterone was demonstrated to have similar anti-inflammatory properties and inhibited the rise in COX-2 mRNA after IL-1α treatment. The pro- and anti-inflammatory paradigm displayed by the OSE can be summarised in figure 1.13.
Figure 1.12. Summary of inflammation-associated events in the OSE at ovulation.

The anti-inflammatory mechanisms within the OSE may explain the rapid resolution of ovulation-associated inflammation in the ovary that exists in order to prepare for the subsequent cycle of follicle maturation and ovulation should pregnancy not occur. However although most of the work has focused on events surrounding ovulation, there is less understanding of how the OSE heals itself. Is there centripetal healing from the wound edge or is there healing by circulating mesothelial cells, as in the peritoneum? The latter would seem to be the quickest method but there is no evidence for this in the OSE. If progenitor cells do repopulate the ovarian wound where do they come from? Are there niches of OSE stem cells close by or could the mesothelial progenitor initially “plug the gap” and then differentiate into an OSE type cell?

1.6 11βHSDs and Mechanism of Glucocorticoid Action

The existence of two isoforms of the 11βHSD enzyme means that levels of cortisol can be controlled at a local level by the balance of the type 1 and 2 isozymes. Therefore tissues expressing high levels of type 2 11βHSD e.g. kidney will have low tissue levels of cortisol (allowing aldosterone exclusive access to the mineralocorticoid receptor), whereas a relative abundance of type 1 11βHSD allows local cortisol to be generated. Levels of 11βHSD2 are also significantly higher in placenta which may protect the fetus from maternal cortisol. Initial studies of the pure 11βHSD1 enzyme were achieved in the liver where it acts to generate glucocorticoids by reductase activity on 11-ketosteroids (figure 1.12). Whereas the 11βHSD1-containing cellular fractions and microsomes appear to have bi-directional catalytic activity, intact cells appear only to show unidirectional metabolism of cortisone to cortisol. It is probable therefore that the latter situation reflects in vivo action in the OSE more accurately. Furthermore it is likely that the unidirectional
activity of 11\(\beta\)HSD1 exists because of its compartmentalised location within the endoplasmic reticulum, where it relies on NADPH as a co-substrate. It is clear that 11\(\beta\)HSD1 levels can be increased in response to inflammation in many other tissues as well as the OSE: aortic smooth muscle (Cai, T.-Q. \textit{et al.} 2001), pre-adipocytes (Tomlinson, J.W. \textit{et al.} 2001), endometrium (McDonald, S.E. \textit{et al.} 2006), trophoblast (Li, W. \textit{et al.} 2006) and fetal membranes (Sun, K. \textit{et al.} 2003). There is growing evidence for a switch in isozyme production in pre-adipocytes (Bujalska, I.J. \textit{et al.} 2002) and endometrium (McDonald, S.E. \textit{et al.} 2006). These studies suggest that tissues are able to switch from 11\(\beta\)HSD1 to 11\(\beta\)HSD2 production given the correct signals. This strengthens the case against dehydrogenase activity of 11\(\beta\)HSD1 occurring \textit{in vivo} as it would appear that if dehydrogenation of the 11-hydroxyl group is required, the tissue preferentially induces 11\(\beta\)HSD2 production rather than alter co-substrate availability in order to induce dehydrogenase activity of 11\(\beta\)HSD1. The reductase activity of the Characteristics distinguishing 11\(\beta\)HSD1 and 11\(\beta\)HSD2 are shown in table 1.2.

Within the ovary, cells other than the OSE have been extensively studied with regard to their ability to influence glucocorticoid levels. In granulosa cells which have not been exposed to LH, mRNA for both the MR and 11\(\beta\)HSD2 are expressed (Tetsuka, M. \textit{et al.} 1999; Tetsuka, M. \textit{et al.} 1997). Following luteinisation however there is no detectable expression of 11\(\beta\)HSD2 mRNA or protein and the predominant isoform is 11\(\beta\)HSD1 (Michael, 1997). In addition the steroid receptor profile changes from MR to GR. This may give an insight to the mechanism of follicular development where water follows sodium transport mediated by the MR.

The hypothesis develops that abnormal accumulation of fluid within pathological ovarian cysts could arise from deregulation of MR or 11\(\beta\)HSD2 expression and an investigation of 11\(\beta\)HSD in abnormal ovaries is warranted.
Table 1.3. Summary of properties of 11βHSD1 and 11βHSD2 after (Draper, N. et al. 2005).

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<thead>
<tr>
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<th>Type 1</th>
<th>Type 2</th>
</tr>
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<tbody>
<tr>
<td><strong>Tissue Distribution</strong></td>
<td>Liver, lung, pituitary, brain, gonad, adipose tissue</td>
<td>Kidney, colon, placenta, salivary glands</td>
</tr>
<tr>
<td><strong>Direction of Reaction</strong></td>
<td>Bi-directional (in vitro) Reductase (in vivo)</td>
<td>Uni-directional (dehydrogenase)</td>
</tr>
<tr>
<td><strong>Enzyme kinetics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Km Cortisol</em></td>
<td>17-27 μmol l-1</td>
<td>14-60 nmol l-1</td>
</tr>
<tr>
<td><em>Km Cortisone</em></td>
<td>300 nmol l-1</td>
<td>∞</td>
</tr>
<tr>
<td><strong>Chromosome location</strong></td>
<td>1q32.2</td>
<td>16q22</td>
</tr>
<tr>
<td><strong>Gene size</strong></td>
<td>30kb, 6 exons</td>
<td>6.2kb, 5 exons</td>
</tr>
<tr>
<td><strong>Protein size</strong></td>
<td>292aa, 34kDa</td>
<td>405aa, 44kDa</td>
</tr>
<tr>
<td><strong>Inhibitor</strong></td>
<td>Glycyrrhetinic acid, carbenoxolone (CBX), CDCA, metyrapone, BVT.14225 (Biovitrum/Amgen)</td>
<td>Glycyrrhetinic acid,</td>
</tr>
<tr>
<td><strong>Regulation of expression</strong></td>
<td>⊕TNFα, IL-1,IL-4, IL-5, IL-6,Leptin,IFNy,cortisol, GH,IGF-I</td>
<td>⊕AVP, dexamethasone, ⊕TNFα, PPARΔ, Hypoxia</td>
</tr>
<tr>
<td><strong>Co-substrate</strong></td>
<td>NADP(H)</td>
<td>NAD(H)</td>
</tr>
<tr>
<td><strong>Clinical associations</strong></td>
<td>Obesity, insulin resistance, Apparent Cortisone Reductase Deficiency (ACRD), osteoporosis, glaucoma.</td>
<td>Apparent mineralocorticoid excess (AME), idiopathic hypertension, cancer.</td>
</tr>
</tbody>
</table>
An important feature that influences 11βHSD1 enzyme efficiency is co-substrate availability. The generation of NADPH is dependent on hexose 6-phosphate dehydrogenase (H6PDH) which catalyses the first two steps of the pentose phosphate pathway (Clarke, J.L. *et al.* 2003) as summarised in figure 1.14. H6PDH lies within the smooth endoplasmic reticulum (ER) (Bublitz, C. *et al.* 1987), which is impermeable to pyridine nucleotides, and is dependent on glucose 6-phosphate (G6P) transport into the ER by glucose 6-phosphate translocase for generation of NADPH. This relationship is not purely one-sided as experiments in rat live microsomes demonstrate not only that H6PDH stimulation (by G6PD) increased cortisol production but addition of cortisone increased H6PDH activity (Czegle, 2006). These data also suggested a significant pool of latent NADPH within the liver as addition of cortisone caused a measurable increase in production of cortisol. However this may be a feature of glucose/glycogen rich tissues such as the liver, and peripheral tissues could conceivably have reductase activity impaired by an absence of NADPH through reduced glucose availability. Thus cell type may be a determinant of whether 11βHSD1 acts as a reductase or dehydrogenase. In cell culture work, omission of glucose from culture medium reduces keto-reductase activity of 11βHSD while there appears to be an increase in dehydrogenase activity (Ferguson, 1999). This fuel deprivation could be exacerbated further in tissues such as cancer where demand as a result of disinhibited growth could exceed supply.

A consequence of the interdependence of 11βHSD1 and H6PDH is that mutations in H6PDH will lead to diminished NADPH production and thus reverse the reaction direction of 11βHSD1, resulting in reduced cortisol production and a syndrome of apparent 11βHSD1 deficiency. This scenario has been elegantly demonstrated by Draper *et al* in a series of 3 patients, the females of which presented with symptoms normally associated with polycystic ovarian syndrome (PCOS) i.e. hirsutism, acne, obesity, oligoamenorrhea and infertility (Draper, N. *et al.* 2003). While 11βHSD1 has long been suggested as a target for anti-obesity agents, perhaps H6PDH should also be considered in equal measure.
Figure 1.13. Mechanism of co-substrate involvement in 11βHSD1 activity. GT, glucose 6-phosphate translocase; G6P, glucose 6-phosphate; 6PG, 6-phosphogluconolactonate.

The generation of cortisol by 11βHSD1 provides a ligand for the glucocorticoid receptor (GR). Due to the high lipophilic natures of cortisol, the GR does not require to be on the cell membrane and instead can be found within the cytoplasm and on the nuclear membrane. Activation of GR can have several different outcomes, depending on the target tissue and its current milieu. The GR is believed to have evolved from a common ancestor around 400 million years ago (Thornton, J.W. 2001) and shares similarities with other steroid receptors from the nuclear receptor
superfamily of transcription factors that also includes receptors for mineralocorticoids, oestrogen, androgen, progesterone, vitamin D and retinoic acid. Through its effect on transcription the GR can influence a vast array of cell processes including proliferation, apoptosis, immune response, neuronal signalling and vascular tone. Rather than there being a simple ligand-receptor interaction with a clear cellular effect, with GR signalling there are myriad possibilities by which the signalling process can be altered. Classically the GR resides in the cytoplasm within a complex of proteins including hsp90, which maintains the ligand binding site, along with several others. Binding of glucocorticoid causes dissociation of this complex and alters the GR complex to allow its translocation to the nucleus. Once inside GR homodimers can interact with Gene Response Elements (GRE) to either promote or inhibit gene transcription. Furthermore it would appear that the activated GR complex can also interact by protein to protein binding with other transcription factors, such as activator protein-1 (AP-1), nuclear factor-κB (NFκB), Sma and Mad related protein (SMAD) and signal transduction and activator of transcription (STAT). While GR to DNA binding occurs within the nucleus, these protein-protein interactions can occur within the cytoplasm. This sequence of events clearly has points at which signalling could be disrupted and there are further mechanisms by which GR function might be upset. The gene for GR is composed of 10 exons and lies within chromosome 5q31-32. Alternative splicing of exon 9 gives rise to two isoforms GRα and GRβ. GRα protein consists of 777 amino acids while GRβ is identical for the first 727 amino acids but the carboxy terminal amino acids are replaced resulting in a protein of 742 amino acids. In the human GRα appears in almost all tissues while GRβ is present at much lower levels but always within the nucleus. GRβ is not transcriptionally active but appears to act as an inhibitor of GRα. There is some evidence that an imbalance in relative amounts of these two isoforms occurs in some disease states, and may play a role in glucocorticoid resistance, particularly in asthma (Hamid, Q.A. et al. 1999). A further splice variant GRγ (an insertion of an additional arginine between exons 3 and 4) has been implicated in childhood acute lymphoblastic leukaemia suggesting that GR dysregulation can play a role in the malignant process (Beger, C. et al. 2003). Further potential disturbance to GR signalling can arise from post-translational
processing of GR such as phosphorylation, ubiquitination and SUMOylation (Duma, D. *et al.* 2006). Therefore in cell-based science any glucocorticoid effect may be the result of many different signalling pathways and be dependent on the immediate cellular environment. This makes cell culture based research using glucocorticoids not only difficult to predict but also difficult to translate to the clinic.

### 1.7 11βHSD And Cancer

Rabbitt et al have suggested that a switch in 11βHSD isozyme production could underlie alterations in cell proliferation and thus be implicated in the development of cancer (Rabbitt, E.H. *et al.* 2003). Using stable transfects of either 11βHSD1 or 11βHSD2, they were able to demonstrate a higher rate of proliferation in cells transfected with the type 2 isozyme (Rabbitt, E.H. *et al.* 2002). While the study is clear in its findings, the overall relevance of transfecting a human gene into a rat osteosarcoma cell line to human pathology is questionable. Nevertheless Rabbitt describes several other studies suggesting a link between overexpression of 11βHSD2 and malignancy, for example in pituitary adenomas (although these are not invasive tumours) (Korbonits, M. *et al.* 2001) and colon cancer (although 11βHSD2 was also present in normal tissues) (Takahashi, K. *et al.* 1998). Rabbitt’s hypothesis would suggest that raised 11βHSD2 levels (and thus low cortisol levels) in tissues would stimulate cell proliferation. This is in keeping with the pro-differentiation and anti-proliferative effects seen with cortisol *in vitro*. However in the OSE glucocorticoids have been shown to have no effect (Karlan, B.Y. *et al.* 1995) or stimulate cell proliferation (Salamanca, C.M. *et al.* 2004). If these studies are relied upon then the tumorigenic effect of 11βHSD2 could instead be explained by a glucocorticoid independent action, perhaps by activation of an oncogene. This thesis examines Rabbitt’s hypothesis directly by assessing the relative quantities of type 1 and 2 11βHSD in normal OSE and samples of EOC. The evidence supporting Rabbitt’s hypothesis has been further added to with regard to ovarian cancer by a study which appeared to suggest higher 11βHSD2 activity in tissue samples of
ovarian cancer compared to normal ovaries (Temkin, S. et al. 2006). Herein lies a major stumbling block for ovarian cancer research using primary tissues: what tissue to refer to as normal? If the source of EOC is the OSE, as these authors imply, then the tissue that should be used for comparison with cancers should be the OSE and not whole ovary. The considerable steroidogenic potential of the ovary invalidates its use as a comparator. An additional criticism of Temkin’s study is that the samples were homogenized and additional NAD was added to the assay mixture. Firstly, the disruption of the cells releases 11βHSD1 from its location in the ER as described above, and secondly addition of NAD artificially raises dehydrogenase activity compared to the intact cell. However this was undertaken for control tissue (post-menopausal ovary) also and no such rise was seen.

The issue of choice of normal tissue as a comparator is an important one as results of any comparative study could be dramatically altered by use of the wrong control tissue. Whole ovary is widely used in studies looking for differences between normal and malignant ovarian tissue (DiFeo, A. et al. 2006; Newton, T. et al. 2006; Szlosarek, P.W. et al. 2006; Welsh, J.B. et al. 2001; Widschwendter, M. et al. 2004), but if the authors believe that EOC arises from the OSE then it is illogical to use whole ovary as the comparator instead of the OSE. As the OSE makes up such a minute contribution to the ovarian volume, the use of whole ovary could influence any comparison due to the considerable amount of stromal tissue in the ovary. In addition the small foci of highly steroidogenic cells within ovarian follicles could also skew data. In a clever microarray study Zorn et al investigated differences in gene expression between different cells used as ‘normal’ comparators with EOC (Zorn, K.K. et al. 2003). Looking at whole ovary, uncultured OSE brushings, and cultured primary and immortalized OSE cells, they demonstrated that gene clustering was found between the primary tissues and also between the cultured cells showing that exposure to tissue culture conditions may affect gene expression in OSE.

Further examination showed the whole ovary samples clustering differently to the OSE brushings showing the effect of large quantities of stromal cells in the sample. Overall it would appear that the most ideal normal control is OSE brushings without any culture. There are considerable practical problems with use of this tissue
however as the numbers of cells obtained at each brushing is of the order of several thousand so the quantity of RNA that can be extracted is tiny.

The discovery of 11βHSD1’s important role in response to inflammation in the ovary coupled with the knowledge that inflammation is a probable causative agent in EOC (Fleming, J.S. et al. 2006) lead Gubbay et al to investigate inflammatory signalling in ovarian cancer cell lines (Gubbay, O. et al. 2005). Looking at baseline levels of mRNA, they demonstrated a significantly lower level of 11βHSD1 in three of the four cancer cell lines selected compared to OSE, but a higher level of 11βHSD2 in all cancer cell lines. Furthermore when the cells were treated with IL-1α, there was no significant induction of 11βHSD1 in three of the four cancer cell lines. Interestingly the one cell line that showed a response to IL-1α, PE-14, was the same cell line with relatively normal 11βHSD basal levels. This cell had been derived from a patient with a histologically well differentiated cancer whereas the three other cell lines were from poorly differentiated tumours. The question then arises – does poor tumour differentiation cause dysregulated 11βHSD expression or does aberrant 11βHSD production determine tumour differentiation?

It is unlikely that the answer to this question will arise from work on ovarian cancer cell lines, as the cells used have been selected for their ability to replicate without differentiation and as such are poorly representative of primary ovarian tumours. In contrast, primary cancer cell cultures are derived directly from histologically proven tumours, have not undergone multiple passages and are therefore characteristic of the “real-life” disease. What is lost by not using cell lines is the predictable, repeatable and uniform behaviour of a particular cell but what is gained in primary cell culture is the increased probability of being able to translate research findings into clinically relevant interventions. Established cell lines do undoubtedly have a place in cancer research, however their ease of use has meant they are often preferred to the more labour-intense process of working with primary tissue. In some situations this has had a detrimental effect on whole bodies of research, in particular when evidence shows that contamination of some cell lines by a more virulent type has meant
researchers have been making conclusions about a different cell type than the one they set out to study (Nelson-Rees, W.A. et al. 1981; van Bokhoven, A. et al. 2001). Furthermore in the field of ovarian cancer research one cell line, SW626, that has been extensively used as an example of ovarian cancer now appears to be of colonic origin (Furlong, M.T. et al. 1999). In deriving a cell line, the process selects a clone of cells that have achieved immortality. However even primary tumour cells are not immortal once placed in culture. Thus the relationship between the primary tumour and the cell line is likely to be remote. It is quite clear that ovarian cancer tissue contains dynamic DNA mutations occurring in different areas of tumour, suggesting that, far from being a monoclonal entity, tumour development is a constantly evolving process (Khalique, L. et al. 2007; Tanaka, K. et al. 1989). This tumour heterogeneity is in contrast to the monoclonal nature of cell lines.

The ability of a cell line to replicate the in vivo tissue becomes even more tenuous when “transformed” cells are used to simulate normal tissue. Widely used techniques to create these “normal” cell lines include insertion of papillomavirus E6 and E7 genes or the human telomerase reverse transcriptase (hTERT). Yet once immortalized the cells cease to be normal (Ziltener, H.J. et al. 1993) and have taken on characteristics of malignancy. Despite this, these cells are often referred to as normal (Choi, J.-H. et al. 2005), in the same way that EOC cell lines are often described as “ovarian cancer”. Not only is it likely that such dependence on these cellular examples of Frankenstein’s monster is misguided, it is probable that the understanding of some biological processes has been set back by over-reliance on results obtained from an immortalized cell system far removed from the in vivo tissue.
The transformation of a normal cell into a cancerous cell can theoretically occur through a variety of mechanisms, but for many patients the precise sequence of events may never be known. The common feature in many cancers is an alteration in the DNA sequence. The change in DNA can be passed through the germline as a susceptibility for developing cancer or may arise spontaneously in the somatic tissue. It has more recently become apparent that another mechanism by which gene expression may be altered in cancer is not by disruption of the gene sequence itself but by an epigenetic effect, DNA methylation, that inhibits gene transcription (Baylin, S.B. et al. 2006). Almost 1% of the genes in the human genome have been implicated with development of cancer (Futreal, P.A. et al. 2004). In ovarian cancer this can involve the activation of oncogenes or the inhibition of tumour suppressor genes. In addition the purity of the genome has to be maintained with cell division so interference with the DNA repair mechanisms may also be an initiating or predisposing step in cancer development.

1.8.1 Hereditary Ovarian Cancer

Most cases of ovarian cancer are stochastic events and less than 10% of cases of EOC are familial (Risch, H.A. et al. 2001). In affected families where genetic profiling has been undertaken, the genes most commonly associated with ovarian cancer are principally BRCA1 (70-75% of familial cases), BRCA2 (20%), hereditary non-polyposis colorectal coli (HNPCC, 2%) and others (5%). Despite BRCA1 first being implicated in breast and ovarian cancers nearly 13 years ago (Miki, Y. et al. 1994), and BRCA2 the following year (Wooster, R. et al. 1995), functions for the gene products are only now becoming clear. Both genes are predicted to give rise to complex yet distinct proteins which appear to localize in the nucleus. BRCA1 spans
80kb on chromosome 17q12-21, encoding multiple protein products, the most commonly studied of which is p220, while \textit{BRCA2} lies within 13q12-13 and encodes a predicted protein of 3418aa. A product of alternative splicing of the \textit{BRCA1} gene has been shown to modify DNA replication. Suppression of the 1399aa gene product \textit{BRCA1-IRIS} reduced rate of cellular DNA replication while its overexpression stimulated DNA replication (ElShamy, W.M. \textit{et al.} 2004).

The precise roles for \textit{BRCA1} and \textit{BRCA2} have not been identified but they are implicated in the maintenance of chromosomal structure (Venkitaraman, A.R. 2002). However given that this role is required in all tissues, it is a mystery as to why mutations in these genes preferentially predispose to breast and ovarian cancer. Some light has been shed on this by Hu \textit{et al} who demonstrated that an inverse relationship between \textit{Brca1} expression and aromatase activity exists in murine ovaries (Hu, Y. \textit{et al.} 2005). In addition they demonstrated that knockdown of \textit{BRCA1} in a granulosa cell line caused significant up-regulation of aromatase mRNA. Thus mutations in \textit{BRCA1} could increase aromatase activity locally in the ovary and in adipose tissue, therefore elevating tissue oestrogens and driving mitosis. A local oestrogenic stimulus may also explain the preference for EOC cells to metastasise to and thrive in the omentum. More recently evidence has arisen that \textit{BRCA1} may cause silencing of the X chromosome in female animals (Ganesan, S. \textit{et al.} 2002; Silver, D.P. \textit{et al.} 2007), although another group has implied that these experiments are not reproducible (Xiao, C. \textit{et al.} 2007).

In comparison to other tumours the cancers that patients with \textit{BRCA1} mutations develop tend to be serous and have better prognoses (Rubin, S.C. \textit{et al.} 1996). It is possible that knowledge of increased risk causes patients to present at an earlier stage than their counterparts thus making their outcomes appear more favourable. However there may be significant differences in the mechanism of onset of EOC in patients with a familial predisposition to developing cancer to those who develop the disease by chance. Yet because they form an easily studiable group patients with a
heritable predisposition to EOC are an ideal cohort with whom studies of preventative interventions can be carried out.

Of course, not all patients with BRCA1/2 mutations will go on to develop cancers and like HRT users discussed earlier it is likely that there are other mechanisms in place to protect some predisposed women from developing EOC.

1.8.2 Sporadic Ovarian Cancer

While there may be overlap with BRCA1 in sporadic EOC, the genetic background to most cancers is unknown. The list of genes that have been associated with sporadic EOC is vast and too extensive for discussion here. However there are a few candidates that require further elaboration.

1.8.3 Putative Oncogenes

1.8.3.1 Met/HGF Receptor

The tyrosine kinase receptor Met is an ideal candidate for an oncogene in EOC. After binding with its ligand hepatocyte growth factor (HGF), it dimerises and is able to induce a variety of pathways to bring about invasive growth (Comoglio, P.M. 2001). In OSE cultures, the phenotype becomes progressively more neoplastic with higher levels of Met and ERK1/2 activation (Wong, A.S.T. et al. 2004). Corps et al described 20-fold higher levels of the met protein in SKOV-3 cells compared to another cell line, CH1 (Corps, A.N. et al. 1997). When HGF was added to the culture motility, migration and $^{3}$[H]-thymidine incorporation were all increased in the SKOV-3 cells but not the CH1 line. This showed that Met/HGF signalling was intact in the SKOV-3 cell line, but this is difficult to translate to the in vivo situation of EOC. The same group also demonstrated that ascitic fluid contains high levels of HGF (Sowter, H.M. et al. 1999). Met appears to be overexpressed in ovarian cancer and is associated with a poor prognosis (Di Renzo, M.F. et al. 1994; Sawada, K. et
NK4, which acts as an inhibitor of Met, suppresses tumour formation and increases survival in mouse xenograft models (Saga, Y. et al. 2001). These findings would therefore suggest that the Met/HGF pathway is ideal for therapeutic targeting.

1.8.3.2 RAS/RAF

The ras family of proteins act as a relay downstream of tyrosine kinase receptors and upstream from the RAS-RAF-MEK-ERK-MAP kinase signalling pathway. The proto-oncogene K-Ras has been implicated in many cancers. Activating mutations have been discovered at codons 12, 13 and 61, but codon 12 is the most common mutation site in gynaecological cancers. The most common histological subtype affected is mucinous EOC, where it is mutated in 50% of cases (Enomoto, T. et al. 1991; Gemignani, M.L. et al. 2003). This is of interest given that it is also a common mutation in gastro-intestinal cancers. RAF genes code for one of three serine/threonine kinases which bind RAS. Mutations in BRAF exist in the same types of tumour as KRAS mutations, but are rarely present simultaneously in the same tumour, suggesting a common mechanism of tumorigenesis. Interestingly one study showed evidence for KRAS mutations in borderline serous tumours but absence of KRAS mutation in invasive serous tumours suggesting that borderline tumours may not be pre-cursors to invasive cancers (Mayr, D. et al. 2006).

1.8.3.3 EGFR/HER2

The epidermal growth factor receptor (EGFR) is a member of the ErbB family of tyrosine kinases that includes HER2 (ErbB2). It is selectively activated by binding its ligands EGF or TGFα, upon which it dimerises or pairs with another member of the ErbB family to stimulate intracellular tyrosine kinase activity. The EGFR has been linked with EOC for over twenty years (Gullick, W.J. et al. 1986) and appears to be overexpressed in some ovarian cancers although mutation of the sequence appears to be uncommon (Lassus, H. et al. 2006). An alternative splice product of EGFR (sEGFR) has been discovered in the serum and if used in conjunction with
CA125 may provide a means for EOC screening (Baron, A.T. et al. 2005). While the link between HER2 and EOC appears weaker than in breast cancer there is evidence that HER2 over-expression is a poor prognostic indicator (Serrano-Olvera, A. et al. 2006; Verri, E. et al. 2005). Reports of HER2 positivity vary widely and depend on whether protein or gene amplification is being measured. However in Phase II trials the response to HER2 monoclonal antibody therapy (trastuzamab) is disappointing with a response rate of only 7% (Bookman, M.A. et al. 2003). As this study was undertaken in patients previously treated for EOC, it does not exclude a future role for anti-HER2 treatment in early cancers or as first line therapy. However this group of receptors does provide an ideal target for drug therapy.

1.8.4 Tumour Suppressor Genes (TSG)

1.8.4.1 p53

The tumour suppressor p53 (encoded by the gene TP53) is one of the most widely investigated in cancer research. It has been implicated in many epithelial cancers, where mutations of p53 have been described in 50% of cases (Levine, A.J. 1997). Missense mutations make up the majority of p53 mutations in 80% of cancers. In EOC, several small studies show high incidences of both over-expression and mutation of TP53 (Kmet, L.M. et al. 2003). p53 is found in low levels in normal cells and it is targeted for degradation by MDM2, so levels are kept low by its relatively short half-life of twenty minutes (Levine, A.J. 1997). Cell stressors such as DNA damage, loss of cell contact signals and hypoxia can all inhibit MDM2 production therefore preventing p53 breakdown and a rapid rise in intracellular p53. This mediates a number of downstream events culminating in cell cycle arrest or apoptosis. A loss of p53 function can therefore result in unimpeded cell proliferation. In many tumours with a p53 mutation, there is an equivalent level of overexpression of that mutated protein, suggesting that p53 production is being driven but its mutant form is unable to become active and switch off the driving signal. In a study of 197 cases of invasive ovarian cancer, Schildkraut et al found that women whose cancers had high p53 expression were more likely to have had a high number of lifetime ovulations (Schildkraut, J.M. et al. 1997). This finding
raises this possibility of different mechanisms of tumorigenesis for different tumour types, because if number of lifetime ovulations was a risk factor for all tumours there should have been no difference between the p53 positive and negative groups. Rather than being a necessary initiating feature however it is possible that p53 mutation is picked up as a tumour develops. Evidence for such a mechanism arises from pooled studies of p53 expression in ovarian cancer (Kmet, L.M. et al. 2003). In this paper, the authors mention that 42 studies examined p53 status and stage of disease and overall p53 was detected in 39% of the early FIGO stage tumours but in 55% of FIGO stage 3 or 4 tumours. Higher grade tumours also had higher frequency of p53 overexpression. In addition, borderline tumours were p53 positive in 17% of cases while benign ovarian cysts were positive in 7%. The authors also highlighted the considerable variation in p53 immunopositivity given that over 7 different antibodies were used to detect p53 across the studies. For such a plethora of investigation, many questions about p53 remain.

The prevalence of TP53 mutations in EOC raises the theoretical possibility of gene therapy where administration of an attenuated adenovirus carrying functional wild type TP53 could be used to restore the p53 status of the cell, thus altering the malignant phenotype. This has also been attempted in head and neck cancers where the treatment is injected directly into the tumour. In the EOC setting results of such an approach are still in the early stages. Trials have failed to demonstrate any significant response to therapy and most patients develop flu-like symptoms with adenovirus therapy (Vasey, P.A. et al. 2002). There are some potential problems with such an approach to gene therapy. Firstly an immune response to the vector may adversely affect the treatment outcome. Secondly, loss of p53 function may not be simply due to gene mutation. Thirdly, the adenovirus itself may alter cellular function so that p53 expression is impaired. Fourthly, even if gene therapy works at a cellular level there are potential problems delivering the agent to the tumour. In intraperitoneal therapy the outermost surface of the tumour is exposed to the treatment however the majority of the tumour mass may be well hidden suggesting that the therapy should also be administered intravascularly, where greater side
effects would be anticipated. Based on current evidence it is difficult to envisage gene therapy for EOC becoming standard treatment in the near future.

Whereas activation of an oncogene can permanently switch on cell replication and hence development of a tumour, inactivation of a tumour suppressor gene is a more complicated process. Traditionally it has been thought that this requires two hits to inactivate such a gene, following Knudson’s classic model (Knudson, A.G. 2000). However it has become clear that inactivation of a gene can occur by inactivating its promoter sequence through DNA methylation of the CpG island (Baylin, S.B. et al. 2006). Thus there are now appearing a whole raft of “new age” tumour suppressor genes where the DNA sequence of the gene is not altered but gene transcription is suppressed. Unlike genetic mutations DNA methylation can occur through environmental agents which may last a lifetime. For example, in gastric cancer, previous exposure to Helicobacter Pylori caused DNA methylation which was maintained after the infection was cleared (Ushijima, T. et al. 2006). This mechanism of cancer development could explain the delay between the inflammatory insult of ovulation in a woman’s reproductive years and the development of EOC later in life.

1.8.4.2 PTEN

PTEN (phosphatase and tensin homolog) was identified as a TSG in several cancers by LOH (Li, J. et al. 1997). This localised PTEN to 10q23. Mutations in PTEN appear to occur in endometrioid and clear cell ovarian cancers but not serous (Obata, K. et al. 1998). In an exceptional study using a mouse model of endometriosis Dinelescu et al. inoculated mouse OSE with K-Ras mutations using an adenovirus vector and were able to induce endometriosis-like lesions (Dinulescu, D.M. et al. 2005). By combining K-Ras and PTEN mutations the lesions that occurred became malignant and spread throughout the peritoneal cavity – much like EOC in humans. This elegant study gives a potential mechanism for development of endometrioid EOC, which may explain why not all patients with endometriosis go on to develop
EOC, as this may be conditional on a second PTEN mutation. In addition it gives a potential target for therapeutic manipulation.

1.8.4.3 OPCML and the IgLONs

LOH studies indicated that a tumour suppressor could reside on chromosome 11q (Davis, M. et al. 1996; Foulkes, W.D. et al. 1993; Gabra, H. et al. 1995). More detailed LOH analysis refined the potential genes to the locus 11q25 and subsequently demonstrated LOH of 49% at D11S4085 (Sellar, G.C. et al. 2003). This lay within the second intron of the gene for opioid-binding cell adhesion molecule-like protein (OPCML or OBCAM). Quantitative RT-PCR demonstrated that OPCML expression was abrogated in a panel of primary ovarian tumours and cancer cell lines but not in normal whole ovary. As previously discussed use of whole ovary as a normal comparator could be criticised in this study as the authors imply that they believe the OSE to be the source of ovarian cancer, yet the OSE contribution to the whole ovary is minimal. Using methylation-specific PCR, Sellar et al went on to demonstrate that OPCML was unmethylated in normal ovaries (although they did not examine OSE specifically), but methylated in 83% of primary ovarian cancers and 82% of ovarian and non-ovarian cell lines. Furthermore when transfected into the ovarian cancer cell line SKOV-3, OPCML caused significant suppression of tumour growth both in vitro and in vivo following inoculation of nude mice. This was the first description of OPCML as a candidate tumour suppressor in EOC, however soon after its publication a similar paper provided evidence that a related family member limbic system associated membrane protein (LSAMP) acted as a TSG in clear cell renal cell carcinoma (Chen, J. et al. 2003).

Following on from this work Ntougkos examined the expression of OPCML and its related family members in primary ovarian cancer tissue, looking for correlation between tumour phenotype and clinical outcomes (Ntougkos, E. et al. 2005). OPCML is a member of the IgLON family of immunoglobulin (Ig) domain containing superfamily comprising Limbic System Associated membrane Protein...
(LSAMP), OPCML, Neurotrimin (HNT) and Neuronal Growth Regulator 1 (NEGR1) also known as Kindred of LON (Kilon). Ntougkos and co-workers demonstrated that OPCML, LSAMP and neuronal growth regulator-1 (NEGR1) expression was reduced in samples of ovarian cancer compared to normal ovary. In addition it appeared that LSAMP expression was an indicator of poor prognosis and negatively associated with survival.

Prior to these studies, interest in the IgLONs was primarily focused on neurological research, particularly in chick brain development. OPCML or OBCAM was first purified from bovine brain in 1986 and subsequent cDNA analysis revealed an amino acid sequence of 345aa (Cho, T.M. et al. 1986). As a consequence of its three immunoglobulin domain-like structures and homology with neural cell adhesion molecule (NCAM) it was named OBCAM. Initial studies hinted that the protein might have a role in opioid binding given that the purified protein showed stereospecific opioid binding, (Cho, T.M. et al. 1986) and monoclonal antibodies to OBCAM and anti-sense cDNA inhibited $^3$H-diprenorphine binding (Ann, D.K. et al. 1992; Roy, S. et al. 1988). Subsequent cloning of the human form of OBCAM revealed a complete open reading frame of 1038bp with 93% homology to the rat cDNA and 98% homology with the rat protein, with similar values for the bovine form. In addition, OBCAM was mapped to chromosome 11 (Shark, K.B. et al. 1995). Using monoclonal antibodies to OBCAM, Hachisuka et al localised the expression of OBCAM across five different species. They also suggested that OBCAM was a glycosyl phosphatidylinositol (GPI)-anchored membrane protein, possibly sited within lipid rafts, as it could be released from the cell membrane by treatment with phosphotidylinositol specific phospholipase C, and not a G protein coupled receptor like the main group of opioid receptors (Hachisuka, A. et al. 1996). They suggested the weight of the protein was 34kDa but following differential glycosylation two isoforms existed, with molecular weights of 51 and 58 kDa.

Following the discovery of OBCAM it became clear that another similar protein existed in primary rat neurones and a further member of the family, called
neurotrimin after its three Ig-like domains, was described in 1995 (Struyk, A.F. et al. 1995). At the same time a protein distributed in the rat limbic system, LSAMP (limbic system associated protein) also appeared to be homologous with OBCAM and neurotrimin (Pimenta, A.F. et al. 1995). A subsequent search for this in the human confirmed that it had 99% homology with the rat protein and that the degree of conservation indicated an important role (Pimenta, A.F. et al. 1996). Due to the high degree of homology between LSAMP, OBCAM and neurotrimin the authors suggested that these proteins were a novel family of proteins and collectively designated them IgLONs. The characteristics of the IgLON proteins can be seen in Table 1.3.
<table>
<thead>
<tr>
<th></th>
<th>OPCML</th>
<th>Neurotrimin/CEPU-1</th>
<th>LSAMP</th>
<th>NEGR1/Kilon</th>
</tr>
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<tbody>
<tr>
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<td>(Homo sapiens)</td>
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<tr>
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</tr>
<tr>
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<td>Human, rat, chick</td>
<td>Human, rat, cow chicken</td>
<td>Human, rat, mouse, chick</td>
</tr>
<tr>
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<td>Thalamus, hippocampus, pontine nucleus, cerebellum, olfactory bulb, retina, dorsal root ganglion (rat)</td>
<td>Limbic system (rat)</td>
<td>Cerebral cortex, hippocampus, diencephalon, skull, intervertebral discs, intestine, ribs (rat)</td>
</tr>
</tbody>
</table>

Table 1.4 Characteristics of IgLON genes and gene products (from NCBI databases and selected references).
A 55kDa protein, GP55, was discovered in chick brain and is likely to be the chick equivalent of OBCAM (Wilson, D.J. et al. 1996) while CEPU-1, also discovered in the chick seems to be a likely candidate for the chick form of neurotrimin (Spaltmann, F. et al. 1996). While a 50kDa protein, AvGp50 was also deemed to be a member of the IgLON family (Hancox, K.A. et al. 1997). A further member of the family was added in 1999 when a Japanese group discovered, Kilon, a 46kDa protein with many similarities to OBCAM (Funatsu, N. et al. 1999).

From much of the work in neurological development it appears that the IgLONs have an important role in neuronal development and can influence cell proliferation, migration and differentiation (Brummendorf, T. et al. 2001). Knowledge of how the IgLONs interacted with each other was scant until Reed et al demonstrated the binding preferences of the IgLONs in cerebellar granule cells (Reed, J. et al. 2004). In a series of experiments examining both homo and heterodimers formation it became apparent that LSAMP/OBCAM had the highest binding affinity and LSAMP/LSAMP dimers, the lowest. It appeared that most of the IgLONs preferentially bound as heterodimers and that homodimerization was weak and uncommon except in the case of CEPU-1 (Neurotrimin). The frequency of dimerization led the authors to suggest that DIgLON (dimeric IgLON) would be an appropriate term to use for the combined molecule.

While the considerable majority of work on IgLON biology has been undertaken in the field of neurological sciences, the question that arises from their association with cancer is; what role do the IgLONs play in normal tissue? While some scepticism might arise for any ovarian role for a group of molecules acting predominantly in the central nervous system, the finding of functional neuropeptides in the ovary is not without precedence (Anderson, R.A. et al. 2002; Dees, W.L. et al. 2006; Spears, N. et al. 2003). A further site in the genital tract where OPCML is found is Fallopian tube (Dr G Sellar, personal communication). The highly complex signalling that occurs when the fimbrial end of the Fallopian tube locates the ovulatory stigma at
ovulation could imply a potential role for IgLON interaction in this process. Furthermore given recent evidence for the Fallopian tube as a potential source of peritoneal carcinomatosis (Crum, C.P. et al. 2007; Kindelberger, D.W. et al. 2007; Lee, Y. et al. 2007) coupled with evidence of TSG function of OPCML and LSAMP, further research into the role of these proteins in reproductive tissues is warranted.

1.9 Project rationale

Epithelial ovarian cancer (EOC) carries a high mortality and little has been achieved to improve the prognosis of women affected by the disease in the past three decades. It provides the biggest challenge of all the gynaecological malignancies where, unlike many other epithelial cancers, the natural history of EOC remains a mystery. The overarching aims of this thesis are to explore the ovarian cancer phenotype and inflammation associated responses of normal tissue.

1.9.1 An investigation of glucocorticoid metabolism in primary ovarian cancer.

There are consistent epidemiological data showing that an increase in the number of ovulations in a woman’s lifetime increases her risk of developing epithelial ovarian cancer. Combining this with evidence that ovulation is an inflammatory process causing repetitive cyclical damage to the ovarian surface epithelium (OSE, the putative source of ovarian cancer) gives credence to the hypothesis that inflammation-associated injury of the OSE is a causal factor in the development of EOC.

Following on from extensive previous research demonstrating the role of 11βHSD1 as an anti-inflammatory mediator in the OSE, it was hypothesised that loss of 11βHSD1 was an important step in the development of EOC. In order to investigate
this, a system for primary culture of EOC was established thus allowing comparison of inflammatory signalling in these cultures with primary cultures of normal human OSE.

1.9.2 Anti-inflammatory signalling in the peritoneal surface epithelium (PSE).

The OSE and PSE share similar embryological origins and are morphologically similar. Their carcinomatous counterparts epithelial ovarian cancer and primary peritoneal cancer are essentially indistinguishable. It was hypothesised that the OSE and PSE would share the ability to increase local cortisol production via upregulation of 11βHSD1. In order to test this, a method for primary culture of human PSE was established, creating cell culture based systems for investigating the effects of the inflammatory cytokine interleukin-1α on 11βHSD1 and inflammation associated genes.

1.9.3 The effect of inflammation on expression of OPCML and the IgLON family in primary cultures of human ovarian surface epithelium.

The OSE is the most likely candidate tissue for the source of EOC. The mechanism by which this transformation occurs is not understood, however one potential route is through the inactivation of a tumour suppressor gene (TSG). OPCML is a candidate TSG lying within 11q25, a chromosomal region with a high rate of loss of heterozygosity in EOC. In light of the OSE being the likely source of most EOCs it was important to investigate the expression of OPCML in primary cultures of human OSE to test the hypothesis that OPCML is switched off by inflammation, thus allowing the OSE to proliferate and aid wound healing at the site of ovulation.
Chapter 2

Materials & Methods
2 Materials and Methods

2.1 Primary Cell Culture

2.1.1 Cancer Tissue Collection and Culture

Patients attending for initial laparotomy for suspected ovarian cancer were approached pre-operatively and asked to donate tissue for research in accordance with good clinical practice guidelines. The study was approved by the local ethics research committee (COREC reference S1103/44, Chief Investigator Dr K S Fegan) and all patients taking part provided written evidence of informed consent. Copies of patient information sheets and consent forms are detailed in Appendix I. Relevant clinico-pathological information is shown in table 3.3.

At initial laparotomy ascitic fluid was collected in a 2 litre sterile vacuum container and transferred to the laboratory at room temperature. Where possible the sample collected was the initial aspirate in order to reduce contamination by blood. During surgery the excised specimens were placed in sterile polythene bags and transferred to the pathology laboratory for specimen dissection by a consultant pathologist. This step was important firstly to ensure that the sample was truly representative of tumour tissue and secondly to prevent interference with the final pathology result. The obtained samples were placed immediately in a sterile universal container containing 20ml PBS (Gibco, Paisley, Scotland, UK) and placed on ice for transfer to the laboratory.

In a Class 2 tissue culture hood in the laboratory, 10 to 15mls of ascites was mixed with an equal volume of HOSE1 (table 2.2) and placed in 75cm² tissue culture flasks (Corning BV Life Sciences, The Netherlands), using a modification of Dunfield’s published method (Dunfield, L.D. et al. 2002). Media were changed twice weekly until confluent.

Primary cultures from solid ovarian cancer tissue were treated using a modification of the method described by Wilson (Wilson, A.P. 2004). In the aseptic environment of a Class 2 tissue culture hood, tumour was placed in a 5cm diameter Petri dish and divided in two. One piece was placed in 4% normal buffered formalin (NBF) for
later histological use and the second piece then minced using two sterile scalpels. The minced tumour was then transferred to a 50ml Falcon tube (Corning) containing 0.25% trypsin (Gibco), 0.004% DNaseI (Sigma), in 20ml cold HOSE2 medium (serum-free to prevent denaturing of enzyme activity), known as “digestion mix”. The flask was left mixing overnight at 4°C. The following morning the solid components were allowed to settle and the supernatant aspirated, mixed with HOSE1 to inactivate the trypsin, centrifuged at 500g for 5minutes and the subsequent pellet resuspended in 15ml HOSE1 medium and placed in a 75cm² tissue culture flask, the sample labelled as IW (initial washings). The remaining minced tumour was mixed with a further 20ml aliquot of digestion mix and placed in an agitated water bath at 37°C for 30minutes. Following this, the supernatant fluid was aspirated, inactivated with equivalent volume HOSE1 and centrifuged as above to form a pellet. The cellular pellet was then resuspended in 15ml and transferred to a 75cm² tissue culture flask. The tumour mince was washed and pelleted a further two times so that a total of four flasks was obtained from every piece of solid tumour. Flasks were left undisturbed for one week in a humidified atmosphere of 5%CO₂, 95% air at 37°C to allow cells to attach. If no cells were attached by this time the culture flask was discarded. Successful cultures then had HOSE1 media changes twice weekly until confluency was reached.

2.1.1.1 Methods Used to Identify a Malignant Cell Culture

Ascitic fluid and solid tumour specimens consist of a variety of cells including mesothelial cells, fibroblasts and immune blood cells. Ascites and solid tumour was examined independently by a consultant histopathologist and the diagnostic pathology result was known in all cases. Thus malignant primary cultures were confirmed as originating from ascites containing malignant cells. Attempts were also made to confirm the malignant nature of (assess the relative tumour cell component in) each culture. These methods included:
1. Monolayer Morphology

Tissue culture flasks were inspected daily and epithelioid colonies were identified. Flasks containing heavy contamination fibroblasts-looking cells were not used for final analysis. Cell and colony characteristics were assessed and features such as disorganised colony formation, large nuclear:cytoplasmic ratio, spheroid formation and absence of fibroblast-looking cells were used to intimate relative quantities of tumour cells in the primary culture.

An example of such a monolayer is shown in figure 2.1.

Figure 2.1. Photomicrograph of uniform colonies of borderline malignant cells from solid tissue (magnification x4).

2. Cytokeratin Immunohistochemistry

Tissue culture flasks containing cell monolayers were washed in 10ml PBS and disaggregated using trypsin/EDTA (0.05% w/v, Invitrogen) at 37°C for 5 minutes. Cells were collected by centrifugation and the pellet washed and resuspended in fresh HOSE 1 medium. Cell number and viability were assessed by Trypan blue staining (Sigma) and counted by haemocytometer. Cells were transferred to individual wells of 8-well chamber slides (VWR International) in 200-500µl volumes
of HOSE-1 medium (40 000 – 200 000 cells) and incubated overnight at 37°C in a humidified incubator. The following day the medium was aspirated and the chambers washed with PBS. The cells were then fixed in cold methanol at -20°C for 10 minutes. After washing in PBS, the chambers were pre-treated with Normal Goat Serum solution consisting of Goat serum (20%v/v) and bovine serum albumin (0.5% w/v) in PBS, at room temperature for 30 minutes. The primary antibody was then added and incubated overnight at 4°C. Primary antibodies were anti-human cytokeratins 5, 6, 8, 7,19 (M0821, Dako, Denmark) and anti-human cytokeratin 7 (M7018, Dako, Denmark). A mouse IgG was used as a negative control. The next day, the secondary biotinylated antibody (goat anti-mouse, Dako) was applied for 30 minutes, followed by an avidin-biotin complex conjugated to horseradish peroxidase for 30 minutes. Staining was achieved using DAB chromogen system (DakoCytomation).

3. CA125 Measurement

By gradually increasing the number of cells and length of time of exposure of medium, CA125 levels could be measured in the primary cultures in 10ml of HOSE1 medium left in contact with a confluent flask monolayer for one week. CA125 was analysed by magnetic separation chemilluminescence on the Siemens Advia Centaur Immunoassay System (Siemens Healthcare Diagnostics, Deerfield, USA). While CA125 was detectable and raised in confluent flasks containing EOC primary cultures, it became clear that CA125 was also measurable in some flasks of OSE (see table 3.2). Indeed on review of the literature most tissues of the female genital tract are capable of secreting CA125, although interestingly it appears absent from the ovarian surface epithelium in one study (Kabawat, S.E. et al. 1983).
Table 2.1  CA125 level in primary cultures of EOC, OSE and HOSE1 culture medium.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>CA125 Serum Level (IU/L)</th>
<th>Source of Culture</th>
<th>CA125 Level in Medium (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 2</td>
<td>4990</td>
<td>Ascites</td>
<td>131.7</td>
</tr>
<tr>
<td>Patient 4</td>
<td>2647</td>
<td>Ascites</td>
<td>184</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tumour</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metastasis</td>
<td>41</td>
</tr>
<tr>
<td>Patient 7</td>
<td>90</td>
<td>Washings</td>
<td>&lt;15</td>
</tr>
<tr>
<td>Patient 6</td>
<td>108</td>
<td>Ascites</td>
<td>118.3</td>
</tr>
<tr>
<td>Patient 8</td>
<td>4264</td>
<td>Ascites</td>
<td>2123.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metastasis</td>
<td>118.2</td>
</tr>
<tr>
<td></td>
<td>HOSE1 Medium</td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Human OSE</td>
<td></td>
<td>140.6</td>
</tr>
</tbody>
</table>

4. Matrigel Invasion Assay

Two experiments were carried out to investigate differences in invasive properties between the invasive cancer cell line SKOV-3 (passage 50), primary cultures of HOSE cells and two separate primary cultures of ovarian serous adenocarcinoma through a Matrigel basement membrane (BD Matrigel Invasion Chambers, BD Biosciences) using non-matrigel coated membranes as controls (BIOCOAT Cell culture Control inserts, Becton Dickinson). Experiments were carried out with the help of Dr Mick Rae. Cell suspensions were prepared at 100 000 cells/ml in serum-free HOSE-2 medium. The chambers were filled with 50 000 cells with and without test reagent and the wells with the chemoattractant serum containing HOSE-1 medium. The plates were incubated for 22 hours in a humidified incubator at 37°C, 5% CO₂, 95% air. Non-invading cells were scrubbed from the membrane using a cotton wool bud. The cells were then fixed in ice-cold methanol for two minutes then stained with haematoxylin. The membranes were air-dried and then mounted in immersion oil on a glass slide. Invading cells were counted under the microscope in six areas across each membrane. As several membranes had uneven distribution of
cells i.e. denser distribution at the periphery, the median of the six counts was used for data analysis rather than the mean. The percent invasion was determined by:

\[
\% \text{ Invasion} = \frac{\text{Median number of cells invading through Matrigel membrane}}{\text{Median number of cells migrating through control membrane}}
\]

Invasion Index was determined by:

\[
\text{Invasion Index} = \frac{\% \text{ Invasion of Test Cell}}{\% \text{ Invasion of Control Cell}}
\]

### 2.1.2 OSE Collection

Normal OSE was collected from patients undergoing surgery for benign gynaecological conditions with local ethics committee approval (COREC reference S1103/36, Chief Investigator Dr K S Fegan) and informed consent (patient information sheet and consent forms are included in Appendix I). Patients were pre-menstrual and over 16 years of age. Exclusion criteria included intercurrent malignancy, gynaecological inflammatory states such as current pelvic inflammatory disease or endometriosis and use of gonadotrophin-releasing hormone agonists or antagonists. While the use of hormonal contraception was not an exclusion criterion, knowledge of exogenous hormone use was documented in patient data files. All samples were allocated a research number and were then anonymised but linked. Following recruitment, OSE was collected by gently brushing the ovarian cortex early in the surgical procedure using a sterile Aylesbury spatula at laparotomy or a Tao cytobrush (Cook, Ireland) at laparoscopy. During surgery handling of the ovaries was minimised by the surgeon to prevent loss of the OSE layer. The collecting instrument was rinsed in 15ml pre-warmed HOSE1 medium (see table 2.2).
to dislodge the cells and then transferred to the laboratory at room temperature. The medium was then aspirated and transferred to a 75cm² tissue culture flask (Corning BV Life Sciences, Schipol-Rijk, The Netherlands) pre-coated with donor calf serum. Flasks were inspected using phase-contrast microscopy to confirm presence of OSE “flakes”, then incubated for up to 42 days in a humidified atmosphere of 5%CO₂, 95% air at 37°C. The flasks were inspected daily and HOSE1 medium was changed weekly until confluency reached.

<table>
<thead>
<tr>
<th>Additive</th>
<th>Quantity</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium 199:MCDB 105</td>
<td>1:1 (v/v)</td>
<td>Gibco, Paisley</td>
</tr>
<tr>
<td>Fetal Calf Serum (heat inactivated)</td>
<td>15% (v/v)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>1mmol/l</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Penicillin</td>
<td>50IU/ml</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>50μg/ml</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>

Table 2.2 Components of HOSE1 Medium. pH was established at 7.30 by titrating with 10M sodium hydroxide.
### Additive Quantity Supplier

<table>
<thead>
<tr>
<th>Additive</th>
<th>Quantity</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium 199:MCDB 105</td>
<td>1:1 (v/v)</td>
<td>Gibco, Paisley</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>0.01% (w/v)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>1mmol/l</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Penicillin</td>
<td>50IU/ml</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>50μg/ml</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>

**Table 2.3 Composition of HOSE2 medium, which was similar to HOSE1 medium but the Fetal Calf Serum was replaced with a solution of Bovine Serum Albumin to achieve similar oncotic pressure. pH was established at 7.30 by titrating with 10M sodium hydroxide.**

### 2.1.3 PSE Collection

Samples of PSE were collected in a similar manner to that previously described for OSE by gentle sweeping of the peritoneal surface using either a cytobrush (Cook, UK) or sterile Aylesbury spatula. Care was taken not to apply significant pressure at sampling to ensure firstly that the underlying tissue was not traumatised and secondly that only surface epithelial cells were collected and not stromal cells which could lead to contamination of the culture. Two sites for sampling were chosen: the anterior abdominal wall (designated ‘A’ cells) and the body of the uterus (designated ‘B’ cells). The reasoning for choosing two separate sites was firstly to investigate whether there was any variation in cell phenotype between visceral and parietal peritoneal cells and secondly whether cells covering a Mullerian tract derived organ (the uterus) differed from peritoneal cells with no contact with any Mullerian structures.
2.1.4 A Comparison of Outcomes of OSE and PSE Cultures

Qualitative inspection of the PSE flasks prior to incubation suggested that the number of cells collected from the anterior abdominal wall was markedly less than that collected from the body of the uterus. In order to investigate whether this affected cell culture growth, the outcomes of PSE cultures (n=121) over the study period were audited and compared to outcomes for OSE cells (n=193) over the same time period. Flasks were considered to be ‘used’ if they were involved in any experimentation, passaging, or freezing. Use was not limited to confluent flasks. The indications for discarding flasks were not specifically examined, however possible reasons include infection, inadequate growth and stromal contamination.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Used</th>
<th>Discarded</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSE Left</td>
<td>57</td>
<td>39</td>
<td>96</td>
</tr>
<tr>
<td>OSE Right</td>
<td>55</td>
<td>42</td>
<td>97</td>
</tr>
<tr>
<td>OSE Total</td>
<td>112</td>
<td>81</td>
<td>193</td>
</tr>
<tr>
<td>PSE ‘A’</td>
<td>19</td>
<td>42</td>
<td>61</td>
</tr>
<tr>
<td>PSE ‘B’</td>
<td>31</td>
<td>29</td>
<td>60</td>
</tr>
<tr>
<td>PSE Total</td>
<td>50</td>
<td>71</td>
<td>121</td>
</tr>
</tbody>
</table>

Table 2.4 Outcomes of primary cell culture according to cell type and site of sampling.

These data are shown in graph format to highlight the rate of attrition in these primary cultures (figure 2.4).
Figure 2.2. Outcomes of PSE Primary Cultures.

The high discard rate of the PSE ‘A’ samples of 69% is likely to reflect the low number of cells initially collected. A two-sided Fisher’s exact test returned a P value of 0.027 suggesting that the difference between the A samples versus B samples is unlikely to be due to chance alone, all other things being equal.

As PSE samples were always collected at the same time and from the same patients as OSE samples it is reasonable to ask whether there is a difference in outcome between OSE and PSE. As it has been established that the PSE A samples have poor growth outcomes compared to the B samples the outcomes of OSE and PSE B samples were compared. Using Fisher’s exact test the P value does not reach significance demonstrating no difference in successful outcome between OSE and PSE.
2.1.5 PSE Morphology

On the day of collection, flakes of PSE a single cell thick could be clearly seen suspended in the culture medium (figure 2.5). These were morphologically indistinct from flakes of OSE. Furthermore following *in vitro* incubation these flakes settled to form epithelial colonies which quickly expanded to cover the base of the flask in a confluent monolayer that also demonstrated the classic cobblestone appearance of the OSE in culture.

Figure 2.3. Morphological similarities between the ovarian surface epithelium and the peritoneal surface epithelium on day of collection (magnification x20) and following incubation (magnification x4).
In addition to these morphological similarities PSE cells stained positive for low-molecular weight cytokeratins, as is documented for OSE (figure 2.6).

Figure 2.4. Chamber slide immunohistochemistry localizing cytokeratin in PSE cells (magnification x40). Inset demonstrates matched IgG negative control. Bars represent 20µm.
2.1.6 Experimental treatment of OSE, PSE and EOC

Once OSE monolayers had become confluent the flasks were rinsed twice with 10ml phosphate buffered saline (PBS) (Gibco, Paisley) then incubated with 5-10ml trypsin/EDTA (0.05%, w/v) (Invitrogen) for 5 minutes at 37°C until cells had detached from the flask base. The cell suspension was then aspirated, mixed with the same volume of warmed HOSE1 to inactivate the trypsin, and sedimented by centrifugation at 800 g for 5 minutes. The cell pellet was resuspended in 2ml of HOSE1 medium and cell number and viability assessed by Trypan Blue (Sigma-Aldrich) and counted with a haemocytometer. The cells were then diluted in HOSE1 medium to a density of 100,000 – 125,000 cells/ml. Four to five mls of this suspension was used to seed each well of a six-well plate (Corning) and the flasks returned to the 37°C incubator overnight. The plates were inspected the following morning to confirm cell attachment and the medium changed to serum-free culture medium (HOSE2), containing 0.01% bovine serum albumin (BSA) (Sigma-Aldrich). Cells were kept in this serum-free environment for a further 24 hours.

Experimental treatments were made up fresh using HOSE2 as the diluting medium. Treatments included interleukin-1α (IL-1α) (0.5ng/ml, unless otherwise stated, R&D Systems Europe Ltd, Abingdon, Oxon), cortisol (1μM) and progesterone (1μM), and a combination of IL-1α and each steroid. Steroid treatments were made from stock solutions of 100μM in ethanol, so control wells received HOSE2 medium containing ethanol at a similar concentration (1% v/v). Concentrations of steroid were derived from previous published data of follicular steroid concentrations, as the experimental milieu was attempting to represent the peri-ovulatory environment (Andersen, 1991; Andersen, 1994). Four ml of treatment solution was added per well and left at 37°C for 48 hours unless otherwise stated. At the end of the experiment the wells were inspected to assess morphological changes due to treatment. The medium was then aspirated and stored and the cells treated as described below. For RNA extraction cells in 6-well plates were lysed with 350μl of lysis buffer made from: RLT (Qiagen RNeasy kit) /β-mercaptopturine (Sigma, UK) (100:1 v/v). Commonly the experiments
took place over a working week, with the cell cultures being set up on day one, the medium changed to HOSE 2 on day 2, the treatments added on day three and then left for 48 hours until day 5, when the samples were placed in RNA lysis buffer, homogenized and frozen at -180°C prior mRNA isolation at the beginning of the next week (see below).

2.2 mRNA Isolation and Quantification

2.2.1 mRNA Isolation

Total RNA was extracted and DNaseI treated using RNeasy minispin columns (Qiagen, Santa Clara, CA) as per manufacturer’s instructions. Once the lysed samples were thawed, there followed a series of ethanol washes, on column DNaseI treatment and final elution in 30μl RNAse free water. One μl aliquots were taken for quantification and quality assessment using the Agilent 2100 Bioanalyzer System for total RNA in combination with RNA6000nano chips (Agilent technologies, Cheshire, UK). Only samples attaining RNA Integrity Numbers (RIN) >9 were used for quantitative RT-PCR.

2.2.2 Rotorgene Light Cycler Quantitative RT-PCR

Quantitative RT-PCR was undertaken with the Rotorgene thermal cycler (Corbett, Australia). The total reaction volume of 15μl constituted 40ng of DNaseI-treated total RNA, 0.3μM of forward and reverse primer and the Quantitect SYBR Green one step RT-PCR kit (Qiagen, UK), as per the manufacturer’s instructions. Primer sequences for genes of interest are given in table 2.5. Primers were intron spanning and designed using Primer3 v0.2 software by Dr E Ntgoukos. Reactions were carried out in triplicate for standard curve samples, quadruplicate for experimental samples and for no template controls and duplicate for no reverse transcriptase controls. Fluorescence was detected using the FAM channel and analysis performed using Rotorgene v5 software. Quantification of RNA was calculated by extrapolating from the standard curve and comparison to β-actin levels within the same sample. Cycling conditions were as follows: 30 minutes at 50°C for reverse
transcription, 15 minutes at 95°C for activation of polymerase, then 40 cycles of 15 seconds at 94°C for denaturation, 30 seconds at 54°C for annealing and 30 seconds at 72°C for extension. After final extension at 72°C for 1 minute, the products were melted by 1°C increments every 5 seconds to a final temperature of 99°C.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPCML Forward</td>
<td>TACCATAGATGACCGGGTAA</td>
</tr>
<tr>
<td>OPCML Reverse</td>
<td>CTATTAGGTGAACCGGGGAC</td>
</tr>
<tr>
<td>β-Actin Forward</td>
<td>CTACGTCGCCCTGGACTTCGAGC</td>
</tr>
<tr>
<td>β-Actin Reverse</td>
<td>GATGGAGCCGCGATCCACACGG</td>
</tr>
</tbody>
</table>

Table 2.5 Primer sequences used in Light cycler RT-PCR.

2.2.3 Taqman Quantitative RT-PCR

DNaseI treated total RNA (200ng) was reverse transcribed (random hexamer kit; Applied Biosciences) and 2μl of cDNA added to a total assay volume of 25μl containing commercially available primers and Taqman hybridisation probes (see Table 2.6). All primers and probes had been previously validated. For the IgLON analyses pre-validated primer/probe mixes were purchased directly from Applied Biosystems (Assays-on-demand, Taqman Gene Expression Assays) and primer sequences were not made available by the company. Sample reactions were set up in triplicate. Controls included no template (water) controls for both cDNA synthesis and Taqman assays, as well as negative controls for cDNA synthesis where reverse
transcriptase was omitted from the reaction. Each experimental plate also contained a standard RNA control, depending on the gene of interest. These RNAs included Liver (11βHSD1, COX2, IL1R), placenta (11βHSD2, GRα) and Fallopian tube (OPCML, NEGR1, LSAMP, HNT). Fluorescence was detected by the Taqman RT-PCR system (ABI PRISM 7900 Sequence Detection System; Applied Biosystems, UK). The mRNA of interest was quantified in relation to the abundance of 18S rRNA in each sample using the delta-delta \( \Delta \Delta C_T \) method.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSD11B1</td>
<td>NM 181755</td>
<td>AAGATGTTCTGCATGGATTTC</td>
<td>AGCTCTGCGCCAAGAGAAGT</td>
</tr>
<tr>
<td>HSD11B2</td>
<td>NM 000196</td>
<td>GGCAGGTTCCTGCCAGTGCAGT</td>
<td>GTTGTGCTCAGGAGGTGTTC</td>
</tr>
<tr>
<td>PTGS2</td>
<td>(COX2)</td>
<td>CCTTCCTCCTGCTGTAGTG</td>
<td>ACAATCTCATTTGATCUAGC</td>
</tr>
<tr>
<td>IL1R1</td>
<td>NM 000877</td>
<td>TGTCACCGCAGTTGAGTCAT</td>
<td>GCACGTGGGTCATCTCATCATA</td>
</tr>
<tr>
<td>NR3C1</td>
<td>(GRα)</td>
<td>GCGATGGCTCAGAAAACAAAC</td>
<td>GCAGAGGATAACTTCTGCTAATC</td>
</tr>
</tbody>
</table>

Table 2.6 Primer sequences for genes of interest used in Taqman quantitative RT-PCR.

### 2.2.4 OPCML Western blotting

Total protein concentration was established by Bradford assay. Twenty \( \mu g \) of protein was mixed with 3X sample buffer and heated at 95°C for 5 minutes prior to separation on a 10% acrylamide gel under standard conditions. Electroblotting was carried out in Tris, glycine and 10% SDS. The membrane was then pre-treated with 0.5% blocking agent in tris-buffered saline with Tween 20 (TBS-T) for one hour then
incubated with the OPCML primary antibody (custom-made IgY polyclonal antibody, raised in chick against an OPCML peptide sequence, Biosource) at 1:2000 dilution overnight at 4°C. The membrane was then incubated with a secondary antibody (goat anti-chick, Abcam) conjugated to horseradish peroxidase for one hour and finally in luminol substrate (Roche) for one minute. Chemiluminescence was detected by X-ray film (Amershams). β-Actin was detected using a commercially available antibody (1:50 000, Calbiochem) with a goat anti-mouse secondary antibody (1:4000, Calbiochem).

2.3 11-Oxoreductase Assay

11-oxoreductase activity was assessed by quantification of conversion of [1,2,6,7-^3^H]cortisone to [1,2,6,7-^3^H]cortisol, as described previously (Yong, P.Y. et al. 2002). Primary cell culture was carried out as described in section 2.1.6. When a culture was ready for use the medium was aspirated and the monolayer disaggregated with trypsin/EDTA as previously described. Following quantification of cells with the haemocytometer, the cells were seeded into 12-well plates (Corning) at a density of 2x10^5 cells/0.5 ml HOSE1 medium. The plates were incubated at 37°C in a humidified atmosphere of 5%CO₂, 95% air overnight. The following morning the culture medium was changed to serum-free HOSE2 medium and incubated for a further 24 hours. Treatment with IL-1α (0.5ng/ml) in HOSE2, or HOSE2 medium alone as control was then added and incubated for 48 hours, as induction of 11βHSD1 has been shown to be maximal at this time (Rae, M.T. et al. 2004). The media were then aspirated and replaced with 0.5ml of HOSE2 containing [1,2,6,7-^3^H]cortisone for a specific activity of 2.5x10^5 Bq per well, and 50pmol cortisol as carrier steroid (total substrate concentration 0.1μM) for eight hours. Tritiated cortisol was prepared by Dr AF Howie as follows. Five MBq of [1,2,6,7-^3^H]cortisol (Perkin Elmer, Cambridge, UK) were dried down under nitrogen and reconstituted in 100% ethanol (Fisher, UK). To this placental microsomes were added (gift from Dr T Bramley) and 100mmol/l potassium phosphate (pH7.3) solution with 10mmol/l
NAD. The solution was vortexed and left overnight at 37°C. The following morning the steroid component was extracted using three diethylether washes. The organic solvent was then dried down under nitrogen and the residual steroids reconstituted in dichloromethane (Fisher, UK), spotted onto a silica-backed gel plate and underwent thin layer chromatography in a 92:8 chloroform:ethanol solvent (both Fisher, UK), with cortisol and cortisone markers. The site of cortisol migration was then identified and excised and dissolved in ethanol. After filtering, the radio-activity of the solution was measured, labelled and stored at -5°C for future use.

Following incubation, the media were collected and vortexed with 5ml dichloromethane (Fisher Scientific, Loughborough, Leicestershire, UK) to extract steroid. The aqueous phase was discarded and the organic phase evaporated to dryness under a nitrogen stream. The dry steroid extract was then reconstituted in a small volume of fresh dichloromethane and spotted onto silica gel precoated sheets (Sigma-Aldrich). These were then placed in chloroform:ethanol (92:8, v/v) and the steroid component separated by thin layer chromatography. The radio-labelled steroid was identified on the chromatogram and quantified using a Bioscan 200 imaging detector (Lablogic Systems, Sheffield, UK).

### 2.4 Immunohistochemistry (IHC)

#### 2.4.1 Chamber Slide IHC

Cells were obtained from 75cm flasks as already described (section 2.1.2). Following viability assessment and quantification cells were seeded into 8-well chamber slides (VWR International, Lutterworth, Leicestershire) at a density of 5-10x10⁴ cells/0.5ml medium and incubated at 37°C overnight. The medium was aspirated and wells rinsed with PBS then the cells were fixed with methanol. Following further triplicate PBS washes the cell monolayer was blocked with non-immune goat serum and then the primary antibody applied (see table 2.7). Negative controls were treated with same concentration non-immune mouse IgG. The wells were next washed with PBS+0.01% Tween 20 (3 washes for 3 minutes each) and the secondary antibody
applied. Further triplicate washes with PBS+ 0.01% Tween were undertaken before incubating with Vector ABC Elite reagents and visualisation with diaminobenzidine (DAB).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Molecular Weight Cytokeratins (5,6,8,17,19)</td>
<td>DAKO, Denmark</td>
<td>1:1000</td>
<td>4°C, overnight</td>
</tr>
<tr>
<td>Cytokeratin-7</td>
<td>DAKO, Denmark</td>
<td>1:1000</td>
<td>4°C, overnight</td>
</tr>
<tr>
<td>Progesterone receptor</td>
<td>DAKO, Denmark</td>
<td>1:50</td>
<td>1hr room temp</td>
</tr>
<tr>
<td>Glucocorticoid receptor α</td>
<td>Santa-Cruz, Germany</td>
<td>1:100</td>
<td>1hr room temp</td>
</tr>
</tbody>
</table>

Table 2.7 Details of Primary Antibodies used in chamber slide immunohistochemistry.

2.4.2 Tissue Immunohistochemistry

Portions of tissue from samples of ovarian and omental tumour to be used for primary cell culture were fixed in 4% neutral buffered formalin (NBF) overnight and soaked in ethanol (70% v/v) prior to paraffin blocking. Serial sections of 3-5μm thickness were transferred to glass slides and dewaxed, rehydrated and washed in PBS. Antigen retrieval was carried out by pressure cooking in citrate buffer at pH 6 for 5 minutes then 20 minutes at rest. Following avidin/biotin blocking (Vector Laboratories, Burlingame, California, USA) slides for 11βHSD1 and GRα were blocked with normal goat serum (NGS, Diagnostics Scotland, Edinburgh, UK) diluted in PBS with bovine serum albumin (BSA, Sigma-Aldrich, 5%w/v) and those for 11βHSD2 with normal rabbit serum/PBS/BSA (NRS, Diagnostics Scotland) for 30 minutes and 120 minutes respectively. Primary antibodies raised against
11βHSD1 (1:50, Cayman Chemical Co.), 11βHSD2 (1:200, in-house) and GRα (1:50, Santa Cruz, Germany) were added and incubated overnight at 4°C. Negative controls were treated with matched rabbit IgG (11βHSD1 and GRα, DAKO) and sheep IgG (11βHSD2, Santa Cruz). Sections were then washed twice in PBST for 5 minutes and secondary antibodies were applied (goat anti-rabbit, diluted 1:200 in NGS/PBS/BSA, DAKO, for 11βHSD1 and GRα; rabbit anti-sheep 1:200, DAKO for 11βHSD2). Fluorochromes streptavidin, Alexa fluor 488 (Molecular Probes, Invitrogen) (11βHSD2 and GRα) and tiramide Cy3 (Perkin Elmer, UK) (11βHSD1) were applied to sections for 60 minutes then washed twice in PBST. Nuclear counterstains propidium iodide (11βHSD2 and GRα) and sytox green (Invitrogen) were applied and sections washed in PBS then coverslips mounted with permafluor (Permafluor; Beckman Coulter, High Wycombe, UK). Images were captured using a LSM 510 Axiovert 100M confocal microscope (Carl Zeiss Ltd., Welwyn Garden City, UK).

2.5 Statistical Analyses

The choice of statistical tests is discussed in individual chapters. Statistical analyses were undertaken using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.
Chapter 3

Glucocorticoid Metabolism in Primary Cultures of Human Ovarian Surface Epithelium and Epithelial Ovarian Cancer
3 Glucocorticoid Metabolism in Primary Cultures of Human Ovarian Surface Epithelium and Epithelial Ovarian Cancer

3.1 Introduction

Inflammation has been implicated as a causative factor in many epithelial cancers (Coussens, L.M. et al. 2002)(table 3.1). Epithelial ovarian cancer is no exception and it has long been suggested that the “incessant” inflammation associated with ovulation may create an oncogenic environment (Fathalla, M.F. 1971). Although the local inflammatory processes that arise in the ovary at the time of ovulation have been elucidated, the mechanisms by which these could give rise to cancer are poorly understood. As described in Chapter 1, the ovarian surface epithelium (OSE) from which most ovarian cancers are thought to be derived, is able to generate an anti-inflammatory response to inflammation by up-regulating $11\beta$HSD1 and therefore increase local cortisol production. The hypothesis that arises is that it is the loss of the OSE’s inflammation-quelling ability through disruption of $11\beta$HSD1 expression that pre-disposes the tissue to malignant transformation.

Work with EOC cell lines has previously suggested that the $11\beta$HSD1 response to inflammation was lost in the malignant cells and that $11\beta$HSD2 levels were raised in cancer compared to OSE (Gubbay, O. et al. 2005). However these differences may have been due simply to the comparison of cell lines with primary human cells. Yet Gubbay’s findings corroborate Rabbitt’s hypothesis that increased cortisol production via raised $11\beta$HSD1 activity gives an “anti-proliferative, pro-differentiation stimulus”, while raised type 2 is “pro-proliferative” (Rabbitt, E.H. et al. 2003). Consequently there is a need to investigate the balance of the $11\beta$HSD isozymes in primary ovarian cancer tissue. Thus the initial focus of this work was to establish primary cell cultures from human ovarian cancers. Subsequently these cells
were then utilised to investigate the status of their responsivity to inflammation, with particular reference to 11βHSD isozyme expression.

<table>
<thead>
<tr>
<th>Pathological Condition</th>
<th>Associated Neoplasm</th>
<th>Aetiological Agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asbestosis, silicosis</td>
<td>Mesothelioma, Lung carcinoma</td>
<td>Asbestos, silica</td>
</tr>
<tr>
<td>Bronchitis</td>
<td>Lung carcinoma</td>
<td>Smoking</td>
</tr>
<tr>
<td>Cystitis</td>
<td>Bladder carcinoma</td>
<td>Urinary catheter</td>
</tr>
<tr>
<td>Gingivitis</td>
<td>Oral carcinoma</td>
<td></td>
</tr>
<tr>
<td>Inflammatory bowel disease,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crohn's disease, chronic ulcerative colitis</td>
<td>Colorectal carcinoma</td>
<td></td>
</tr>
<tr>
<td>Lichen sclerosis</td>
<td>Vulval carcinoma</td>
<td></td>
</tr>
<tr>
<td>Chronic Pancreatitis</td>
<td>Pancreatic carcinoma</td>
<td>Alcohol, trypsinogen gene mutation</td>
</tr>
<tr>
<td>Reflux oesophagitis, Barrett's oesophagus</td>
<td>Oesophageal carcinoma</td>
<td>Gastric secretions</td>
</tr>
<tr>
<td>Sialadenitis</td>
<td>Salivary gland carcinoma</td>
<td></td>
</tr>
<tr>
<td>Sjogren syndrome, Hashimoto's thyroiditis</td>
<td>MALT lymphoma</td>
<td></td>
</tr>
<tr>
<td>Skin erythema</td>
<td>Melanoma</td>
<td>Ultraviolet irradiation</td>
</tr>
</tbody>
</table>

**Table 3.1. Cancers with a probable inflammatory aetiology (Coussens, L.M. et al. 2002).**
Table 3.2 Clinico-pathological data from patients donating tissue.
<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age</th>
<th>Serum CA125 (IU/l)</th>
<th>Pathological Diagnosis</th>
<th>Histological Grade</th>
<th>FIGO Stage</th>
<th>Sample Collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>50</td>
<td>4990</td>
<td>Serous Adenocarcinoma</td>
<td>3</td>
<td>IV</td>
<td>Ascites</td>
</tr>
<tr>
<td>3</td>
<td>77</td>
<td>&lt;30</td>
<td>Ovarian fibrothecoma</td>
<td>N/A</td>
<td>N/A</td>
<td>Washings/OSE/Ovarian tumour/ Omentum</td>
</tr>
<tr>
<td>4</td>
<td>55</td>
<td>2647</td>
<td>Papillary Serous Carcinoma</td>
<td>3</td>
<td>IIIc</td>
<td>Ascites/Ovarian tumour/ Omental metastasis</td>
</tr>
<tr>
<td>6</td>
<td>56</td>
<td>108</td>
<td>Mixed serous and clear cell carcinoma</td>
<td>3</td>
<td>IIIc</td>
<td>Ascites/OSE/Ovarian tumour/ Omenttal metastasis</td>
</tr>
<tr>
<td>7</td>
<td>42</td>
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<td>Ovarian papillary carcinoma</td>
<td>2</td>
<td>IIib</td>
<td>Washings/metastatic tumour</td>
</tr>
<tr>
<td>8</td>
<td>69</td>
<td>426</td>
<td>Papillary Serous Carcinoma (and mixed melanotic tumour on left ovary)</td>
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<td>IIIc</td>
<td>Ascites/OSE/Ovarian tumour/metastatic tumour</td>
</tr>
<tr>
<td>10</td>
<td>79</td>
<td>544</td>
<td>Primary Peritoneal Papillary Serous Carcinoma</td>
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<td>IIIc</td>
<td>Ascites/OSE/metastatic tumour</td>
</tr>
<tr>
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<td>55</td>
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<td>Ovarian tumour</td>
</tr>
<tr>
<td>13</td>
<td>74</td>
<td>305</td>
<td>Mixed Serous and endometrioid. Poorly differentiated</td>
<td>3</td>
<td>IIIc</td>
<td>Ascites/metastatic tumour</td>
</tr>
<tr>
<td>14</td>
<td>61</td>
<td>21480</td>
<td>Papillary serous</td>
<td>3</td>
<td>IIIc</td>
<td>Ascites/Ovarian tumour/ Omental metastasis</td>
</tr>
<tr>
<td>15</td>
<td>67</td>
<td>206</td>
<td>Primary peritoneal serous adenocarcinoma</td>
<td>3</td>
<td>IIIc</td>
<td>Ascites/metastatic tumour</td>
</tr>
<tr>
<td>16</td>
<td>53</td>
<td>569</td>
<td>Poorly differentiated serous adenocarcinoma</td>
<td>3</td>
<td>IIIc</td>
<td>Ascites/Ovarian tumour/ Omental metastasis</td>
</tr>
<tr>
<td>17</td>
<td>61</td>
<td>2233</td>
<td>Endometrioid adenocarcinoma</td>
<td>1</td>
<td>Ic</td>
<td>Washings/Ovarian tumour</td>
</tr>
<tr>
<td>18</td>
<td>43</td>
<td>17912</td>
<td>Mixed adenocarcinoma</td>
<td>3</td>
<td>IIIc</td>
<td>Ascites/Ovarian tumour</td>
</tr>
<tr>
<td>19</td>
<td>65</td>
<td>154</td>
<td>Ovarian fibroma</td>
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<td>N/A</td>
<td>Ovarian tumour/omentum</td>
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<tr>
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<td>65</td>
<td>150</td>
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<td>N/A</td>
<td>Washings</td>
</tr>
<tr>
<td>21</td>
<td>55</td>
<td>42</td>
<td>Serous borderline tumour</td>
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<td>IIIA</td>
<td>Washings/Ovarian tumour</td>
</tr>
<tr>
<td>22</td>
<td>63</td>
<td>15</td>
<td>Cystadenofibroma</td>
<td>N/A</td>
<td>N/A</td>
<td>Washings/Ovarian tumour</td>
</tr>
<tr>
<td>23</td>
<td>41</td>
<td>46</td>
<td>Metastatic high grade breast carcinoma</td>
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<td>N/A</td>
<td>Ascites/Ovarian tumour</td>
</tr>
<tr>
<td>25</td>
<td>74</td>
<td>&lt;15</td>
<td>Serous cystadenofibroma</td>
<td>N/A</td>
<td>N/A</td>
<td>Washings/Ovarian tumour/ Omentum</td>
</tr>
<tr>
<td>26</td>
<td>75</td>
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<td>Mucinous cystadenoma</td>
<td>N/A</td>
<td>N/A</td>
<td>Ovarian tumour</td>
</tr>
<tr>
<td>27</td>
<td>71</td>
<td>&lt;15</td>
<td>Mature cystic teratoma</td>
<td>N/A</td>
<td>N/A</td>
<td>Ovarian tumour</td>
</tr>
<tr>
<td>28</td>
<td>59</td>
<td>1387</td>
<td>Poorly differentiated serous adenocarcinoma</td>
<td>3</td>
<td>IIIc</td>
<td>Ascites/Ovarian tumour/ Omental metastasis</td>
</tr>
<tr>
<td>29</td>
<td>67</td>
<td>353</td>
<td>Serous Adenocarcinoma</td>
<td>3</td>
<td>IIIc</td>
<td>Ascites/Ovarian tumour</td>
</tr>
<tr>
<td>30</td>
<td>59</td>
<td>3065</td>
<td>Poorly differentiated serous papillary adenocarcinoma</td>
<td>3</td>
<td>IV</td>
<td>Ascites/Ovarian tumour/ Omental metastasis</td>
</tr>
<tr>
<td>31</td>
<td>88</td>
<td>202</td>
<td>Mixed Serous and endometrioid carcinoma</td>
<td>2</td>
<td>IIib</td>
<td>Washings/Ovarian tumour</td>
</tr>
<tr>
<td>32</td>
<td>53</td>
<td>3270</td>
<td>Serous carcinoma</td>
<td>3</td>
<td>IV</td>
<td>Ascites/Ovarian tumour/ Omental metastasis</td>
</tr>
<tr>
<td>33</td>
<td>73</td>
<td>447</td>
<td>Serous carcinoma</td>
<td>3</td>
<td>IV</td>
<td>Ascites/Ovarian tumour/ Omental metastasis</td>
</tr>
<tr>
<td>35</td>
<td>66</td>
<td>3101</td>
<td>Endometrioid adenocarcinoma</td>
<td>3</td>
<td>IIIc</td>
<td>Ascites/Ovarian tumour/ Omental metastasis</td>
</tr>
</tbody>
</table>
3.2 Results

3.2.1 Effects of Interleukin-1α and Cortisol (F) on mRNA expression.

Having successfully established methods for primary culture of EOC, these cells were then used to compare the response to inflammation with primary ovarian surface epithelial cells.

3.2.1.1 Comparison of EOC and OSE Primary Cell Cultures

Using samples from 16 different EOC patients (n=26) (further details presented in table 3.3) and 11 different patients with no or benign pathology donating OSE, the effects of the pro-inflammatory cytokine interleukin-1α (IL-1α) and anti-inflammatory steroid cortisol (F) were examined (figure 3.2). Taking all experiments together, cortisol alone caused a significant up-regulation of 11βHSD1 in both OSE and EOC compared to untreated control samples (8.5-fold and 4.2-fold respectively, P<0.001). Treatment with 0.5ng/ml IL-1α induced an up-regulation of 11βHSD1 in both OSE and EOC (28.4-fold and 6.8-fold respectively, P<0.001). There was a significant difference in fold change after IL-1α between the two cell types with OSE giving the higher degree of induction (P<0.05). The combination of IL-1α and cortisol caused an up-regulation of 11βHSD1 in OSE and EOC greater than IL-1α alone (63.3 and 16.4-fold respectively, P<0.001). Again the fold change for OSE was significantly higher than for EOC (P<0.05).

There was no statistically significant change in 11βHSD2 mRNA expression compared to untreated cells after any treatment in EOC and OSE (figure 3.2b). As a consequence there was no significant difference in fold-change of 11βHSD2 between the two cell types.

The anti-inflammatory steroid cortisol had no effect on COX2 expression in either OSE or EOC (figure 3.1c). The pro-inflammatory cytokine IL-1α, however,
significantly up-regulated COX2 expression in both OSE and EOC (11.6-fold and 29.2-fold, P<0.001). There was no significant difference in magnitude of these cell types’ fold-change response to IL-1α. By combining IL-1α with cortisol the fold-change of COX2 expression was not significantly raised over control for OSE (2.5-fold) but was raised slightly for EOC (3.16-fold, P<0.01). There was a significant difference in COX2 mRNA expression for both OSE and EOC when comparing IL-1 treatment to the IL-1+F treatments (P<0.001).

Data (C_T values) were analysed using a repeated measures one-way ANOVA for each cell type, with Bonferroni post-test correction, having tested that the data were normally distributed and were effectively matched using an F test. As the experimental set-up involved the comparison of OSE and EOC cells on each occasion, the question was then asked whether there were significant differences in fold change for each treatment. These data were analysed using unpaired t-tests.
Figure 3.1. The 11βHSD1 response to inflammation is attenuated in EOC compared to OSE. 11βHSD1, 11βHSD2 and COX2 mRNA expression by quantitative RT-PCR comparing responses to 1μM cortisol (F) +/- 0.5ng/ml interleukin-1α (IL-1α) between OSE (n=11) and EOC primary cultures (n=27). Dissimilar superscripts indicate statistically significant
effects of treatment compared to control samples within cell type. NS = not significant. Asterisk indicates significant differences in fold change between cell types (*=P<0.05).

3.2.1.2 Analysis of OSE versus EOC from Ascites and Solid Tumour

Type 1 11βHSD mRNA levels were significantly increased by cortisol compared to control in primary cultures derived from both ascites (4.4-fold, P<0.01) and solid tumour (4.2-fold, P<0.01) (figure 3.2). There were no significant differences in fold change amongst these two cell types and OSE. IL-1α caused significant increases in 11βHSD1 in both ascites (8.6-fold, P<0.001) and solid tumour (4.5-fold, P<0.01) derived cells. Again there was no difference in fold change in 11βHSD1 to that seen with normal OSE. The combination of cortisol and IL-1α significantly raised 11βHSD1 mRNA levels in ascites (19.3-fold, P<0.001) solid tumour (14.9-fold, P<0.001) and OSE (63.3-fold, P<0.001), however there were no significant differences in fold change amongst the three cell types.

Type 2 11βHSD mRNA levels were not significantly altered by cortisol, IL-1α or both combined in any of the cells treated. There was no significant difference in response amongst OSE, ascites or solid tumour preparations.

COX2 mRNA expression was not changed by cortisol treatment alone in any of the three cell types examined. IL-1α caused a significant up-regulation of COX2 mRNA expression in OSE, ascites and solid tumour cultures (mean fold induction 11.5, P<0.001; 29.2, P<0.001; and 26.5-fold, P<0.05 respectively). Co-treatment of all 3 cell types with cortisol abrogated the ability of IL-1 to up-regulate the expression of COX-2 to the extent that it no longer differed significantly from the control.
Figure 3.2. Histograms of quantitative RT-PCR results showing changes in 11\(\beta\)HSD1, 11\(\beta\)HSD2 and COX2 in response to 1\(\mu\)M cortisol (F), 0.5ng/ml IL-1\(\alpha\) and IL-1\(\alpha\)+F in primary cultures of EOC derived from ascites and solid tumour in comparison to OSE. Bars indicate
Mean/SEM. Dissimilar superscripts indicate significant difference due to treatment. NS=not significant.

3.2.1.3  IL-1α Treatment Time Course

The effect of increasing length of exposure of the malignant cell cultures to IL-1α (0.5ng/ml) on mRNA expression was investigated using five different time points (0, 6, 12, 24, and 48 hours) (figure 3.4).

These data demonstrate that 11βHSD1 mRNA expression increased with time and reached maximal levels at 48 hours. 11βHSD2 mRNA expression was unchanged over time while COX2 message reached a peak at 6 hours then quickly tailed off.
Figure 3.3. The effect of time exposed to 0.5ng/ml IL-1α in an EOC primary culture. Line graphs showing 11βHSD1, 11βHSD2 and COX2 mRNA expression changes with time exposed to IL-1α (0.5ng/ml) by quantitative RT-PCR (n=1).
3.2.1.4 IL-1α Dose Response

These experiments were initially attempted (n=4 patients) with IL-1α concentrations of 0.01, 0.05, 0.1, 0.5ng/ml. Thereafter further experiments were carried out using equivalent IL-1α concentration escalations (0.02, 0.1, 0.5ng/ml)(n=4 patients). The first set of data (figure 3.4a) suggests that 1μM IL-1α achieves the highest response in 11βHSD1 mRNA (51.2-fold). In addition COX2 mRNA was greatest above 0.1ng/ml (51-fold at 0.5ng/ml and 28-fold at 1.0ng/ml). However these data have large variances and thus statistical testing does not achieve a P-value <0.05. Therefore type II statistical errors may have been present as it is biologically likely that there is a rise in both 11βHSD1 and COX2 with IL-1α at concentrations above 0.1ng/ml. The second set of data demonstrates that there is an increase in 11βHSD1 with increasing doses of IL-1α with maximal effect at 0.5ng/ml (21.2-fold mean induction) (figure 3.5a). These data also suffer from the same problem with variance and the experiments did not include samples treated at 1ng/ml. Data were analysed using one way ANOVA having tested for normal distribution, with Bonferroni post test comparison. The absence of statistical significance reflects the low number of samples used. There are no significant changes in type 2 11βHSD mRNA expression with increasing dose of IL-1α, whereas COX2 mRNA expression rises significantly from control with 0.1 and 0.5ng/ml IL-1α (17.8 and 27.4-fold mean induction respectively).
Figure 3.4 Quantitative RT-PCR data indicating the effect of increasing dose of on a) $11\beta$HSD1, b) $11\beta$HSD2 and c) COX2 mRNA expression after 48 hours of exposure in EOC primary cultures (n=4). Bars indicate mean/SEM. NS indicates $P > 0.05$. 
Figure 3.5. Quantitative RT-PCR data indicating the effect of increasing dose of on a) $^{11}\beta$HSD1, b) $^{11}\beta$HSD2 and c) COX2 mRNA expression after 48 hours of exposure in EOC primary cultures (n=4). Bars indicate
mean/SEM. Asterisks indicate significant effect due to treatment (**=P<0.001).

3.2.1.5 Cortisol Dose Response

The effect of three different cortisol concentrations on the inflammatory genes of interest was examined in the presence and absence of IL-1α (n=2) (figure 3.6). Data were unable to be analysed using statistical software due to the inadequate number of samples. Cortisol itself had little effect at the lowest concentration (0.01 μM), but caused a minor rise in 11βHSD1 mRNA expression at 0.1 μM and 1 μM (2.3 and 2.2-fold, respectively). However in the presence of IL-1α, cortisol caused a greater rise in 11βHSD1 expression (2.9-fold at 0.01 μM, 4.8-fold at 0.1 μM, and 6.3-fold at 1 μM.) Neither cortisol at any dose nor IL-1α had any effect on 11βHSD2 mRNA expression.

COX2 mRNA expression was highest in the presence of IL-1α and absence of cortisol (13-fold induction over untreated control), at 0.01 μM cortisol plus IL-1α the COX2 mRNA level was only moderately raised over the control sample, and at higher doses of cortisol COX2 was suppressed.
Figure 3.6. Effect of increasing concentration of cortisol. Quantitative RT-PCR of a) $11\beta$HSD1, b) $11\beta$HSD2 and c) COX2 mRNA expression in the presence and absence of IL-1$\alpha$ (0.5ng/ml). n=2.
### 3.2.1.6 IL-1R mRNA

To investigate whether the IL-1 receptor (IL-1R) mRNA was present in both EOC and OSE and whether there was any difference in IL-1R mRNA response to inflammation in both tissues, quantitative RT-PCR was undertaken on a subgroup of samples (figure 3.7). Data were analysed after testing for normal distribution using a one way ANOVA and Bonferroni post-test comparison. These data indicated that firstly there was no significant effect of any treatment on either cell type and secondly that there was no significant difference in fold change between the two cell types (figure 3.7a). Although the data sets were small any change in IL1R appears to have been maximal at 6 hours where a rise in mRNA level was noted (figure 3.7b). However after 48 hours incubation IL-1α suppressed IL-1R mRNA with a greater effect at lower dose. Incubation with cortisol after 48 hours did not appear to have a significant effect on IL-1R mRNA expression.
Figure 3.7. IL-1R mRNA expression in EOC primary cell cultures. Quantitative RT-PCR data demonstrating a) effect of 1μM cortisol (F), 0.5ng/ml IL-1α and IL-1α+F on IL-1R expression in OSE (n=2) and EOC (n=14), b) response of IL-1R mRNA expression following IL-1α treatment over time (n=1), c) effect of 5-fold changes of IL-1α
concentration (n=3), and d) effect of 10-fold variation in cortisol concentration in the presence and absence of IL-1α (n=1).

3.2.2 Absolute mRNA levels in primary cultures of EOC and OSE.

Whilst quantitative RT-PCR has been helpful in revealing dynamic changes in cell culture samples following IL-1α and cortisol treatments over time, the reference sample for each of the test samples is the untreated control samples from that cell type. Therefore it was not possible to deduce from the experiments described above whether there was any difference in absolute mRNA quantities between different patient’s samples, only whether a treatment had an effect on any one particular cell culture. To investigate whether basal messenger RNA levels varied between the cancers and normal samples and indeed between the individual samples themselves, cDNA was synthesised from a standard quantity of RNA from a selection of control tissue samples, and quantitative RT-PCR performed. These data then gave some idea of the relative levels of the genes of interest amongst samples from different patients and thus an idea whether there was any gross derangement of any of these genes between the normal and malignant cells. Samples were collected prospectively therefore two such studies were undertaken during the research period and the combined data are presented.
A) 11βHSD1

HSD11B1 mRNA Expression (relative to liver standard)

RNA Sample

B) 11βHSD2

HSD11B2 mRNA Expression (relative to placenta standard)

RNA Sample
Figure 3.8  Quantitative RT-PCR demonstrating variation of a) $11\beta$HSD1, b) $11\beta$HSD2 c) COX-2 and d) IL1R mRNA expression between OSE samples ($n=9$, blue bars) and EOC samples ($n=27$, red bars) relative to a standard RNA sample. EOC samples are labelled by patient ID number and letter indicating source of cell culture (A=ascites, T=primary tumour, M=omental metastasis).

These data indicate that mRNA quantity ranges widely between tumours and also between normal OSE samples (figure 3.8). The samples from patient 13 stand out for their low $11\beta$HSD1, high $11\beta$HSD2 and absent COX-2 mRNA levels compared to the other tissues. There appears to be a trend that samples with high type 1 levels have low type 2 levels and vice versa, however investigation of these data for correlation fails to demonstrate any statistical significance. There was further evidence of a switch in $11\beta$HSD isozyme expression with samples with low type 1 having the highest levels of type 2 (sample 32M) and samples with low type 2 having higher type 1 levels (sample 23A).
A) $11\beta$HSD1

B) $11\beta$HSD2
C) COX2

![COX2 mRNA Expression](image)

D) IL1R

![IL1R mRNA expression](image)
As shown in figure 3.9, there are no statistical differences in mean relative expression between EOC and OSE for 11βHSD1, COX2, IL1R and GR. The data were Log transformed to allow normal distribution and subsequent parametric testing. An unpaired t-test indicated a small but significant difference in 11βHSD2 mRNA expression with EOC having the higher expression (P=0.0464). This could be explained by type 1 error however this finding agrees with Rabbitt’s hypothesis. The numbers of samples used in the analysis of IL1R and GR is explained by insufficient quantities of mRNA being available to examine both messages across all samples.
There was highly significant correlation between 11βHSD1 and COX2 mRNA expression in EOC specimens (Spearman correlation co-efficient $r_s = 0.90$ (95%CI = 0.78 to 0.96), $P<0.001$) (figure 3.10). There was no other statistically significant correlation between the other genes of interest other than a positive correlation between COX2 mRNA and GR mRNA expression ($r_s = 0.76$, CI=0.35 to 0.93, $P<0.01$)(figure 3.12).

Figure 3.10  COX2 mRNA against 11βHSD Type 1 mRNA quantity in samples of EOC (n=27).
3.2.3 11β-Hydroxysteroid dehydrogenase enzyme activity

3.2.3.1 11-oxoreductase activity

Seven separate experiments were undertaken to compare the relative reductase activities of 11βHSD1 in OSE and EOC. Every experiment included one OSE sample and up to three EOC samples (all in triplicate), an example of the read-out given in figure 3.15.
The results indicate that 11-oxoreductase activity was equivalent in both OSE and EOC untreated cultures (mean activities 1.58pmole/hour and 1.71pmole/hour respectively, P>0.05). Furthermore both OSE and EOC significantly increased levels of 11βHSD1 activity in response to IL-1α treatment (2.09pmole/hour, P=0.0024, and 2.28pmole/hour, P<0.0001, respectively, see figure 3.16).
Figure 3.13. 11-oxoreductase activity in OSE (n=7) and EOC (n=13) +/- IL-1α per well of 2x10^5 cells. Bars indicate Mean+SEM. Asterisks indicate significant difference due to treatment (**=P<0.01, ***=P<0.001).

3.2.3.2 11-dehydrogenase activity

11-dehydrogenase activity was also assessed using tritiated cortisol. Despite varying cell density, length of time of incubation and carrier steroid concentration the only sample where definite dehydrogenase activity could be demonstrated was from patient 28 (figure 3.17). There did not appear to be any increase in activity in response to IL-1α treatment. There was no measurable activity from the OSE samples.
3.2.4 Immunohistochemistry

Selected tumour representative of different histological subtypes and normal ovary samples were used to investigate expression of $11\beta$HSD1, $11\beta$HSD2 and GR (figure 3.18). Positive controls included liver ($11\beta$HSD1 and GR) and kidney ($11\beta$HSD2). In addition there was a tumour negative control sample included where antibody was substituted by matched IgG.
The 11βHSD1 antibody was assessed by Western blotting, where two separate antibodies were assessed for suitability, an in house polyclonal antibody (kind gift from Dr K Chapman) and a commercially available polyclonal antibody (Cayman Chemical, Bambridge, UK). Both identified a band at around 34kDa the predicted size of 11βHSD1, however the in house antibody appeared to show additional bands. The Cayman antibody was chosen due to its better specificity.

**Figure 3.15** Western blots of protein from Lane 1: CHO cells transfected with 11βHSD1, Lane 2: CHO cells transfected with 3βHSD2, Lane 3: Liver, with two antibodies raised against 11βHSD1.
Controls

Patient 13 Omental Metastasis. Poorly differentiated mixed serous & endometrioid ovarian adenocarcinoma
Patient 31 Ovarian Tumour Serous/endometrioid carcinoma

Ovarian Surface Epithelium

H&E

11βHSD1

11βHSD2

GR
Figure 3.16. Immunohistochemistry collages from control samples, tumour samples and normal OSE. 40x Magnification. 11βHSD1 is shown by red and nuclear counterstain is green. 11βHSD2 is shown by green with red nuclear counterstain. GR is shown by red with green counterstain. Some GR images show nuclear counterstain (top left), GR (top right) and merge (bottom left) given nuclear distribution of GR.
3.3 Discussion

3.3.1 Tissue Collection and Culture

Only two patients of the 37 who were approached to donate ovarian cancer tissue declined to take part in the study. Tissue was not obtained from four patients who had consented to the study; two because a pathologist was not available to dissect tissue, one due to the theatre list running late and one because there was no overt pathology. Of the 29 samples collected, eight were benign tumours and there was one borderline tumour with non-invasive implants. As the goal of tissue collection was to maximise numbers of malignant cell cultures these data emphasise the difficulty of predicting malignant pathology pre-operatively.

Inadvertently these findings highlight the difficulties that exist in screening for ovarian cancer. Many of these patients would still have been scheduled for surgery if ultrasound and CA125 were used as a screening modality. However particularly in the elderly population morbidity and also mortality from abdominal surgery is significant. If a sensitive and specific test was available to distinguish benign from malignant ovarian cysts then some of these elderly women could be managed conservatively and therefore escape the risks associated with laparotomy. In the PLCO study of ovarian cancer screening of the 1706 patients who went forward for surgery 541 did not have cancer (Buys, S.S. et al. 2005). However the authors did not disclose the morbidities associated with surgery in this group. While patients presented in this thesis with grossly elevated CA125 levels (>1000) always had malignancy, women with low CA125 levels (patients 6, 7 and 23; 108, 90 and 46iu/l respectively) had cancer while some women with mildly raised CA125 levels did not (patients 11, 19 and 20; 337, 154 and 150iu/l respectively). This is in agreement with the conclusion that serum CA125 alone is a poor screening tool for ovarian cancer.

In this study, of the 19 ovarian or primary peritoneal malignancies 17 were stage III or IV (90%). The mean age of women with ovarian or primary peritoneal malignancies was 61.5 years (range 42-88 years). There was one patient who was
discovered to have a breast primary malignancy which had metastasised to the ovaries (patient 23).

With regard to the primary cultures used in this study, criticisms could be made of the inability to provide cultures of pure tumour cells. Indeed in many instances cell morphology suggested the presence of more than one phenotype. However in other cases the tumour cells were the only cell type present (this was especially noticeable in samples from patients 13 and 21). Furthermore it may have been helpful to attempt to quantify cell components of primary culture flasks using a technique such as flow cytometry. Newer techniques have become commercially available which allow purification of cell cultures by targeting cells expressing a particular antibody. For example immunomagnetic microbeads that select cells expressing the human epithelial antigen-125 (HEA-125) have been used to successfully purify primary cell cultures from ascites in patients with EOC (Chan, J.K. et al. 2007). In Chan’s study some of the starting samples prior to purification consist of 60% tumour cells which would suggest that the malignant cells contribute most to the culture characteristics. Immunohistochemistry studies of the cultured cells suggested a high percentage of cytokeratin-positive cells. While some of these cells could have been mesothelial in origin, the irregular shaped cells and high nuclear:cytoplasmic ratio suggests that malignant cells made up a significant proportion of the culture.

The rationale for using primary cultures of EOC in this study is that they are more likely to reflect the primary tumour and disease than cell lines which have undergone myriad genetic mutations since their initiation. Therefore the presence of some non-tumour cells that have nevertheless been isolated from the initial cancer material may be important in providing a supportive role for the malignant cells. Indeed, there is evidence of communication between tumour and host immune cells in EOC (Gordon, I.O. et al. 2006; Hagemann, T. et al. 2006). This may be of more relevance in the glucocorticoid metabolism studies than the RNA studies, where a heterogeneous population may be more representative of the in vivo tumour environment.
3.3.2 Comparison of EOC and OSE Response to Inflammation

Looking at mRNA levels, primary cultures of EOC were tested for their responses to inflammation in 27 separate experiments. In eleven of these experiments primary cultures of OSE were simultaneously available to provide a direct comparison in the response to cortisol and IL-1α. The nature of prospective tissue collection meant that confluent cultures of OSE were not always available for experimentation when EOC cultures were confluent. In addition as cells sourced from different tissues from the same patient were often confluent at different times, experiments on same-patient samples were not always able to be conducted concurrently. In light of these issues of timing, the results were initially analysed with each experiment taken independently. In addition it may have been erroneous to assume that samples from the same patient would give identical responses. It is for these reasons therefore that the in toto analysis was undertaken.

These results show that although 11βHSD1 mRNA was significantly up-regulated by F, IL-1α and F+IL-1α in the EOC samples, the degree of fold change with the IL-1α treatments was significantly less than in the OSE cell population. This implies that the EOC phenotype may be less responsive to IL-1α than the untransformed OSE cell. These data are also in agreement with Gubbay et al.’s findings that EOC cell lines have significantly less induction of 11βHSD1 with IL-1α than OSE, although the difference was less marked in this current study (Gubbay, O. et al. 2005). These new data hint at a difference between cell lines and primary cultures, where the cell lines have reached immortality and may have acquired further genetic mutations and therefore do not reflect the true in vivo characteristics of cancer.

Separation of the data by origin of cell culture into solid and ascitic components was important to assess whether there was any marked difference in cellular response due to the cell culture technique. This revealed that ascitic and solid tumour cultures behaved in a similar manner with respect to 11βHSD1, 11βHSD2 and COX2. However on statistical analysis the difference between malignant and normal cells in the 11βHSD1 response to IL-1+/-F was lost. This can be explained by a reduction in statistical power as a result of reducing “n”, but also hints at there perhaps being little real difference between these tissues and perhaps type II error could have arisen.
The relatively low number of specimens used in these experiments does raise the possibility of statistical tests being insufficiently powered to demonstrated significant effects of treatments. While the data were tested for normality prior to the use of parametric testing, it is accepted that the low number of samples has the potential to mislead. This notwithstanding, the level of statistical significance is likely to equate with biological significance. It can be more confidently stated that IL-1α increases 11βHSD1 and COX-2 mRNA levels in EOC and OSE (where P<0.001), than the fold-change in 11βHSD1 is greater in OSE than EOC (where P<0.05). It is certainly possible that the difference between OSE and EOC is an example of a type I error, where a significant difference is seen but this may be the result of a sampling error. Conversely the small sample size could also lead to a type 2 error for the 11-oxoreductase assay, which is discussed in more length below.

Following the mRNA studies, 11βHSD1 protein expression was assessed by functional studies of enzymic activity. 11βHSD1 acts primarily in vivo as a reductase enzyme and metabolism studies using tritiated cortisone as substrate gave a direct quantification of enzyme quantity. Overall there was no significant difference in 11βHSD1 activity between OSE and EOC untreated samples. Both OSE and EOC had significantly higher enzyme levels in response to IL-1α compared to their untreated controls, which was predicted from the mRNA analysis. However the difference in 11βHSD1 seen at the mRNA level was not demonstrated at the enzyme level, and the normal and malignant tissues had equivalent enzyme activities. One explanation for this could be that Taqman quantitative analysis is a more sensitive test and is better able to highlight differences. The 11-oxoreductase assay may be more prone to variation due to the larger number of biological variables that influence enzyme activity.

While attempts were made to keep some experimental conditions the same for both mRNA and protein studies, there were slight differences. Firstly, the mRNA studies used fresh primary cells that had not undergone any trypsinisation or passage. In order to guarantee a constant supply of OSE and to allow synchronisation of OSE and EOC cultures reaching confluency at the same time, a stock of frozen OSE was created and divided into multiple cryovials. These were then thawed and cultured to
achieve confluence at the same time as the EOC cultures. This had the added benefit of increasing the reproducibility of the assay, as each OSE sample used had the same patient origin, and allowed a quality control assessment of the enzyme assay. Secondly, the density and number of cells used in each type of experiment differed, the enzyme assay using 2 x 10^5 cells per well (2 x 10^5 per ml) but in triplicate while the mRNA studies included 4-5 x 10^5 cells per solitary well (1.0-1.25 x 10^5 per ml). While it is unlikely that this difference in cell number had any significant effect, there remains the possibility that the lower density of cells could influence cell response. Enzyme activity is also influenced by other significant variables including co-substrate availability, temperature and carrier steroid concentration. As steroid extraction and TLC techniques were identical for all experiments any inter-experimental variation was more likely to be related to differences in co-substrate availability. One way of overcoming this would have been to drive the reaction by providing an abundance of NADPH. However this would detract further from the attempt to emulate the in vivo actions of this enzyme and limited co-substrate availability itself could be characteristic of some tumours. Furthermore the proliferative nature of malignant tissue in vivo results in high oxygen demands which often outstrips the ability of the local vasculature to supply. This explains the areas of necrosis that is commonly found within tumour tissue. It seems likely therefore that the redox state of cancerous tissue is altered and as such co-substrate availability which is dependent on adequate oxygenation may be disrupted. This reasoning raises a caveat to the 11-oxreductase assay performance. In the setting of the in vitro assay the numbers of cells is equivalent between normal and malignant cultures as well as all other tissue culture variables. The situation in the human body may be quite different. While normal OSE is likely to be well perfused, malignant tissue will have a gradient of oxygenation from capillary to tumour centre. The poor oxygenation in the malignancy may reduce NADPH availability in these tissues and 11βHSD1 may not function as a reductase. Thus although levels of type 1 enzyme may be equivalent in the normal and malignant tissues it is highly probable that weight for weight activities in the tissues are not. This probable imbalance in co-substrate in malignant tissue could result in the enzyme acting as a dehydrogenase, thought to be solely an in vitro action. There are also limitations in the 11-
oxoreductase assay which could explain why no difference was seen between the tissues. The assay followed previously published methods where OSE cells had been investigated for cortisol activity (Yong et al., 2002). A time course of 1, 4, 8 and 12 hours was set up initially to assess the best time point to end the reaction. At one and four hours there was no detectable reductase activity and it only became detectable at 8 hours. This was therefore chosen as the standard time for all experiments. The difficulty with this is that by this time a large proportion of the substrate may have already been utilised and the enzyme kinetics no longer first order. It would have been ideal to have identified the linear part of the reaction prior to commencing the study, with multiple concentrations of steroid. Analysis of the 11βHSD1 enzyme is complicated further by its low affinity for cortisol and potential to catalyse both directions of the reaction, as well as its dependence on the cosubstrates NADP⁺ and NADPH. Thus once the reaction has been running for some time, there is less cortisone but also less NADPH and the redox status of the tissue culture flask may favour the conversion of cortisol to cortisone. The enzyme assay can be set up to investigate this additional step by the deuterated cortisol tracer method described by Andrew et al. (Andrew et al., 2002).

A further factor affecting tissue levels of cortisol is cortisol binding globulin (CBG). In the circulation over 90% of cortisol is bound to CBG, with lesser amounts bound to albumin and sex hormone binding globulin (SHBG) (Andersen, 2002). Cortisol has 10-fold higher binding affinity than cortisone to CBG. In addition progesterone is able to dissociate cortisol from CBG by competing for the active binding site. It is hypothesised that the protective effect of the contraceptive pill is through the displacement of cortisol by exogenous progestins, further reducing inflammation within the ovary. Therefore relative levels of albumin, CBG and progesterone could all affect tissue glucocorticoid distribution in malignancy.

As predicted from earlier studies the type 2 11βHSD isoform is unlikely to play a significant role in normal OSE function, as the level of mRNA is low and its levels are not affected by inflammatory stimuli (Rae, M.T. et al. 2004). Furthermore, it is difficult to detect any 11-dehydrogenase activity in cultured OSE cells (Gubbay, O.
et al. 2005). Both these observations are held up by these studies comparing EOC and OSE. The absence of any induction of 11βHSD2 mRNA in OSE and EOC means that any alteration in tissue glucocorticoid is consequent to alteration in 11βHSD1 expression. This is analogous to the expression of the cyclo-oxygenase enzymes where COX1 is a constitutionally expressed enzyme while COX2 can be rapidly induced and therefore alter tissue prostaglandin concentration when signalled to do so. In a large series of primary cultures of both OSE and EOC only one sample gave any positive evidence of 11βHSD2 enzyme activity. This suggests that 11βHSD2 over-expression is not a necessary requisite of EOC development, yet it remains possible that it might be of importance in some cancer subtypes. The tissue which appeared to have measurable 11βHSD2 activity (28T, a poorly differentiated serous adenocarcinoma) had the fourth highest type 2 11βHSD mRNA levels in the series (figure 3.10). Assuming this is not a spurious result (as it was replicated in triplicate), the increased activity could be explained by increased availability of co-substrate.

Other approaches could have been to homogenize the tissue and drive the reaction with additional co-substrate. This is less appealing as, although it might represent the potential enzymic activities of each sample, it is far removed from actual tissue metabolism especially as it would disrupt intracellular compartmentalization of the enzymes and their co-factors. One explanation for the 11βHSD2 enzyme assay findings could be that the assay was simply not sensitive enough to detect differences in enzyme activity between tissues. In retrospect it would have been prudent to include a positive control sample such as placenta to assess the assay’s performance.

3.3.3 Comparison of Absolute mRNA quantities

Having established the response of primary cancer cell cultures to inflammation, it was then important to investigate whether there was any difference in absolute mRNA levels between EOC and OSE. The study highlighted that one patient’s samples (patient 13) contained very low levels of type 1 11βHSD and COX-2 but the highest quantities of type 2 11βHSD. Of note was the histological finding that this patient’s tumour was poorly differentiated (figure 3.15). Whilst this is case study
evidence, it was also noted that cultures form this patient’s samples were highly proliferative and morphologically identical, producing confluent homogeneous monolayers of small rounded cells. The reassuring feature of these results is that the samples from each tissue source (i.e. ascites, primary tumour and omental metastases) behaved identically providing justification that similarly malignant cells can be cultured from both ascites and solid tumour.

Analysis of the median quantities of mRNA in EOC and OSE did not reveal any significant differences between the two tissue types, although there was a trend towards higher levels of both 11βHSD1 and 11βHSD2 in the cancer samples. Importantly there was no significant difference in IL-1R mRNA expression between the two samples suggesting that the reduced responsivity to IL-1α in EOC compared to OSE was not due to IL-1R expression, although it would be necessary to quantify the receptor protein before this can be stated with absolute confidence. In the dynamic studies, IL-1 receptor (IL1R) mRNA was unchanged by IL-1α or cortisol at 48 hours in both EOC and OSE. The time course of IL-1 effect however suggests that IL1R mRNA expression in response to IL-1α may be maximal at 6 hours, therefore any effect at 48 hours post-treatment may have been lost.

It was also apparent that the sample with the highest type 2 11βHSD (patient 32) also had one of the lowest levels of type 1 11βHSD mRNA. Furthermore there was evidence of a switch in isozyme expression when the samples were ranked by mRNA quantity. Thus those samples with high type 2 11βHSD mRNA levels tended to have low type 1 mRNA levels and vice versa. Correlation analysis showed that despite switches in expression of 11BHSD1 and 2 mRNA in some individual cancer cases there is no statistical correlation.

There is a reproducible correlation between 11βHSD1 and COX2 mRNA expression in the EOC specimens (figure 3.1). This might have been anticipated by the demonstration of IL-1α up-regulating both 11βHSD1 and COX2 in the cell culture experiments. The pro-inflammatory pathway may be dominant in these malignant cells as a primary rise in 11βHSD1 would be anticipated to reduce levels of COX2, assuming that COX2 expression remains responsive to anti-inflammatory agents in
malignant tissue. However looking at the time profile of the up-regulation of these genes shows that COX-2 is maximally up-regulated at 6 hours whereas the 11βHSD1 response takes 48 hours to reach its highest level. This is in keeping with previously published data showing that prostaglandin synthesis is closely linked to 11βHSD1 production in human granulose cells (Jonas et al., 2006). Furthermore this could explain the events surrounding ovulation where acute inflammation is allowed to occur then switched off by a slower rising anti-inflammatory mechanism via 11βHSD1. In addition if this anti-inflammatory response is primed by prostaglandin production the size of the response will be proportional to the preceding pro-inflammatory stimulus and a homeostatic feedback cycle initiated. In the context of malignancy, if the type 1 11βHSD response is impaired then only the pro-inflammatory mechanism is allowed to dominate, and if the type 2 enzyme dominates there will be a further decrease in tissue cortisol. These finding therefore provide further impetus to Rabbitt’s hypothesis that type 2 11βHSD predominance predisposes to the development of malignancy.

There is evidence that COX2 expression is deregulated in several cancers as reviewed by Zha (Zha, S. et al. 2004). In EOC studies have examined COX2 expression and survival with conflicting results. In advanced serous EOC Ali-Fehmi and co-workers concluded that increased COX2 expression was associated with tumour angiogenesis and poor survival (Ali-Fehmi, R. et al. 2005). However in a subsequent study COX2 negativity was associated with poorer survival (Steffensen K.D. , M.W.U.J.E.I.B.A.J. 2007). This Danish study included all histological subtypes where the rate of serous tumours was 61%, perhaps explaining these differences. An increase in COX2 expression, like 11βHSD1 expression, does not appear to be a pre-requisite for cancer development, although the fact that many cancers over express this enzyme may implicate it in the host response to disease. COX2 overexpression is likely to play a role in tumour angiogenesis (Kuwano, T. et al. 2004).

On analysis of the mRNA quantities in OSE and EOC, there was no significant difference in 11βHSD1, COX2 or GR expression. However there was a significantly higher quantity of 11βHSD2 mRNA in the EOC samples compared to OSE (P<0.05).
This is in agreement with Gubbay’s study comparing OSE and EOC cell lines. In addition there is evidence from other malignancies that type 2 11βHSD is up-regulated in cancer (as summarised by Rabbitt, (Rabbitt, E.H. et al. 2003)) and work examining 11βHSD enzyme expression in homogenised tumour material has also previously suggested higher levels of type 2 11βHSD in the cancers (Temkin, S. et al. 2006). One weakness of the study presented here is the strength of the statistical difference which could have been improved by increasing the numbers of OSE samples used. However primary tissue is a rare and valuable resource and the OSE samples were limited.

### 3.3.4 Tissue distribution of 11βHSDs

The immunohistochemistry (IHC) studies were of value in examining the distributions of the 11βHSD isozymes. Reassuringly, the positive control samples indicated that the antibodies were specific for the proteins of interest. In addition Western blotting had been undertaken prior to IHC and had confirmed specificity of the 11βHSD1 antibody. Past local experience meant that the 11βHSD2 antibody could be used with confidence and indeed it appeared to demonstrate localization of the enzyme to the renal glomeruli as previously reported (Kataoka, S. et al. 2002).

In general, type 1 11βHSD appears to be expressed in most of the tissue tested but absent in patients 13, 14, 23 and 29. This is of particular interest as the mRNA studies indicated that patient 13 had a particularly low expression of type 1 11βHSD. Strong signals for 11βHSD1 were noted in samples from patients 16, 31, 32, and 35. There appears to be little evidence therefore that switching off of 11βHSD1 is necessary for EOC to develop. Indeed the presence of the enzyme and reductase activity suggest that local cortisol production is not likely to be impaired in these tissues.

11βHSD2 protein could also be demonstrated in some tumour samples. It was clearly expressed in patients 28,29, 32 and 33. The high expression of type 2 11βHSD in patient 28 is corroborated by the finding of 11-dehydrogenase activity in this tumour also. Whilst most of the other tumours did not demonstrate the presence
of 11βHSD2 its high expression in some cancers does not exclude a “pro-
proliferative” role for it in some tumours.

GR appeared to be widely distributed within the nuclei of most tumour samples.  
This implies that cortisol signalling could occur in the malignant tissue. However 
pre-receptor effects of 11βHSD1 & 2 would also be expected to influence the cellular 
response.

3.3.5 General Discussion

In summary, the data in this chapter demonstrate that it is possible to collect and 
culture primary ovarian cancer cells from ascites and solid tumour, that enables 
comparison to normal untransformed primary OSE. These results hint at the 
11βHSD1 mRNA response to IL-1α-induced inflammation being attenuated in EOC 
compared to OSE. However 11βHSD1 enzyme activity appears to be similar in the 
two cell types. 11βHSD2 mRNA levels are unaffected by inflammation in normal 
and malignant ovarian tissues and the only cell type with any demonstrable 
11βHSD2 enzyme activity was from EOC. 11βHSD1 and 2 isozymes are found in 
inverse proportions in EOC samples and there is some suggestion that high 
11βHSD2 expression is associated with higher grade tumours. Both isozymes can be 
detected by IHC in tissue sections of malignant ovarian tissue. The final analysis of 
these data has revealed limitations of the experimental set-up and provides 
suggestions for the future.

From the beginning of the project there was uncertainty as to how successful primary 
culture of EOC was going to be. The creation of such cultures required the early 
institution of a protocol for tissue handling and this then had to be maintained 
throughout the research time to avoid variation between the cell cultures. 
Experiments were performed on the healthiest and most quickly confluent flasks, so 
some degree of selection bias may have been introduced. As the project advanced 
knowledge of the patient’s pathology results were known which again could 
introduce bias.
It could be argued that OSE was an inappropriate cell type to use for comparison because samples were not from the same patient and OSE was collected from pre-menopausal women whereas nearly all the cancers came from post-menopausal women. Thus the sex steroid backgrounds are likely to have been quite different for both tissues. Nevertheless, OSE is a strong candidate tissue for most EOCs and in light of the primary nature of EOC cultures, primary OSE is more relevant as a comparator than OSE cell lines. These suppositions are held up by evidence from gene expression profiles of ovarian cancers, primary OSE and cell lines (Hough, C.D. et al. 2000).

Having been satisfied that the correct tissues for comparison had been chosen, the evidence presented here hints that deregulation of type 1 11βHSD may occur in the development of ovarian cancer, and eventually type 2 11βHSD becomes predominant. The exact mechanisms by which this occurs are not yet clear however it may be that there is a mismatch in pro-inflammatory activity and the anti-inflammatory response, in favour of inflammation.

The relative expression of the pre-receptor glucocorticoid modulators is likely a consequence of tumour differentiation and not a cause. As evidenced by patient 13 in this study, tumours which are high grade and poorly differentiated are associated with low expression of 11βHSD1. In addition the cell type of origin may be important in determining tumour phenotype. The fact that epithelial ovarian cancers can show such wide heterogeneity could be explained by either each histiotype having a separate cell of origin, or alternatively that the different histologies arise from the same progenitor or stem cell which has diverse mechanisms of differentiation, for example by HOX gene activation. A unifying cell of origin is an appealing concept and potentially provides a target for therapy and preventative medicine. No matter how attractive this idea is, the bottom line is that the natural history of EOC remains unknown and until these early steps can be illuminated the ability to target the disease at its earliest stages will remain elusive.
Chapter 4

Anti-inflammatory steroid activity in the human peritoneal surface epithelium
4 Anti-inflammatory steroid activity in the human peritoneal surface epithelium

4.1 Introduction

Ovulation has long been considered a naturally occurring inflammatory event (Espey, L.L. 1980). However in 2002 evidence for a corresponding anti-inflammatory reaction in the OSE was presented (Yong, P.Y. et al. 2002). This paper showed an increase in 11βHSD1 in primary OSE cells following stimulation by IL-1α and subsequently increased local conversion of inactive cortisone to the more active anti-inflammatory steroid cortisol (see figure 1.10). It was hypothesised that the creation of this anti-inflammatory milieu would then allow the ovarian wound to heal in preparation for the next round of ovulation. A unique property of the ovary is its ability to recover from injury without the formation of scar tissue on its external surface. This may reflect the fact that ovulation-associated injury is a natural phenomenon rather than inflammation associated with trauma, infection or disease processes such as endometriosis or cancer. The peritoneum is not exposed to frequent episodes of “natural” inflammation so it may have alternative ways of responding once an insult has occurred.

Examination of other tissues has revealed that up-regulation of 11βHSD1 mRNA in response to cytokine stimulation is not unique to the OSE. While the first description of this phenomenon was in the ovary (Tetsuka, M. et al. 1999; Tetsuka, M. et al. 1997), subsequent studies have shown that 11βHSD1 is increased following inflammatory stimuli in renal mesangeal cells (Escher, G. et al. 1997), aortic smooth muscle (Cai, T.-Q. et al. 2001), osteoblasts (Cooper, M.S. et al. 2001), pre-adipocytes (Bujalska, I.J. et al. 2002; Tomlinson, J.W. et al. 2001), endometrial stroma (McDonald, S.E. et al. 2006), fetal membranes (Sun, K. et al. 2003),
trophoblast (Li, W. et al. 2006) and macrophages (Gilmour, J.S. et al. 2006). The ability of a cell population to increase 11βHSD1 after inflammation therefore appears to be a widespread characteristic. However the paracrine effect of increased 11βHSD1 will only result in increased local cortisol if there is no alteration in, or down-regulation of 11βHSD2, which catalyses the dehydrogenation of cortisol to the inactive metabolite.

The ovarian response to inflammation has been closely inspected not least because of links between ovulation, inflammation and the development of epithelial ovarian cancer (Espey, L.L. 1994; Fleming, J.S. et al. 2006; Ness, R.B. et al. 1999; Ness, R.B. et al. 2000). In the OSE, 11βHSD1 is up-regulated at the protein level also as evidenced by the enzymic conversion of radio-labelled cortisone through hydroxylation of the 11-keto group (Yong, P.Y. et al. 2002). Furthermore inflammation in the OSE as demonstrated by increased cylooxygenase-2 (COX2) is inhibited in vitro by addition of cortisol and to a lesser extent progesterone (Rae, M.T. et al. 2004).

The similar embryological origins of the peritoneal surface epithelium (PSE) and OSE as well as the fact that the OSE is a continuation of the PSE at the hilum of the ovary raises the question; how similar are these two mesothelial cell types? Specifically does the PSE share the OSE’s response to inflammatory cytokines and are there also anti-inflammatory mechanisms present in the peritoneal as well as the ovarian mesothelium?
4.2 Cell Collection and Culture

4.2.1 Ethics and Patient Data

Ethical approval for this study was granted by Lothian Regional Ethics Committee 03 (REC Reference 04/S1103/36 Chief Investigator Dr KS Fegan). All patients gave written informed consent prior to donating samples (see appendix). Relevant clinical data are shown in table 4.1.
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Table 4.1. Relevant clinical details of women donating samples of PSE. DMPA = depo-medroxyprogesterone acetate, Lap Ster = laparoscopic sterilisation, Diag Lap = diagnostic laparoscopy, COCP = combined oral contraceptive pill, PID = pelvic inflammatory disease, LLETZ = large loop excision of the transformation zone, TAH = total abdominal hysterectomy, HMB = heavy menstrual bleeding, dysmen = dysmenorrhoea, Fem ster = female sterilisation, IVF = in vitro fertilisation, Lap Chole = laparoscopic cholecystectomy, LNG-IUS = levonorgestrel intrauterine system, BSO = bilateral salpingo-oophorectomy, MEA = microwave endometrial ablation.
4.3 Effects of Interleukin-1α and anti-inflammatory steroids on 11βHSD1, 11βHSD2 and COX-2 in primary cultures of PSE.

In order to examine for effects of the inflammatory cytokine interleukin-1α (IL-1α) an experimental cell culture system was established as previously described using 6 well plates (Chapter 2.1.2). This ensured consistent and repeatable numbers of cells for each experiment and for each treatment within each experiment. Data (CT values) were analysed using a repeated measures one-way ANOVA for each treatment, with Bonferroni post-test correction, having tested that the data were normally distributed and were effectively matched using an F test. The first investigation assessed the effect of IL-1α on inflammation-associated genes.

4.3.1 Dose-dependent effects of IL-1α

To confirm that there was a specific interleukin-1α effect on the PSE cells and to establish the maximally effective dose, five-fold increasing concentrations of IL-1α were used and corresponding genes of interest examined (n=5) (See figure 4.4).

4.3.1.1 11βHSD1

11βHSD1 mRNA expression was increased in a dose-responsive manner by increasing concentrations of interleukin-1α. Maximum up-regulation of 11βHSD1 occurred at a concentration of 0.5ng/ml (8.2-fold change relative to untreated control, P<0.001).

4.3.1.2 11βHSD2

11βHSD2 remained unresponsive to interleukin-1α treatment at any concentration.
4.3.1.3 COX-2

COX-2 mRNA expression was increased in a dose-dependent fashion by interleukin-1α. Maximum mean expression was also at a concentration of 0.5 ng/ml (9.6-fold change relative to untreated control, P<0.001).
Figure 4.1. Quantitative RT-PCR data demonstrating the effect of increasing concentrations of interleukin-1α on expression of a) 11βHSD1, b) 11βHSD2 and c) COX-2 (n=5). Bars indicate mean/SEM.
Asterisks indicate significant difference from untreated control (**=P<0.01, ***=P<0.001).

4.3.2 Changes in 11βHSD1, 11βHSD2 and COX-2 mRNA Expression with IL-1α as a function of time

An examination of IL-1α effects on time dependent gene expression was undertaken in PSE from two different patients. The regulation of the mRNA of interest by IL-1α treatment was assessed by comparing the IL-1α sample with the same time-point untreated control sample. Limited access to clinical material curtailed the number of these experiments to two and subsequently statistical tests could not be applied. See figure 4.5.

4.3.2.1 11βHSD1

11βHSD1 mRNA expression increased over time with maximum expression occurring at 48 hours of IL-1α treatment (mean fold change 8.75 compared to untreated 48 hour sample).

4.3.2.2 11βHSD2

There did not appear to be any change in 11βHSD2 mRNA expression over time.

4.3.2.3 COX-2

In contrast to 11βHSD1, COX-2 mRNA levels appeared to peak at 6 hours (mean fold change 43.8) following administration of IL-1α at 0.5ng/ml. While the extent of COX-2 mRNA up-regulation reduced with time, it was still considerably elevated at 48 hours compared to the untreated control sample (mean fold change 9.2).
Figure 4.2. Effect of duration of IL-1α treatment on a) 11βHSD1, b) 11βHSD2 and c) COX-2 mRNA expression in PSE as assessed by quantitative RT-PCR.
4.3.2.3.1 Effects of Cortisol in the PSE

Having established that 11βHSD1 was up-regulated maximally by 0.5ng/ml IL-1α for 48 hours, the effect of cortisol as an anti-inflammatory agent was then assessed using this dose and time paradigm.

4.3.2.4 Dose-dependent Cortisol Interactions

PSE cells from two separate patients were treated with ten-fold increasing doses of cortisol in the presence and absence of IL-1α at 0.5ng/ml. The number of samples available for this study precluded statistical analysis. See figure 4.6.

4.3.2.4.1 11βHSD1

11βHSD1 mRNA was successively increased by increasing dose of cortisol alone to a maximum mean up-regulation of 23.7 times the untreated control. This effect was enhanced at every cortisol concentration by the presence of IL-1α (at 0.5ng/ml) where the maximum mean fold change of 58.2 was reached at 1μM cortisol.

4.3.2.4.2 11βHSD2

11βHSD2 mRNA was unchanged neither by cortisol treatment at any concentration nor by the addition of IL-1α.

4.3.2.4.3 COX-2

Cortisol alone had no effect on COX-2 expression. As also shown above IL-1α caused a large increase in COX-2 expression (8.4-fold mean up-regulation).
However this effect was gradually abrogated by increasing concentrations of cortisol, until at 1μM the mean level of COX-2 was 2.7-fold that of the untreated control.

![Graphs showing mRNA expression of 11βHSD1, 11βHSD2, and COX2](image)

Figure 4.3. Dose dependent effects of cortisol +/- IL-1α (0.5ng/ml over 48 hours) on a) 11βHSD1, b) 11βHSD2 and c) COX-2 mRNA expression as assessed by quantitative RT-PCR.
4.3.2.5 Cortisol as an anti-inflammatory agent in the PSE

Thus a system was established whereby the interactions of cortisol at a concentration of 1μM and IL-1α at 0.5ng/ml at 48hours incubation could be assessed (figure 4.7). Data (CT values) were analysed using a repeated measures one-way ANOVA for each treatment, with Bonferroni post-test correction, having tested that the data were normally distributed and were effectively matched using an F test.

4.3.2.5.1 11βHSD1

Using PSE cultures from five different patients, levels of 11βHSD1 were increased by both cortisol (mean fold induction 12.6, P<0.001) and IL-1α alone (mean fold induction 9.75, P<0.01). The simultaneous addition of cortisol and IL-1α caused up-regulation of type 1 11βHSD (mean fold change 67.9, P<0.001) which was also statistically different from cortisol (P<0.01) and IL-1α (P<0.001) alone.

4.3.2.5.2 11βHSD2

There was no statistically significant effect of any of the treatments on 11βHSD2 mRNA expression.

4.3.2.5.3 COX2

While cortisol alone had no effect on COX2 mRNA expression the addition of IL-1α induced a significant rise (mean fold induction 10.6, P<0.001). This was returned to levels similar to control by co-incubation of IL-1α with cortisol (mean fold induction over untreated control 1.07, P=not significant).
Figure 4.4. Quantitative RT-PCR showing the effect of interleukin-1α (IL-1, 0.5ng/ml) +/- cortisol (F, 1μM) on expression of 11βHSD1 (a), 11βHSD2 (b) and COX2 (c). n=5. Bars represent mean/SEM. Asterisks indicate significant difference due to treatment. **=P<0.01, ***=P<0.001, NS=not significant.
4.3.3 A Comparison of the Anti-inflammatory Effects of Progesterone and Cortisol

Progesterone is predicted as an anti-inflammatory agent in the PSE from its reported effects in the OSE (Rae, M.T. et al. 2004). Having demonstrated the anti-inflammatory effect of cortisol in the PSE, next, progesterone was used alongside cortisol in a direct comparison of their anti-inflammatory effects in PSE (figure 4.8).

4.3.3.1 11βHSD1

In a separate group of patient samples, results from six different individuals demonstrated that while treatment with cortisol alone again increased 11βHSD1 mRNA expression (10-fold induction, P<0.001), progesterone treatment alone had no effect on 11βHSD1 expression. IL-1α at 0.5ng/ml caused a significant increase in 11βHSD1 message (7.25-fold, P<0.001) which was increased further by the co-incubation with cortisol (28.3-fold, P<0.01). Concomitant treatment of PSE with IL-1α and progesterone appeared to further increase 11βHSD1 expression over IL-1α alone (11.8-fold) but this difference was not statistically significant.

4.3.3.2 11βHSD2

Once again, there was no statistically significant change in 11βHSD2 mRNA expression in response to any of the treatments applied.

4.3.3.3 COX-2

COX-2 expression was unaffected by treatment with cortisol alone. IL-1α caused a significant increase in COX-2 mRNA (mean fold induction 20.3, P<0.001) which was returned to near control levels by the addition of cortisol (mean fold change 4.5 compared to control). Similarly, progesterone alone had no significant effect on COX-2 expression, and comparing IL-1α treatment with IL-1α and progesterone combined, reduced the effect of IL-1α (20.3 to 12.8-fold), though in the case of progesterone this effect was less marked compared to cortisol.
Figure 4.5. Quantitative RT-PCR comparing effects of progesterone (P4) and cortisol (F) (both at 1μM) +/- interleukin-1α (IL-1) on expression of a) 11βHSD1, b) 11βHSD2 and c) COX-2 (n=6). Differing superscripts indicate significant difference due to treatment.
4.3.4 The Response to Inflammation in PSE is not affected by Site of Sampling.

As discussed in 4.2.2, two separate sites of the peritoneum were chosen for collection of PSE cells. While the success of cell culture was lower in the anterior abdominal wall (A) samples due to lower cell numbers at the time of collection, if the sample gave rise to a confluent monolayer there was no distinguishable morphological difference to the cultures collected from the body of the uterus (B sample). Separate analysis of the data for A and B samples in the experimental cell culture system did not demonstrate any difference in $11\beta$HSD1, $11\beta$HSD2 or COX-2 responses to IL-1$\alpha$ in the presence or absence of cortisol or progesterone as a consequence of cell source (figure 4.9).
Figure 4.6. Quantitative RT-PCR results demonstrating the similar responses to IL-1α (0.5ng/ml), cortisol (F) or progesterone (P4) (both at 1μM) in PSE cells sampled from the anterior abdominal wall (A samples, n=6) or the surface of the uterus (B samples, n=8). Bars indicate mean/SEM.
4.3.5 Expression of Glucocorticoid and Progesterone Receptor mRNA and Protein in OSE and PSE.

To demonstrate that both cortisol and progesterone were likely to be biologically active in PSE cells, it was important to demonstrate expression of their respective receptors; glucocorticoid receptor-α (GRα) and the progesterone receptor (PR). Furthermore as the up-regulation of 11βHSD1 by cortisol alone had not been previously noted in the OSE a comparison of GRα in these two cell types might explain their different responses to cortisol.

4.3.5.1 GRα and PR mRNA expression in the OSE and PSE.

Quantitative RT-PCR was used to investigate the relative quantities of GRα and PR mRNA in OSE (n=4) and PSE (n=6). There was no statistical difference in the relative expression of GRα and PR between the two cell types (figure 4.10).
Figure 4.7. Relative quantities of GRα and PR mRNA in OSE (n=4) and PSE (n=6) by quantitative RT-PCR.
4.3.5.2 Glucocorticoid Receptor-α and Progesterone Receptor Protein Expression in OSE and PSE.

4.3.5.2.1 Glucocorticoid Receptor-α

As demonstrated by immunohistochemistry (IHC) in figure 4.11, GRα was expressed in both OSE and PSE.

Figure 4.8 Photomicrograph demonstrating expression of GRα in OSE (A) and PSE (C). Magnification x40, bar represents 20μm. Corresponding negative controls are shown in panels B (OSE) and D (PSE).
4.3.5.2.2 Progesterone Receptor

Figure 4.12 demonstrates the presence of nuclear PR in both OSE and PSE by IHC analysis.

Figure 4.9. Photomicrograph demonstrating nuclear localisation of PR in OSE (A) and PSE (C). Magnification x40, bar represents 20μm. Corresponding negative controls are shown in panels B (OSE) and D (PSE).
4.3.6 Relative quantities of 11βHSD1, 11βHSD2 and COX-2 mRNA in OSE and PSE

An attempt was made to assess the relative levels of mRNA in OSE (n=3) and PSE (n=4) by quantitative RT-PCR where the level of mRNA was compared to a standard RNA sample; liver for 11βHSD1 and COX-2 and placenta for 11βHSD2. When statistical tests were applied there were no differences between PSE and OSE for any of the genes studied, however the mean level of 11βHSD1 in OSE was over two times greater than that in PSE (0.055 vs. 0.023), whereas the mean level of 11βHSD2 in OSE was just over half that of PSE (0.007 vs. 0.014) (figure 4.13). The data were too few to be assessed by a normality test, however using non-parametric tests there were no significant differences between the median values.
Figure 4.10. Relative quantities of a) 11βHSD1, b) 11βHSD2 and c) COX-2 mRNA in OSE (n=3) and PSE (n=4) by quantitative RT-PCR. Values for individual samples are represented by squares (OSE) and triangles (PSE). Bars represent mean values.
4.3.7 Effects of Interleukin-1α on 11βHSD1 11-Oxoreductase Activity in primary cultures of PSE.

The changes in 11βHSD1 mRNA in the PSE in response to pro- and anti-inflammatory agents thus appeared to be remarkably similar to previous reported responses in the OSE. However it could not be assumed that these changes in mRNA were also manifested in protein changes, specifically enzymic activity. As the principal action of 11βHSD1 in the ovary is as a reductase, a glucocorticoid metabolism assay was established where 11-oxoreductase activity was measured by conversion of tritium-labelled cortisone to cortisol. Results from four separate experiments comparing 11-oxoreductase activity in OSE and PSE are shown in figure 4.14. Data are presented as mean cortisol production over 8 hours per 2x10^5 cells. The data were too few to be able to analyse for normality using the D’Agostino and Pearson normality test. However statistical significance persisted whether parametric or non-parametric testing was used.

Enzyme activity was consistently and significantly higher in untreated OSE samples compared to PSE (mean conversion 6.3 pmoles/8hours vs. 1.1 pmoles/8hours, P<0.01). In response to IL-1α treatment, 11βHSD1 activity was significantly increased in the OSE (6.3 pmoles/8hours to 10.4 pmoles/8hours, P<0.05) while there was no statistically significant increase in mean activity in the PSE (1.1 pmoles/8hours vs. 2.4 pmoles/8hours).
Figure 4.11. Comparison of 11-oxoreductase activity in OSE and PSE in presence and absence of IL-1α (0.5ng/ml) (n=4). Bars indicate mean/SEM. Dissimilar superscripts indicate significant difference due to treatment (b=P<0.05). Asterisks indicate significant differences between cell types (**=P<0.01).
4.4 A Comparison of PSE and OSE Response to Inflammation by PCR Microarray

As a result of the above investigations it was hypothesised that the response to IL-1α would be no different in OSE and PSE for genes other than those examined above. A similar inflammatory signature would provide further evidence that the OSE and PSE are essentially the same tissue.

OSE and PSE samples were collected from six patients and experimental plates set up as described in Chapter 2.2. Experimental plates were treated with control medium or IL-1α at 0.5ng/ml as previously described and total RNA extracted and DNaseI treated. The remaining steps were undertaken with the assistance of Mrs Deborah Price and Dr Mick Rae. Two μg RNA was reverse transcribed using the manufacturer’s First Strand Kit (tebu-bio, Peterborough, UK) then mixed with PCR Mastermix and added to the wells of a customised PCR array plate (RT² Profiler PCR Arrays, tebu-bio, Peterborough, UK), where 48 genes of interest were chosen in advance (see table 4.3). Quantitative RT-PCR analysis was undertaken and differences in gene expression examined. Average Ct results from the gene of interest and housekeeping gene from each plate were combined and the relative expression calculated (using the formula $2^{\Delta\Delta Ct}$). Samples were run in quadruplicate to examine differences in basal gene expression between OSE and PSE, then attempts were made to examine the effect of IL-1 on gene expression in the six experiments. One sample of PSE RNA was degraded and one plate was lost due to machine error. However the analysis was able to compare six samples of OSE and three of PSE.
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<td>Mucin 1, cell surface associated</td>
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172
Table 4.2. Gene expression in OSE and PSE by PCR based microarray. Genes up-regulated by IL-1α (0.5ng/ml) are in red and down-regulated in blue.

There appeared to be some differences in basal expression of some genes between OSE and PSE, specifically OPCML, PCOLCE, HNT, LOXL2, AKR1C3, IL8, COX2, HGF and CSF2 being higher in OSE and E-cadherin, MUC16 and FSHR being higher in PSE. β-Actin was chosen as the reference house-keeping gene as its expression was more reproducible than the Ct values for 18s RNA. Two of the samples gave no read out for 18s RNA, and the statistical software analysing the data gave a value of zero to these wells rather than ignoring the data; therefore it could not be used as a housekeeping gene with confidence. This also occurred for IL4 giving an apparent 172812-fold lower value in PSE. The cause of these errors is unexplained but could have been related to the plate integrity or human pipetting error. On treatment with IL-1α, the genes that were up-regulated were the same for both cell types; OPCML, CCL2, LOX, ERβ, HSD11B1, IL8, COX2 and CSF2, as were the down-regulated genes E-Cadherin, HSD3B2, AR, IL10, FSHR, HGF, LHCGR and EGF.
4.5 Discussion

This study introduces a novel method for the collection and culture of human peritoneal mesothelial cells. The most commonly described method for primary culture of human mesothelial cells is by extraction of cells from samples of omentum (Pronk, A. et al. 1993; Robson, R.L. et al. 2001; Stylianou, E. et al. 1990). This strategy carries the risk of including pre-adipocyte, adipocytes, stromal and inflammatory cells in the culture. In addition the use of collagenase and digestive enzymes could prejudice the structural microenvironment of the mesothelium which is likely to play an important role in mesothelial cell physiology. The advantage of the method described here is that it avoids enzymatic digestion and samples only the mesothelial layer, thus maximising the opportunity for pure mesothelial cell monolayers with minimal chance of contamination by non-mesothelial subtypes.

An interesting and unexpected finding was the significant difference in “take-rate” between the PSE samples sampled from the anterior abdominal wall (A samples) and the serosal surface of the uterus (B samples). Direct visualisation of the tissue culture flasks on the day of collection often revealed a clear difference in cellular content between the two types of culture. The scant numbers of PSE flakes seen in the A samples is also reflected by the poor outcomes of cultures compared to the B samples (31% versus 58%). It is not possible from these data to deduce why the numbers of cells were so much lower in the A samples compared to the B samples but this question deserves an answer. As the technique to collect both types of cell was identical, the phenomenon could be explained by either a greater adherence of PSE to the underlying stroma or alternatively a greater number of cells being present on the uterine peritoneal surface. This may suggest subtle differences in the peritoneum covering Mullerian or non-Mullerian structures or more simply be a difference in parietal versus visceral peritoneum. One further possibility is that the mesothelial cell sub-population, as described by Mutsaers (Mutsaers, S.E. 2002) is differentially sited within the peritoneum with a higher density of more loosely applied columnar cells situated over the uterus and more densely adherent squamous
cells on the abdominal wall. Yet a difference in cell populations also requires an explanation from a functional point of view. The uterus occupies a central position within the pelvis and it must be allowed to increase in size when gravid. A generous supply of mesothelial cells on its exterior would therefore minimise adhesions to bowel and other intra-peritoneal viscera therefore minimising complications as the uterus rises out of the pelvis in pregnancy.

The considerable similarities between the PSE and OSE morphology are also reflected in the cellular response to inflammation. The patterns of the 11βHSD1 response to IL-1α in relation to both dose and time are identical to those previously reported for OSE (Rae, M.T. et al. 2004; Yong, P.Y. et al. 2002). A multitude of cytokines could have been chosen to simulate inflammation but as previous work on the OSE had used IL-1α it would not have been logical to stray from its use in PSE, particularly as a direct comparison to OSE was being examined.

Results from the time course of IL-1α action provide an insight into the chronological events seen at ovulation. The rapid rise of COX-2 mRNA reaching a maximum level within 6 hours shows that the pro-inflammatory effects of IL-1α are the first to appear. 11βHSD1 mRNA levels do not peak until 48 hours following IL-1α treatment indicating that the anti-inflammatory pathway shown in Chapter 1 (figure 1.12) lags behind the pro-inflammatory pathway. Thus in the ovary cortisol generation is likely to peak some 48 hours following the initial inflammatory stimulus, suggesting that inflammation is tolerated and even encouraged in the acute phase.

One subtle difference between PSE and OSE is that cortisol alone was able to induce 11βHSD1 mRNA in PSE which has not been previously reported for the OSE. An explanation for this difference is difficult but the OSE studies described in Chapter 3 do hint at this effect of cortisol in OSE also. Rae’s paper from 2004 clearly states that cortisol alone had no effect on 11βHSD1 expression. However the studies were performed some years apart with different reagents. The presence of GRα in PSE means that this phenomenon is feasible, but further enquiry of GR signalling,
including receptor and second messenger inhibitor studies, is required to investigate this effect further.

The other steroid examined in this study was progesterone given its previously reported anti-inflammatory and pro-apoptotic effects in the OSE (Rae, M.T. et al. 2004; Rodriguez, G.C. et al. 2002; Syed, V. et al. 2003). The concentration used in the experiments was 1μM, which is equivalent to that present in follicular fluid at the time of ovulation (Andersen, C.Y. 1991). There was no effect on any of the genes studied of progesterone alone and although there is evidence of a trend progesterone did not conclusively affect 11βHSD1 expression. The down-regulation of IL-1α induced COX-2 expression is in keeping with the anti-inflammatory effect of progesterone seen in the OSE. However progesterone does not appear to be as potent an anti-inflammatory as cortisol at the same concentration. The biological likelihood of progesterone acting on the PSE is enhanced by evidence of the progesterone receptor in these cells (figure 4.12). This finding is important from several angles. Firstly, it is likely that the peritoneum has the potential to be influenced by the fluctuations in serum progesterone that occur with the menstrual cycle. Secondly the presence of progesterone in follicular fluid may be another mechanism preventing adhesion formation after ovulation. Thirdly progesterone is implicated in endometriosis where progesterone resistance may be a feature (Burney, R.O. et al. 2007) and where exogenous progesterone itself is one of the mainstays of treatment.

A complicating factor of progesterone however is that it acts through one of two receptor isoforms designated PR-A or PR-B, the products of a single gene. The isoforms are structurally identical except that the B form has an additional 164 amino acids at its NH₂ terminus. This makes immunohistochemical distinction difficult and it is not possible to state which isoform is being demonstrated in figure 4.12. Functionally, the additional region on PR-B encodes a transactivation function (AF3) specific to PR-B. PR-A on the other hand, suppresses PR-B function. In the presence of progesterone the isoforms dimerise and the relative ratios of PR-A and PR-B will determine the type of dimer formed (A: A, A:B, B:B) and thus influence the physiological response to P4. This may explain Syed’s finding that P4 at low
concentrations stimulates OSE growth but causes inhibition of proliferation at higher doses (Syed, V. et al. 2001). Several polymorphisms of the PR have been sequenced and one, +331A, appears to protect against the development of clear cell and endometrioid ovarian cancers (OR 0.46, CI 0.23-0.92)(Berchuck, A. et al. 2004): the subtypes associated with endometriosis (Brinton, L.A. et al. 1997; Ness, R.B. 2003).

Although there is still debate as to whether endometriosis is the result of explantation of endometrial glandular tissue from reflux menstruation or true metaplasia of the mesothelium, both theories implicate the PSE in the development of the disease. There is also evidence from subfertile women with endometriosis that an upset in the biochemical environment of the pelvis alone may disturb the reproductive physiology through ovarian and tubal mechanisms. There are several studies demonstrating increased levels of inflammatory cytokines in the peritoneal fluid of women with endometriosis including IL-1α (Odukoya, O.A. et al. 1997), IL-1β and TNFα (Taketani, Y. et al. 1992), IL-4 (Hsu, C.C. et al. 1997), IL-5 and IL-6 (Koyama, N. et al. 1993), IL-8 (Ryan, I.P. et al. 1995), IL-10 (Ho, H.N. et al. 1997) and VEGF (McLaren, J. et al. 1996). The interplay of these molecules with 11βHSD1 in the PSE would be an interesting avenue of research.

Several of these cytokines were specifically investigated using a PCR-based microarray to compare OSE and PSE. The advantages of such a technique were that it used small amounts of RNA compared to standard genome microarray, that the results could be generated in a shorter space of time and the costs were lower. The product also allowed comparison of cell responses across a large number of chosen genes that would have been prohibitive to examine individually using Taqman PCR because of cost, time and tissue resource. The first set of data generated compared basal gene expression in unstimulated OSE and PSE samples. These data suggested that there were some differences in gene expression between the two cell types. As the OSE and PSE samples were paired, there would be little difference expected, yet the analysis showed some small fold differences. Whether these differences are biologically important remains to be seen. The results of the fold changes with IL-1α treatment are likely to be more relevant. The array compared gene expression in
control and IL-1α-treated samples in PSE and OSE from the same patient. The results demonstrate that eight genes are significantly up-regulated in both OSE and PSE, while one gene, ERβ, is significantly up-regulated in OSE with IL-1α treatment and in PSE is also up-regulated although to a lesser extent (1.66-fold). There were four genes where the down-regulation was significant in both PSE and OSE and in the four other genes where the down-regulation occurred in OSE there was also a down-regulation in PSE. In all cases the fold changes were in the same direction for OSE and PSE. Another reason that suggests these data are biologically significant is that several have been reported in the literature before. IL-1α is known to increase 11βHSD1, COX2 (Rae, M.T. et al. 2004; Yong, P.Y. et al. 2002), lysyl oxidase (LOX), IL-6, IL-8 (Rae, M.T. et al. 2004) in the OSE so validating the results seen in this study. In studies of normal OSE and malignant epithelial cells CCL2 expression was induced and sustained by TNF-α (Szlosarek, P.W. et al. 2006). Its role involves recruitment of macrophages to sites of inflammation which is of clinical importance as described below. The modest rise in LOX in both OSE and PSE may suggest that these cells respond to inflammation by preparing to lay down collagen given LOX’s main function is to catalyse the cross-linking of elastin and collagen fibrils. Modest collagen synthesis may be important in basement membrane synthesis but if it goes unchecked could result in adhesion formation.

The finding of a down-regulation in E-Cadherin with IL-1 in both OSE and PSE is likely to be significant. Sundfeldt et al. first noted a difference in E-Cadherin expression in the normal human ovary with reduced expression in the OSE but greater expression within ovarian cortical inclusion cysts (Sundfeldt, K. et al. 1997) suggesting that increased expression might be related to the oncogenic process. This increase in E-Cadherin around inclusion cysts was confirmed by Maines-Bandiera et al (Maines-Bandiera, S.L. et al. 1997). Furthermore both these papers suggest that E-cadherin expression is more associated with OSE metaplasia. Interestingly the addition of IL-1α down-regulated E-cadherin in this study whereas IL-1α would be anticipated to induce metaplasia and morphologically cause epithelio-mesenchymal transition. Therefore there may be a difference in studying OSE in vitro or the location of the OSE may be more relevant in E-cadherin expression by interaction with other cell adhesion molecules. It is known to play a role in determination of cell
polarity which is important in the single layer of the mesothelium. E-cadherin has multiple functions other than the formation of tight junctions. Although it lacks intrinsic enzymatic activity it is able to influence cellular behaviour by interacting with cell signalling molecules including tyrosine kinases and tyrosine phosphatases. Many of its actions arise through intermediary proteins, often β-catenin. Its expression appears to be increased in many epithelial tumours, not least EOC although this is lost in poorly differentiated tumours (Jeanes, A. et al. 2008). So as suggested by these physiological studies the response to IL-1α in normal mesothelial cells is a down-regulation of E-cadherin. As E-cadherin may act as a tumour suppressor and inhibit mitosis, the loss of its expression could allow cellular proliferation in the OSE/PSE and thus resolve the cell loss caused by inflammation.

The down-regulation of 3βHSD1 by IL-1α was previously demonstrated by microarray and confirmed on RT-PCR in OSE by Rae et al. (Rae, M.T. et al. 2004). This was further explored in the OSE and evidence presented that IL-1α may be acting via the NFκB pathway (Papacleovoulou, G. et al. 2008). As 3βHSD1 is involved in the metabolism of all classes of steroid precursors, its down-regulation could significantly reduce steroid production in the mesothelium. The physiological advantage of this is not clear but could remove the growth inhibiting effects of progesterone and cortisol (Ivarsson, K. et al. 2001) and allow cell proliferation and repair to the mesothelial wound. Intriguingly 3βHSD2 expression is unchanged by IL-1α. The expression of progesterone receptor is unchanged following IL-1α treatment indicating mesothelial sensitivity to progesterone is likely to be unchanged.

ERβ is up-regulated 3-fold in OSE (only 1.66-fold in PSE), while ERα expression is unchanged by IL-1α. What is not revealed is the relative expression of these receptors in the mesothelium. While Hillier et al. have previously shown that both isoforms are present in OSE they were also unable to determine the relative abundances of the ER subtypes (Hillier, S.G. et al. 1998). There is very little in the literature regarding ER expression in normal human mesothelial cells but one study examined fibroblasts from normal and adhesion associated peritoneal tissue and discovered that both ERα and ERβ mRNA expression was greater in the tissue
derived from adhesions (Detti, L. et al. 2008). Lau et al. identified the ERα mRNA but not ERβ in a mesothelial cell line while examining differences in ER and PR expression between OSE and EOC (Lau, K.M. et al. 1999).

These data demonstrate for the first time that the PSE - like the OSE - is able to respond to inflammation by regenerating local cortisol by up-regulation of 11βHSD1. The pattern of 11βHSD1 response in both cell types is similar in dose, time, and exaggerated effect with cortisol. However the enzymic activity of 11βHSD1 in the PSE is considerably less than that of the OSE, which has important consequences. In most epithelial tissues inflammation leads to influx of inflammatory cells including monocyte/macrophages and fibroblasts which mediate tissue remodelling and laying down of scar tissue. In the peritoneum this can lead to the formation of intra-abdominal adhesions which under some conditions carry significant risks. However an idiosyncrasy of the ovary is its ability to heal without a scar and this could be explained by its relatively potent ability to regenerate local cortisol via up-regulation of 11βHSD1 which would be expected to inhibit local inflammatory cytokine and eicosanoid synthesis, thus minimising attraction of inflammatory cells and subsequent scar tissue formation. In contrast, while the PSE is able to up-regulate 11βHSD1 in response to inflammation, its ability to regenerate cortisol is rather more feeble, hence the brake on inflammatory cell infiltration is weaker and scar tissue (adhesions) more likely to result. The difference in 11βHSD1 enzyme activity between OSE and PSE was not replicated by relative levels of mRNA. However as shown in figure 4.13, there is a trend towards higher levels in the OSE and use of more samples may have allowed statistical significance to be reached. As 11βHSD2 expression is expressed at such low levels in the PSE and OSE, in addition to the fact that its levels are unchanged by any of the agents used in this study, any alteration in glucocorticoid shuttling is most likely to be due to swings in 11βHSD1 expression. Thus although there are no changes in the type 2 enzyme it was nevertheless important to demonstrate its expression in this set of experiments. The model for prevention of scar tissue formation in the ovary is realistic as it has been clearly demonstrated that OSE cells are able to convert cortisone to cortisol and that the rate of conversion is increased by IL-1α. However it is not clear what cellular component the glucocorticoid may be acting on. Candidates include the
monocyte/macroage fraction, fibroblasts and the mesothelial cells themselves. Bachus et al have provided evidence that proliferation of fibroblasts is inhibited by co-culture with macrophages which was overcome by administration of hydrocortisone (cortisol). Mesothelial cells however had increased proliferation rates in the presence of macrophages and this was not affected by hydrocortisone or IL-1 (Bachus, K.E. et al. 1995). Translation of this into the human peritoneum would suggest that fibroblast activation is held in check by a balance of macrophage and peritoneal cell populations. Indeed macrophages appear to have an important role in the prevention of adhesions. Adhesions were more common following chemical peritonitis in a macrophage Fas-induced apoptosis (Mafia) transgenic mouse model, in which macrophages are depleted, compared to the wild type (Burnett, S.H. et al. 2006). It may be that macrophages prevent adhesions by increasing ingestion of apoptotic neutrophils facilitating resolution of inflammation (Liu, Y. et al. 1999). In the mouse where 11-dehydrocorticosterone is converted to the active glucocorticoid corticosterone by 11βHSD1, mice deficient for the enzyme take longer to resolve an episode of acute inflammation (Gilmour, J.S. et al. 2006).

It is likely that macrophages have an important role in the inflammatory events at ovulation. These functions include secretion of proteolytic enzymes and phagocytosis of apoptotic cells but they may promote follicle development and atresia (Wu, R. et al. 2004). Macrophage numbers correspond with the menstrual cycle and inhibition of macrophage function by clodidronate reduces ovulation rate (Van der Hoek, K.H. et al. 2000). One reason that the ovary does not frequently produce adhesions with the pelvic peritoneum may be because the resolution of inflammation originates from the interior of the ovary and by the time ovulation occurs the anti-inflammatory cells and mechanisms have been primed allowing swift resolution and minimal scar tissue deposition.

In their mildest form peritoneal adhesions may have no clinical importance however in the female adhesion formation from whatever cause, be it infection, surgery or endometriosis, is associated with infertility. More extensive adhesions carry more serious and potentially life-threatening sequelae for example bowel obstruction.
Therefore in responding to an intra-abdominal inflammatory insult it is important that an organism responds to that insult appropriately allowing resolution of the inflammatory process but without over-reacting and creating potentially harmful peritoneal adhesions.

The discovery of dissimilar mesothelial cells in distinct areas of the peritoneum then broaches the prospect that it is the underlying tissue than influences mesothelial phenotype. Evidence for this concept is provided in this study by differential $^{11}$βHSD1 enzyme activities in OSE and PSE. What is not clear is whether the mesothelial cell arises from a single progenitor cell then differentiates once attached to the visceral surface or whether there are separate progenitor cell types for different organs. Evidence for the former is supplied by Foley-Comer et al and has been discussed in Chapter 1 (Foley-Comer, A.J. et al. 2002). The possibility that a defect in the ovarian surface could be filled by a mesothelial progenitor cell which then differentiates into the OSE phenotype (which is not so different from PSE) is highly appealing and describes an efficient and rapid mechanism for healing in a tissue where scar formation is often unhelpful and harmful. The isolation and characterisation of the mesothelial stem cell could have important clinical implications. Given that absence of mesothelial cells leads to adhesion formation, it is conceivable that administration of mesothelial cells, as an autologous transplant could be a pragmatic method for prevention of post-surgical adhesions and treatment of endometriosis and even ovarian cancer.

While the OSE is widely held as the source of epithelial ovarian cancer (EOC) the reaction of the peritoneum to the disease is likely to play an important role in its trans-coelomic spread. Mesothelial cells have been shown to be able to inhibit attachment of ovarian cancer cells to cell matrices (Kenny, H.A. et al. 2007) suggesting that autologous mesothelial cell transplants could also help prevent metastasis. Furthermore the striking similarities between EOC and primary peritoneal cancer (PPC) make it likely that they have a common cell of origin. It has been estimated that 10% of EOCs could be classified as PPC (Chu, C.S. et al. 1999) – though whether this matters clinically is contentious as both conditions are
managed similarly with surgery and the same chemotherapeutic regimens. Like EOC, BRCA1 and BRCA2 mutations increase the risk of developing the disease and PPC can still occur in women who have undergone prophylactic surgery. If these women had had their ovaries in situ would the disease have been called EOC instead?

In conclusion, this novel method of culturing human peritoneal surface epithelium has demonstrated that these cells closely resemble the cells covering the surface of the ovary morphologically and in their response to inflammation. In addition both cell types are receptive to progesterone which appears to act as an anti-inflammatory agent. However one major difference is the enhanced ability of the OSE to regenerate cortisol via up-regulation of 11βHSD1 which may explain the ovary’s ability to heal without a scar after injury and the peritoneum’s predisposition towards adhesion formation.
Chapter 5

IgLON Expression in Primary Cultures of Human Ovarian Surface Epithelium
5 IgLON Expression in Primary Cultures of Human Ovarian Surface Epithelium

5.1 Introduction

In any epithelial tissue cellular proliferation is a tightly regulated process. An excess of cellular material could impair tissue function while a shortage leaves the underlying stroma exposed and initiation of fibrosis and scarring could occur. Tissue homeostasis is dependent on signals informing progenitor cells when to divide and when to remain in a non-proliferative state. While there are multiple signals, many of these are transduced by the switching on and off of tumours suppressor genes (TSGs) and oncogenes. When a TSG is permanently switched off or an oncogene permanently activated, uncontrolled cellular division may result leading to tumour formation. One such TSG linked to ovarian cancer is opioid binding cell adhesion molecule-like (OPCML or OBCAM), introduced in Chapter 1 (Section 1.7.4.2).

OPCML is one of a family of four proteins which appear to have similar roles as immunoglobulin domain containing GPI-anchored cell adhesion molecules. The other molecules include limbic system associated membrane protein (LSAMP) and neuronal growth regulator-1 (NEGR1/Kilon), and neurotrimin (HNT). Little is known about the expression of these molecules outwith the central nervous system, but here they appear to form hetero- and homomeric dimers and play an important role in neuronal growth and development (Gil, O.D. et al. 2002; Lodge, A.P. et al. 2000; McNamee, C.J. et al. 2002; Miyata, S. et al. 2003; Reed, J. et al. 2004).

Evidence for OPCML’s role in ovarian cancer arises from studies showing loss of heterozygosity (LOH) in EOC at 11q25 (Sellar, G.C. et al. 2003). On further investigation the defining marker D11S4085 was found to lie within the second intron of the gene for OPCML. Furthermore transfection of OPCML DNA into an ovarian cancer cell line that did not endogenously express OPCML demonstrated
tumour suppressor activity in both *in vitro* and mouse *in vivo* models. In addition the majority of EOC tumours were found to have undergone epigenetic inactivation of *OPCML* and the discovery of only one *OPCML* mutation in over 200 tumours suggested that *OPCML* CpG island methylation, rather than gene mutation, was the most likely cause of malignant transformation. Thus *OPCML* acquired the label of a “new-age” TSG that includes CpG island methylation as a recognised method of gene inactivation; an updated definition of Knudson’s two-hit hypothesis. Having established this role for *OPCML* in malignant tissue it was important to then uncover its pattern of expression in normal tissue and in particular the ovarian surface epithelium, given its role as likely source of EOC. Interestingly Sellar *et al* chose whole ovary rather than OSE for quantitative RT-PCR analysis so the contribution of the OSE to these samples is likely to have been minimal given that the predominant tissue in whole ovary is stroma. Also their RT-PCR analysis included samples which had the OSE dissected from the ovary at surgery. Given that the OSE is only one cell thick it is not possible to dissect this layer off at surgery even with the aid of a microscope and it is highly likely that their samples would have consisted of mainly stromal cells. Thus once again the need to assess *OPCML* expression in pure OSE becomes clear. There is a paucity of data looking at hormone response elements in the IgLONs. However by examining the promoter sequences of each of the genes, transcription factor binding sites can be predicted (figure 5.1).
A) OPCML

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</tr>
<tr>
<td>IRF-1</td>
<td></td>
</tr>
<tr>
<td>PPAR-gammaZ</td>
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<td>Max1</td>
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</table>

Legend: ─ Transcription Starting site of OPCML | Transcription factor binding site
Scale: 13700 bp

B) NEGR1

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<td>CP2</td>
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<td>IRF-7A</td>
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Legend: ─ Transcription Starting site of NEGR1 | Transcription factor binding site
Scale: 9700 bp
Evidence has been presented in Chapter 1 suggesting a link between inflammatory damage of the OSE and development of EOC. Ovulation dramatically disrupts the
OSE at ovulation but the exact sequence of events at the OSE has never been definitively described, in particular how the OSE is reconstituted following wound formation. The author hypothesised that inflammation causes a down-regulation of the \textit{OPCML} tumour suppressor gene, allowing proliferation of OSE surrounding the damaged area of the ovary and subsequent repair. Having developed expertise in collection and culture of OSE the aim of this study was to establish whether OPCML was expressed in OSE, whether inflammation affected its expression and whether expression of other members of the IgLON family altered in tandem.

In order to test this hypothesis, samples of OSE were collected from premenopausal women undergoing surgery for benign gynaecological conditions and cultured in \textit{vitro}. In order to recreate the inflammatory stimulus associated with inflammation IL-1\textsubscript{\alpha} was used to treat the cells. IL-1\textsubscript{\alpha} is closely linked to inflammation in the ovary (Gerard, N. \textit{et al.} 2004) and its production is stimulated in the mouse by the luteinising hormone surge or HCG injection (Simon, C. \textit{et al.} 1994). IL-1\textsubscript{\alpha} initiates a pro-inflammatory cascade including cyclo-oxygenase (COX2) production, matrix metalloproteinase (MMP) activity and Nitric Oxide (NO) synthesis and as such is an ideal agent for mimicking ovulation in \textit{vitro}. The effects of IL-1 can be abrogated by the potent anti-inflammatory steroid cortisol and OSE was also treated with this agent to identify whether OPCML and IgLON expression was controlled by pro- and anti-inflammatory mediators.

\textbf{5.2 Methods}

\textbf{5.2.1 OSE Collection and Culture}

Samples were collected as described in Chapter 2. Relevant clinical details for patients donating OSE for this study are shown in Table 5.1.
Table 5.1 Relevant clinical details for patients donating OSE. TAH = total abdominal hysterectomy, BSO = bilateral salpingo-oophorectomy, DMPA = depo-medroxyprogesterone acetate, LAVH = laparoscopic assisted vaginal hysterectomy.
<table>
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<th>Patient Number</th>
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<th>Parity</th>
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<th>Indication for surgery</th>
<th>Operation</th>
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<td>TAH</td>
<td>Spatula</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>6+0</td>
<td>31</td>
<td>Fibroids, menorrhagia, dysmenorrhoea</td>
<td>TAH+BSO</td>
<td>Spatula</td>
</tr>
<tr>
<td>3</td>
<td>39</td>
<td>3+1</td>
<td>21</td>
<td>Menorrhagia, dysmenorrhoea, benign left ovarian cyst</td>
<td>Laparotomy+ Left oophorectomy</td>
<td>Spatula (Right ovary)</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>5+0</td>
<td>17</td>
<td>Menorrhagia</td>
<td>LAVH</td>
<td>Cytobrush</td>
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<tr>
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<td>Dysmenorrhoea, pelvic pain</td>
<td>Diagnostic laparoscopy</td>
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</tr>
<tr>
<td>6</td>
<td>34</td>
<td>4+1</td>
<td>Uncertain</td>
<td>Unwanted Fertility</td>
<td>Laparoscopic Sterilization</td>
<td>Cytobrush</td>
</tr>
<tr>
<td>7</td>
<td>34</td>
<td>4+0</td>
<td>18</td>
<td>Pelvic pain</td>
<td>Diagnostic laparoscopy</td>
<td>Cytobrush</td>
</tr>
<tr>
<td>8</td>
<td>45</td>
<td>2+1</td>
<td>15</td>
<td>Fibroids, menorrhagia</td>
<td>TAH+BSO</td>
<td>Spatula</td>
</tr>
<tr>
<td>9</td>
<td>25</td>
<td>0+1</td>
<td>Uncertain</td>
<td>Pelvic pain</td>
<td>Diagnostic laparoscopy</td>
<td>Cytobrush</td>
</tr>
<tr>
<td>10</td>
<td>38</td>
<td>0+0</td>
<td>No cycle - DMPA</td>
<td>Fibroids, menorrhagia, dysmenorrhoea</td>
<td>TAH</td>
<td>Spatula</td>
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<tr>
<td>11</td>
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<td>Diagnostic laparoscopy</td>
<td>Cytobrush</td>
</tr>
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</table>
5.2.2 Cell Experiment & RNA Extraction

Methods were as described in Chapter 2.

5.3 Results

5.3.1 Comparison of OPCML mRNA detection by Light Cycler and Taqman RT-PCR

5.3.1.1 Rotorgene Light Cycler

Analysis of the data from the light cycler was hampered by the low levels of OPCML message in the samples. On plotting the C_{T} fluorescence signal versus quantification as compared to a standardised RNA sample, the OSE sample data lay outside the standard curve, beyond the lowest concentration (figure 5.1). This meant that RNA quantification could not be accurately calculated with these data.
Figure 5.2  Light cycler RNA standard curve demonstrating $C_T$ standard samples (blue) and OSE samples (red).

Whilst these $C_T$ values lay outside the standard curve analysis of the melt curve for the DNA strands revealed identical features to the melt curve of the standards suggesting that the DNA products were due to the presence of the OPCML transcript and not because of primer-dimer formation (figure 5.2).

Figure 5.3  Melt curve analysis of OPCML PCR products following quantitative RT-PCR. Samples shown include RNA standard (uppermost curve) and six OSE samples. The bottom curve represents a no template control (water).
However if the CT values are extrapolated from the straight line obtained from plotting the RNA standards the results generated are shown in figure 5.3. Analysis of these data demonstrate a significant increase in OPCML mRNA expression with IL-1\(\alpha\) at 0.5ng/ml for 48h (P<0.01) compared to untreated cells, however there is no significant difference between IL-1\(\alpha\) treatment and IL-1\(\alpha\) in the presence of progesterone (at 1\(\mu\)M). In contrast there is a significant difference in OPCML mRNA expression between IL-1\(\alpha\) treated cells and IL-1\(\alpha\) in the presence of cortisol (also at 1\(\mu\)M) (P<0.01).

Figure 5.4  OPCML mRNA expression in OSE (n=3) using the Rotorgene light cycler in OSE cells treated with progesterone (P4), cortisol (F) (at 1\(\mu\)M) and interleukin-1\(\alpha\) (IL-1, 0.5ng/ml).

5.3.1.2 Taqman quantitative RT-PCR
Taqman RT-PCR analysis of OPCML mRNA expression using an assay-on-demand primer/probe assay mix gave quantifiable C_T values suggesting up-regulation of OPCML expression in OSE with IL-1α (see figure 5.4).

Figure 5.5  OPCML mRNA Expression in OSE using Taqman quantitative RT-PCR (n=3). Treatments include progesterone (P4), cortisol (F) (both 1μM) and interleukin-1α (IL-1) (0.5ng/ml for 48h).

Treatment with IL-1α caused a mean 4-fold up-regulation of OPCML expression in these OSE cell samples (figure 5.4). Treatment effects failed to reach statistical significance.

5.3.2 OPCML mRNA expression in human OSE
Having demonstrated that quantification of OPCML mRNA in OSE was more easily quantified with Taqman assay this became the method of choice for the remainder of the study.

5.3.2.1 Effect of time on OPCML mRNA expression induced by IL-1α

Four separate time course experiments were set up to investigate the profile of IL-1α induced OPCML expression over time (figure 5.5). Data were analysed by paired t-tests for individual time points.

Figure 5.6 Mean fold change in OPCML mRNA expression (compared to same time point control) in IL-1α treated OSE cells (0.5ng/ml) from 4 separate individuals, as a function of length of IL-1α treatment. Error bars indicate SEM, **=P<0.01.
The fold change in OPCML mRNA expression did not begin to rise until 24 hours incubation of IL-1α (2.4-fold), although this did not reach statistical significance. OPCML mRNA expression was significantly higher in IL-1α-treated cells at 48 hours (2.0-fold, P<0.01).

5.3.2.2  IL-1α Dose Response

![Figure 5.7 Dose response effect of IL-1α on OPCML mRNA expression by quantitative RT-PCR (n=8). Data were analysed using repeated measures ANOVA. Bars represent Mean / SEM. Asterisks indicate significant difference due to treatment (P<0.001).](image-url)
OPCML mRNA expression was significantly increased by each of the doses of IL-1α used, reaching a maximum fold induction of 3.1 with 0.5ng/ml. There was no significant difference in OPCML expression between the IL-1α treatments.

5.3.2.3 Cortisol Dose Response

The effect of cortisol on IL-1α induced OPCML expression was investigated using ten-fold changes in cortisol concentration (figure 5.7) in OSE cells from five different patients. IL-1α (at 0.5ng/ml for 48h) significantly increased the quantity of OPCML mRNA in the absence of cortisol (7.3-fold, P<0.001). OPCML mRNA expression was not significantly increased by IL-1α in the presence of cortisol at any concentration.
Figure 5.8 Effect of cortisol (F) in the presence and absence of IL-1α on OPCML mRNA expression by quantitative RT-PCR in OSE cells from five different patients. Points represent mean fold induction and error bars indicate SEM.

5.3.2.4 Interleukin-1α up-regulates OPCML mRNA expression

The next questions to be answered were whether there was any effect of inflammation using interleukin-1α (IL-1α) on OPCML expression, and whether cortisol had any opposing effect, as would be hypothesised by its anti-inflammatory nature.

Samples from seven different patients were used in experiments to assess the effects of cortisol and interleukin-1α on OSE cells. The results demonstrate a significant up-regulation of OPCML following treatment with IL-1α (4.7-fold, P<0.01) and a significant reduction in OPCML expression in IL-1α treated cells in the presence of cortisol (P<0.05) (figure 5.8).
Figure 5.9 OPCML mRNA expression by Taqman quantitative RT-PCR (n=7). Treatments included cortisol (F) (1 μM) and interleukin-1α (IL-1) (0.5 ng/ml). Different superscripts indicate significant differences due to treatment.

5.3.2.5 Cortisol, but not progesterone, abrogates IL-1α induced OPCML expression

Given the previously demonstrated anti-inflammatory effect of progesterone on cyclo-oxygenase expression in OSE cells (Rae, M.T. et al. 2004), experiments using OSE from a further nine separate patients were undertaken to compare the effects of cortisol and progesterone on IL-1α-induced OPCML expression (figure 5.9). The results demonstrate that neither cortisol nor progesterone have any effect on OPCML mRNA expression alone. There is a significant increase in OPCML expression with
addition of IL-1α (at 0.5 ng/ml for 48h) (3.8-fold, P<0.01). Compared to IL-1α alone, OPCML mRNA expression is significantly lower in IL-1α plus 1μM cortisol treated cells (1.4-fold, P< 0.01) but not in IL-1α plus 1μM progesterone treated cells (2.7-fold, P>0.05).

![Figure 5.10 OPCML mRNA expression by Taqman quantitative RT-PCR (n=9). Bars represent Mean/SEM. Differing superscripts indicate significant differences due to treatment.]

5.3.2.6 A Comparative study of OPCML mRNA expression in EOC, OSE and PSE.

Control RNA samples from thirteen EOC, three OSE and four PSE cell cultures were analysed on the same Taqman plate in duplicate. RNA quantity was expressed
relative to a standard quantity of Fallopian tube RNA (discovered to contain high levels of OPCML mRNA). The relative quantities are shown in figure 5.10. As OPCML level was not normally distributed, non-parametric statistical testing was used.

Figure 5.11  OPCML mRNA expression in EOC, OSE (open) and PSE (hatched). EOC sample number refers to table 3.1, A indicates ascites, T primary tumour and M metastatic tumour sources of cell culture.

There was no statistical difference in OPCML mRNA expression between the cell types (figure 5.11).
5.3.3 OPCML Protein expression in human OSE

Western blotting, using protein lysates of cultured OSE, was used to assess presence of OPCML in normal human OSE (figure 5.12). Despite several attempts, no band was seen corresponding to OPCML in the OSE samples, yet the positive control sample of an OPCML over-expressing SKOV-3 cell line BKS2.1 confirmed antibody binding at correct molecular weight corresponding to OPCML. Subsequent staining for β-actin demonstrated even protein loading of these samples.
Figure 5.13 Western blot of OPCML and β-actin expression in human OSE by SKNV3.3 cells were used as a negative control and the SKOV-3 OPCML over-expressing cell line BKS2.1, as a positive control for OPCML.

5.3.4 *IgLON mRNA expression in human OSE*

Previous studies have suggested correlations between expression of OPCML and the other IgLON proteins. In order to examine whether such a relationship existed Taqman quantitative RT-PCR was used to analyze mRNA expression of other IgLONs in a selection of OSE RNA samples.
5.3.4.1 NEGR1

5.3.4.1.1 IL-1α Effects on NEGR1 expression

OSE samples from four separate patients were analysed to investigate the effect of five-fold increments in IL-1α dose on NEGR1 expression. While there was a statistically significant increase in NEGR1 mRNA expression observed at each IL-1α concentration (1.6, 1.8 and 1.6-fold for 0.02, 0.1 and 0.5ng/ml respectively, figure 5.13), there was no difference in magnitude of effect between the different concentrations.

![Figure 5.14](image)

*Figure 5.14* Histogram showing the effect of IL-1α (over 48h) on NEGR1 mRNA expression by quantitative RT-PCR (n=4). Bars represent Mean / SEM. Asterisks indicate significant difference due to treatment (*=P<0.05, **=P<0.01).
5.3.4.1.2 Effect of time on NEGR1 expression

To investigate whether IL-1α might have an effect on NEGR1 mRNA expression at time point other than 48 hours, Taqman quantitative RT-PCR was used to analyse OSE samples from four different patients after 6, 12, 24 and 48 hour exposures to IL-1α. The only significant effect of IL-1α over untreated control occurred at 12 hours where the fold induction of NEGR1 was 1.4 (P<0.05) (figure 5.14).

![Figure 5.15 Fold change in NEGR1 mRNA expression compared to untreated control after different lengths of exposure to IL-1α (0.5ng/ml)(n=4). Error bars indicate SEM, **=P<0.01.](image-url)
5.3.4.1.3 IL-1α in the presence of cortisol down-regulates NEGR1 mRNA expression

Taqman quantitative RT-PCR was used to investigate NEGR1 expression in OSE from six separate patients treated with cortisol, IL-1α and both together (figure 5.15). Cortisol treatment alone caused a small but significant rise in NEGR1 expression (1.3-fold, P<0.05) but IL-1α alone caused a similar size of mean rise in NEGR1 mRNA that failed to reach statistical significance (1.3-fold, P>0.05). The combination of IL-1α and cortisol however caused a significant down-regulation of NEGR1 (0.56-fold, P<0.01).

![Figure 5.16 Histogram of NEGR1 mRNA expression in OSE (n=6) treated with cortisol (F) (1μM) and interleukin-1α (IL-1) (0.5ng/ml) over 48h. Different superscripts indicate significant differences due to treatment.](image-url)
5.3.4.1.4 Progesterone does not significantly influence NEGR1 mRNA expression

A comparison of the effects of cortisol and progesterone was undertaken using OSE samples from five separate patients (figure 5.16). Neither cortisol nor progesterone had any significant effect on NEGR1 mRNA expression, nor did IL-1α alone. In the presence of IL-1α, progesterone had no significant effect but cortisol caused a significant down-regulation of NEGR1 (0.53-fold, P<0.05).

![Figure 5.17](image)

**Figure 5.17** Histogram of NEGR1 mRNA expression in OSE (n=5) treated with cortisol (F) (1μM), progesterone (P4) (1μM) and interleukin-1α (IL-1) (0.5ng/ml, over 48h). Asterisk indicates significant difference due to treatment (P<0.05).
5.3.4.1.5 NEGR1 Cortisol Dose Response

Experimental culture media with ten-fold differences in cortisol concentration were used to investigate the effect on NEGR1 mRNA expression (figure 5.17). Experimental data were obtained from two experiments therefore statistical analysis could not be undertaken. However in the absence of cortisol, mean NEGR1 expression was increased by IL-1α (to 1.6-fold). On addition of cortisol there was a down regulation of NEGR1 expression when concentrations of $10^{-7}$M and $10^{-6}$M were used, but not at $10^{-8}$M.

![Graph showing the effect of increasing cortisol concentration on NEGR1 expression in the presence and absence of IL-1α (0.5ng/ml, over 48h).](image)

**Figure 5.18** Effect of increasing cortisol concentration on NEGR1 expression in the presence and absence of IL-1α (0.5ng/ml, over 48h).
5.3.4.2 LSAMP

5.3.4.2.1 LSAMP levels are unchanged by IL-1α

LSAMP mRNA levels are static in OSE cells when challenged by increasing doses of IL-1α and when incubation time is increased up to 48 hours (figure 5.18).
5.3.4.2.2 In the presence of cortisol, IL-1α down-regulates LSAMP

While neither cortisol nor IL-1α have any independent effect on LSAMP mRNA expression they appear to act together and significantly down-regulate LSAMP (0.25 compared to untreated control, p<0.001, n=7). In contrast progesterone does not have significant effect on LSAMP on its own or in combination with IL-1α (n=5) (figure 5.19).
LSAMP mRNA expression (fold induction over untreated control)

- **Control**
- **F**
- **IL-1**
- **IL-1+F**

**Control P4 F IL-1 IL-1+P4 IL-1+F**

LSAMP mRNA expression (fold induction over untreated control)

- **Control**
- **P4**
- **F**
- **IL-1**
- **IL-1+P4**
- **IL-1+F**

**Control F IL-1 IL-1+F**

**Control P4 F IL-1 IL-1+P4 IL-1+F**
Figure 5.20  Histograms showing LSAMP mRNA expression after treatment with progesterone (P4), cortisol (F) and Interleukin-1α (IL-1α). Bars represent mean / SEM. Asterisks indicate significant differences due to treatment (**= P<0.01, ***=P<0.001).

5.3.4.2.3 Dose dependent effects of cortisol on LSAMP mRNA expression

While the mean fold change in LSAMP with cortisol was lower than the untreated control values this did not reach statistical significance (figure 5.20). However paired t-tests indicated that cortisol at 1μM in the presence of IL-1α significantly down-regulated LSAMP (P<0.05), compared to cortisol alone.

Figure 5.21  LSAMP mRNA expression in OSE cells (n=5) with ten-fold changes of cortisol (F) concentration, in the presence and absence of IL-1α (0.5ng/ml, over 48h). Points indicate mean expression compared to untreated control and bars = SEM. *=P<0.05.
5.3.4.3 Neurotrimin (HNT)

5.3.4.3.1 IL-1α up-regulates HNT – an effect obliterated by cortisol

Seven separate OSE samples were analysed to assess the effect of cortisol and IL-1α on HNT expression. Cortisol and IL-1α were both found to up-regulate HNT mRNA expression (1.4 fold, \(P<0.01\) and 3.3-fold, \(P<0.05\), respectively). HNT mRNA expression following treatment with the combination of cortisol and IL-1α was not significantly different to the control sample (figure 5.21).

Figure 5.22  HNT mRNA expression in OSE treated with cortisol (F), IL-1α or both F and IL-1α over 48h\((n=7)\). Bars represent mean/SEM. Asterisks indicate significant difference due to treatment \(\ast=P<0.05\), \(\ast\ast=P<0.01\), \(\ast\ast\ast=P<0.001\).
5.3.4.3.2 Absence of Progesterone effect on IL-1α induced HNT expression

Analysis of OSE samples from four experiments revealed a non-significant increase in HNT mRNA expression following IL-1α treatment (4.1 mean fold change). Although no treatment achieved a statistically significant effect, IL-1α induced HNT expression was suppressed by cortisol and not by progesterone (figure 5.22).

![Graph showing HNT mRNA expression in OSE cells (n=4) following treatment with progesterone (P4) (1μM), cortisol (F) (1μM), IL-1α (0.5ng/ml) or in combination over 48h. Bars represent mean/SEM.](image)

**Figure 5.23** HNT mRNA expression in OSE cells (n=4) following treatment with progesterone (P4) (1μM), cortisol (F) (1μM), IL-1α (0.5ng/ml) or in combination over 48h. Bars represent mean/SEM.

5.3.4.3.3 Absence of IL-1α Dose Response Effect

Analysis of three OSE specimens where the concentration of IL-1α had been escalated in a five-fold manner did not demonstrate any significant corresponding up-regulation of HNT mRNA (figure 5.23).
5.3.4.3.4 Cortisol Dose Response

Taqman quantitative RT-PCR analysis was used to define HNT mRNA expression in two separate OSE experiments where there was a ten-fold difference in cortisol concentrations. In the absence of cortisol but presence of IL-1α, the HNT mRNA level was nearly three times that of the control sample. However even at low cortisol concentrations HNT expression was similar in the presence and absence of IL-1α. (figure 5.24).

Figure 5.24 HNT mRNA expression with increasing concentration of IL-1α at 48h(n=3). Bars represent mean/SEM.
Figure 5.25  HNT mRNA expression in the presence and absence of IL-1α (0.5ng/ml) after 48h with increasing cortisol concentration (µM) (n=2). Closed squares indicate HNT expression with IL-1α absent and open squares with IL-1α present.

5.3.4.3.5 Time course of IL-1α treatment on HNT expression

Quantitative RT-PCR analysis of four separate OSE experiments did not show any significant difference in HNT expression with IL-1α treatment (0.5ng/ml) over time intervals up to 48 hours (figure 5.25).
Figure 5.26  HNT mRNA expression following IL-1α treatment relative to same time point control in OSE (n=4). Bars indicate SEM.

5.3.4.4 Cyclooxygenase 2 mRNA expression

The OSE samples were used to determine COX2 mRNA expression also. As COX2 mRNA expression data in OSE have been previously published in the literature and its behaviour more predictable, it is a good candidate for reflecting the inflammatory status in this tissue, with high COX2 indicating an inflammatory response.

5.3.4.4.1 Dose dependent effect of IL-1α

Data were obtained from two experiments confirming a dose dependent effect of IL-1α on COX2 mRNA expression (figure 5.26).
5.3.4.4.2 Cortisol Inhibits IL-1α induction of COX2 mRNA in normal human OSE

In five separate experiments OSE cells were treated with IL-1α (0.5ng/ml), cortisol (1μM) and a combination of both (figure 5.27). IL-1α alone caused a significant up-regulation of COX2 mRNA expression (mean 45.2-fold, P<0.001). COX2 mRNA expression after treatment with cortisol and IL-1α together was not significantly different from control.
Figure 5.28  COX2 mRNA expression in OSE cells (n=5) after treatment with cortisol (F)(1μM), IL-1α (0.5ng/ml) and in combination over 48h. Bars represent mean/SEM. Asterisks indicate significant difference due to treatment (***=P<0.001).

5.3.4.4.3 Progesterone does not show any anti-inflammatory effect in OSE

In a further five different experiments, OSE cells were treated with progesterone (1μM), cortisol (1μM), IL-1α (0.5ng/ml) or a combination of IL-1α plus steroid (figure 5.28). These data demonstrated that steroid alone had no effect on COX2 mRNA expression. However IL-1α caused a significant up-regulation of COX2 mRNA expression (mean 14.4-fold induction, P<0.01). The presence of cortisol (2.1
mean fold change over untreated control) but not progesterone (mean fold change 15.3, P<0.01) inhibited IL-1α induction of COX2 mRNA.

Figure 5.29  COX2 mRNA expression in OSE cells (n=5) following treatment with progesterone (P4) (1μM), cortisol (F) (1μM) and IL-1α (0.5ng/ml). Bars represent mean/SEM. Dissimilar superscripts indicate significant difference due to treatment.

5.3.4.4.4 The rise in COX2 mRNA following IL-1α treatment is seen from 6 hours post-treatment

Data from two separate experiments indicate that COX2 mRNA rises from 6 hours following treatment (mean fold induction 5.5) and remains persistently elevated for 48 hours (mean fold induction 6.9) (figure 5.29).
Figure 5.30  COX2 mRNA expression following treatment with IL-1α (0.5ng/ml) (n=2).
5.4 Discussion

Scientific advances in ovarian cancer research have not yielded significant improvements in clinical outcomes and the disease is still associated with a poor prognosis for most patients. Thus identification of novel genes that could influence EOC development is often accompanied by much enthusiasm, optimism and expectation. The discovery of OPCML as a potential tumour suppressor gene for epithelial ovarian cancer in 2003 was no different with national and international scientific and lay press interest. While Sellar’s paper identified epigenetic inactivation of OPCML as a mechanism for development of ovarian pathology (Sellar, G.C. et al. 2003), there are no publications highlighting a role for OPCML in normal ovarian physiology. Indeed, until 2003 there was no evidence for a role for OPCML outside the nervous system. The data presented in this chapter are the first to evaluate OPCML’s role in the normal human ovarian surface epithelium.

Using previously described methods for OSE culture and collection (Hillier, S.G. et al. 1998; Rae, M.T. et al. 2004; Rae, M.T. et al. 2004; Yong, P.Y. et al. 2002), experiments were initiated where the effects of inflammation on OPCML and IgLON expression could be studied. While it is likely that during ovulation the OSE is exposed to a variety of inflammatory mediators, in these experiments IL-1\(\alpha\) alone was used. The rationale for this was firstly to minimise the number of variables in any experiment. Secondly, IL-1 is likely to have a dominant role in ovulation induced inflammation; IL-1 activity has been identified in human follicular fluid (Barak, V. et al. 1992) and influences key components of the inflammatory cascade including prostaglandin, nitric oxide and steroid hormone production as reviewed by Gerard (Gerard, N. et al. 2004). Thirdly there was extensive local experience of IL-1\(\alpha\) effects on OSE, in particular on 11beta hydroxysteroid dehydrogenase type 1 (11βHSD1).
Initially work was carried out in two separate laboratories. OSE cell culture experiments were undertaken in the laboratory of Professor Hillier, Centre for Reproductive Biology, University of Edinburgh. The RNA from these experiments was extracted, quantified and quality assessed then transferred to the University of Edinburgh Cancer Research Centre where RNA quantities were assessed using Rotorgene light-cycler quantitative RT-PCR technology. As highlighted in 5.3.1.1 this system was unable to provide meaningful values for OPCML mRNA due to the low quantities of this messenger RNA in the sample, with OPCML signal only appearing late in the reaction and almost always after the most dilute standard. Attempts were made to improve detection of OPCML in OSE samples by increasing the number of cycles, increasing the extension time in the PCR reaction, doubling the quantity of RNA used per reaction to 20ng/μl and finally using a sixth and seventh RNA standard. None of these alterations had a significant impact on the ability to detect OPCML in these specimens and drawing the conclusion that OPCML mRNA levels in cultured human OSE was nearly undetectable using this technique. Because the primers and RNA standards were being used extensively in cell line work from the same laboratory group that first published data on OPCML in ovarian cancer it was likely that OPCML RNA levels were extremely low in normal OSE, rather than there being an inability to quantify the RNA. Indeed melt curve analysis of the DNA products from OSE samples demonstrated an identical profile to the OPCML standard (figure 5.2), suggesting that the signal that was obtained was a true representation of OPCML DNA and not due to primer dimer formation. It could be argued that cultured OSE was not a true representation of OSE in vivo but the time spent in culture was relatively short (certainly compared to cell lines) and other mRNAs are not lost following culture (e.g. 11βHSD1).

The mechanism of fluorescence in the Rotorgene light-cycler is via SYBR green dye which selectively binds double stranded DNA(dsDNA) (Zipper, H. et al. 2004). An alternative method of RNA/DNA quantification is Taqman quantitative RT-PCR (Applied Biosystems). Here fluorescence remains the method of DNA quantification
but rather than indiscriminate binding of dye to dsDNA, fluorescent “reporter” dye is released from a “quencher” when DNA polymerisation occurs at the site of the probe for the gene of interest. Thus specific probe binding is required before a fluorescent signal can be generated. This enabled detection of OPCML at a level that could be quantified and as the primer/probe assay was commercially generated quality control was guaranteed. Hence the Taqman assay being the preferred method of RNA quantification in this study.

The discovery of a rise in OPCML mRNA in response to IL-1α was contrary to the initial hypothesis. It was predicted that in order for OSE cells to recover from the inflammatory damage caused by ovulation, the OPCML TSG would require to be switched off, thus allowing cellular proliferation. Furthermore, the only previously published study describing a change in OPCML following IL-1 treatment reported a 4-fold reduction in OPCML(OBCAM) in a microarray study of IL-1 on a chondrocyte cell line (SW1353) (Vincenti, M.P. et al. 2001). In Vincenti’s study IL-1 exposure was only two hours but 48hour exposure used for the OSE study is considerably longer than would be encountered in vivo. Indeed activation and phosphorylation of transcription factors may occur within minutes of IL-1 administration but this was not a primary endpoint of this study. The dose that was used for experiments in this thesis (0.5ng/ml) was 20 times less concentrated than in Vincenti’s study (10ng/ml). The decision to use 0.5ng/ml and for 48 hours was borne out by the finding that OPCML mRNA only reached significant up-regulation at 48 hours (like 11βHSD1). Moreover the minimal concentration of IL-1α required to up-regulate OPCML was 0.02ng/ml and thus could have been lowered further. The endogenous IL-1 receptor (IL1R) and IL-1 receptor antagonist (IL1RA) were not measured in this study but using lower dose IL-1 and more prolonged incubation times would increase the likelihood of their levels being important. A short exposure of high dose IL-1 such as in Vincenti’s study would likely be so overwhelming as to negate the importance of these mediators of IL-1 bioactivity and therefore be less physiological in the context of chronic inflammation. Conversely it could be argued that as ovulation is a rapidly resolving episode of acute inflammation, perhaps Vincenti’s model is a rather good model of the inflammatory events surrounding ovulation. Furthermore a chondrosarcoma cell line is likely to behave quite
differently to a primary cell culture of normal untransformed cells. Nevertheless any extension of this thesis’ work should include an investigation of higher dose IL-1 over shorter time periods.

With regard to IL-1 responsiveness it was noted that for individual OSE experiments there was an all or nothing effect where some individuals failed to mount an IL-1 induced rise in OPCML mRNA. In 7 of the 17 patient samples the OPCML fold change following IL-1 treatment was less than 2. This may indicate that OPCML is non-functional in these individuals. What it also suggests is that OPCML is not a necessary mediator in the response to IL-1α. However in those women whose OSE cells did respond to IL-1 by up-regulating OPCML there was a consistent abrogation of this effect by cortisol (section 5.3.2.5). Whether OPCML responsivity could be utilised as a marker for disease development remains to be elucidated but the cohort of patients here could be used for such a prospective study if ethical approval was obtained. It can be concluded that in the OSE of most women OPCML shows characteristics typical of an inflammation-associated gene.

Studies by Rae et al have indicated that progesterone may have an anti-inflammatory role in the OSE (Rae, M.T. et al. 2004). These findings were not replicated in this study and the addition of progesterone to IL-1-containing media appeared to have little effect on IL-1 induced up-regulation of both OPCML and COX2 (sections 5.3.2.5 and 5.3.4.5.3 respectively). Reasons for this disparity were not investigated in the interests of time and resources but the progesterone used came from a laboratory stock solution and was biologically active in other experiments.

Quantitative RT-PCR had indicated that levels of OPCML mRNA were low in samples of cultured OSE. An investigation of OPCML protein expression failed to demonstrate its presence in OSE. While OPCML protein was readily identifiable in the positive control sample, it was not detectable in any of the OSE samples. Adequate protein loading of the OSE wells was demonstrated by bands for β-actin,
suggesting that OPCML protein is not likely to be present in OSE in significant amounts. The fact that even in the IL-1 treated samples (where OPCML levels would be anticipated to be higher) OPCML was not present suggests that either OPCML protein detection was not sensitive enough or OPCML is absent in the human OSE. Another explanation could be that the OPCML antigen in the OSE is altered by IgLON dimerization (discussed later in this chapter) and thus not “visible” to the antibody. Western blotting was attempted from two different patients therefore it may be that OPCML expression varies between individuals and that the two chosen were simply low expressors of OPCML. A large proteomic study could be used to demonstrate OPCML expression in the general population.

Quantitative RT-PCR was also used to determine the quantity of OPCML mRNA amongst samples of EOC primary culture, OSE and peritoneal surface epithelium (PSE). The number of samples was limited by the number of wells on the PCR plate. However for the panel of samples selected there was no difference in mean OPCML level between the samples. For such a small number of samples however it is possible that statistical testing would not be able to distinguish a significant difference hence the need to expand the number of samples in each group for a definitive answer. However in Sellar’s original paper OPCML mRNA was present in normal ovary but absent in a panel of ovarian cancer cell lines. Here the primary EOC cell cultures had some of the highest levels of OPCML. This could be explained by cellular contamination of primary EOC cultures by cells other than malignant tumour cells. Alternatively the OSE sample in the published paper may not contain pure OSE. The only other study examining OPCML in normal ovary and EOC also demonstrated significant overlap of OPCML quantity in the two tissue types (Ntougkos, E. et al. 2005). Finally as already highlighted the quantity of OPCML in OSE appears to be very low overall so the quantities may be extremely low compared to other tissues. Indeed evidence for this arises from the comparison with Fallopian tube RNA that was used as the standard. While the same quantity of total RNA per sample was used to make cDNA, values for the test samples are $10^5$ fold lower than that seen in the Fallopian tube RNA.
The discovery of high levels of OPCML mRNA in Fallopian tube may provide an avenue for further research. OPCML protein has been discovered in murine and human Fallopian tube (Dr G Sellar, personal communication). Given OPCML’s likely extracellular domain and role in directing neuronal growth, it is highly probable that it plays an important role in cell to cell signalling. The oviduct’s ability to identify and transport ova following ovulation is poorly understood, but is an area which has significant implications for understanding human reproductive physiology and pathology. It has been suggested that dysfunction of the fimbrio-ovarian relationship could be the cause of some cases of unexplained infertility (Roy, K.K. et al. 2005). With regard to ovarian cancer, there is increasing evidence for the Fallopian tube being the source of some cases of EOC, discussed in Chapter 1 and reviewed by Piek (Piek, J.M. et al. 2008). In summary while a role for OPCML in the OSE has yet to be fully defined, the role of OPCML in tubal function and disease is an area ripe for study.
The current state of knowledge of OPCML function suggests that in the central nervous system it forms dimers with itself and other members of the IgLON family (Reed, J. et al. 2004). With regard to the IgLONs other than OPCML, Ntougkos’ paper suggested that HNT was increased in ovarian cancer compared to normal ovary, while LSAMP and NEGR1 were lower. The data presented in this thesis are the first to describe the effect of IL-1 on the other IgLON family members in human OSE.

The emerging theme from these studies is that IL-1α alone has no effect on expression of NEGR1 and LSAMP, but that there is a significant down-regulation of both when IL-1α and cortisol are used in combination (sections 5.3.4.1.3 and 5.3.4.2.2). This down-regulation is not seen when IL-1α is used in the presence of progesterone. The mechanism behind this synergistic effect requires further study but it is possible that the pro-inflammatory effect of IL-1α primes the cells thus enhancing the effect of cortisol. In contrast both OPCML and HNT are up-regulated by IL-1α, an effect that is lost when cortisol is added. The configuration of IgLON expression in OSE places the individual members in quite separate categories and the data suggest similarities between OPCML and HNT, and the other pairing of NEGR1 and LSAMP. It seems likely that NEGR1 and LSAMP are constitutionally expressed while OPCML and HNT are more dynamic members of the IgLON family. The concern raised earlier of OPCML levels being so low to suggest lack of function may be countered by considering that small changes in OPCML levels might have amplified effects on dimer function and thus enhancing cell-cell adhesion or communication. The functional relevance of these IgLON expression patterns in OSE now requires further attention.
Chapter 6

General Discussion and Future Directions
6 General Discussion and Future Directions

The underlying theme of this thesis is the exploration of mechanisms in primary cell cultures that may be relevant to the origin of epithelial ovarian cancer (EOC). For a relatively prevalent disease about which so much is known, it is remarkable how little is known about its natural history. While much epidemiological evidence supports the incessant ovulation theory of damage to the ovarian surface epithelium (OSE) as the initiating event, the direct evidence for this is lacking. There is much debate even about the tissue from which EOC originates, with several groups favouring the Fallopian tube over the OSE, with further evidence that endometrioid histiotypes may arise within endometriosis. Nevertheless the OSE remains a likely candidate for some, if not all, types of EOC and worthy of research.

While there is extensive knowledge of glucocorticoid metabolism in the OSE, there is little published literature regarding glucocorticoid metabolism in EOC. It was this deficiency that stimulated the work presented in Chapter 3, where it was hypothesised that $11\beta$HSD1 expression is deregulated in cancer and that an imbalance in $11\beta$HSD1 and $11\beta$HSD2 isozymes alters generation of local cortisol, favouring proliferation of malignant cells. Previous published work had suggested this to be the case in ovarian cancer cell lines (Gubbay, O. et al. 2005) and a group from Birmingham, UK had reviewed evidence for a predominance of $11\beta$HSD2 in some cancers (Rabbitt, E.H. et al. 2003). The mRNA studies described in Chapter 3 would appear to agree with Gubbay’s data and suggest an abrogation of the $11\beta$HSD1 mRNA response to inflammatory stimulation by IL-1α in EOC. However on reviewing the basal unstimulated mRNA levels in OSE and EOC primary cultures, there was no discernible difference. Furthermore using radio-labelled cortisone there was no significant difference in $11\beta$HSD1 enzyme activity between normal OSE and EOC primary cultures. Yet these metabolism assays were missing from Gubbay’s paper. For comparison it would perhaps have been useful to have
included some EOC cell lines in the metabolism assays here. The biologically significant finding is that levels of type 2 11βHSD mRNA appear to be higher within the malignant cultures compared to normal. These findings suggest that within malignant tissue not only is there a deficiency in production of cortisol (by dysfunction of 11βHSD1) but there is also a shift in glucocorticoid metabolism towards dehydrogenation of cortisol by higher levels of type 2 11βHSD. As target tissue effects of cortisol include a drive towards cell differentiation and less proliferation, it would be reasonable to assume that within the malignant tissue cells are more likely to proliferate and less likely to differentiate. It is therefore possible that these changes in 11βHSD dynamics could form an important step in the genesis of ovarian cancer.

There are many advantages of working with primary cell cultures over cell lines however. The first is that it is likely that the primary culture reflects the original tumour much more realistically than a cell line as it is derived directly from the patient and as such makes the research more clinically relevant. Secondly cell lines have been selected for their ability to persist in culture and as such may have lost many features relating to the original tumour. Thus the endurance of cells over many years and passages promotes further genetic mutation and divergence from the original cell line isolated. The assertion that the cell line behaves just like a cancer is potentially misleading. In contrast the cell line does offer more reproducibility than the primary cell culture which not only carries the inter-patient genetic and histological differences, but which may contain more than one cell type. Timing of experiments can be much more closely controlled with cell line work whereas primary cell culture is dependent on clinical cases presenting and being recruited. There are obvious ethical and bureaucratic constraints with primary cell culture, where ethical approval and compliance with good practice guidelines is essential; an aspect of research which plays much less heavily in cell line experimentation. In the work presented here the comparison of like with like was felt to be important and thus normal OSE was collected from patients and compared directly with EOC primary cultures rather than cell lines. A further advantage of the prospective
method of primary cell culture used here was that each sample could be considered a
case study and indeed there were some interesting findings at individual sample
levels such as the switch in 11βHSD1 and 11βHSD2 isozymes seen in a patient with
highly proliferative undifferentiated cells (patient 13, figure 3.8).

With regard to future directions for the EOC primary cultures, it is important to
establish the complement of cells within these cultures, and if possible encourage
growth of malignant cells only. However this may further remove the culture from its
original primary nature, where the malignant cell component may be dependent on
the supporting stromal cell network. Taking this further it may be more clinically
relevant to assess growth of clumps of malignant cells (spheroids) seen in ascites, or
tumour explants, in three dimensional culture, than dispersed monolayers of cells.
Another aspect of primary cell culture that warrants investigation is cell co-culture
where normal mesothelial cells could be cultured in the same medium but in a
separate chamber from malignant cells. The signalling between the cell types could
be investigated by analysis of culture medium or treatments added to the culture
medium and responses in the primary cells investigated. The response of the
peritoneum to malignant cells is an important step in dissemination of ovarian cancer
and inhibition of this process could lead to improvements in the clinical course.

The search for a cell of origin of EOC may ultimately turn out to be fruitless as the
cancer could arising from a stem cell, which may have potential for differentiation
into different histological subtypes of EOC. Evidence for the concept of cancer
stem cells (CSC) in EOC is accumulating. One study from India suggested that
CSCs can be derived from EOC ascitic spheroids (Bapat, S.A. et al. 2005). Their
reasons for concluding that there were stem cells present were that:

“(a) they self-renew and are clonogenic, (b) differentiate in vitro to form organized
spheroids in suspension, (c) express multipotency and tissue-specific differentiation
markers, (d) express self-renewal mechanisms in vivo (sequential tumorigenicity),
and (e) undergo *in vivo* differentiation to produce a disease similar to that in the patient”.

The evidence for CSCs is drawn from clinical observations that not all malignant cells need to be destroyed by chemo or radiotherapy for a cancer to be cured, and even if some malignant cells remain they do not proceed to clinical disease recurrence. In development of serial tumour transplantation whereas inoculation of mice requires large numbers of cells initially, fewer numbers are needed for transplant with each passage suggesting selection of a self renewing stem cell. In addition the frequent relapse seen in EOC following treatment might suggest a quiescent group of stem cells, that may have elevated multi-drug resistance (MDR) pump activity (a feature of stem cells) that undergo renewal once the chemotherapy has finished. However there remains some debate as to the existence of CSCs reviewed by (Hill, R.P. 2006) and further analysis of primary tumours and ascites looking for stem cell markers would be useful.

The concept of a mesothelial stem has already been discussed but the similarities between the OSE and PSE revealed in Chapter 4 would support a common cell of origin. The OSE has long been heralded as a unique type of cell capable of differentiation into multiple histological subtypes when it undergoes malignant transformation. However if a disease indistinguishable from EOC (i.e. primary peritoneal carcinoma) can arise in women whose ovaries and tubes have been removed, the PSE deserves attention as a possible source of malignancy. In the studies described in Chapter 4, evidence is presented that the OSE and PSE may be more alike than has previously been acknowledged. Certainly both are able to up-regulate 11βHSD1 mRNA and enzyme activity in response to IL-1α-induced inflammation. However the only distinction is that the degree of enzyme response is greater in the OSE. While it could be conceded that the metabolism assays might have benefited from a larger number of samples to provide greater statistical confidence, the result appears to demonstrate a difference in cortisol generation according to site of mesothelial cell sampling. This may explain why the ovary heals
without adhesion formation following ovulation, whereas the peritoneum, which has less 11βHSD1 activity, is more prone to adhesion formation. Unanswered questions are: where do the cells that repopulate the OSE following ovulation arise from, is there a free-floating circulating population of mesothelial progenitor cells that are able to quickly repair damaged mesothelium, and do the OSE and PSE share a common stem cell? The successful primary culture of PSE demonstrated here will allow further comparison of the cell types. One relevant experiment would be to assess whether there is a difference in wound healing between the cell types, and whether this process is dependent on induction of 11βHSD1 expression or affected by glucocorticoids. These types of study could open the door to investigation of putative anti-adhesion products and mechanisms e.g. inhibitors of collagen deposition.

The PCR array study raises several candidate genes for further research. CCL2 is up-regulated by IL-1α in both cell types and may play an important role in the initiation of adhesion formation and the response to inflammation and cancer by drawing in macrophages from the circulation and surrounding tissues. The initial host-tissue reaction is a potential target for inhibition of EOC metastasis and elucidation of the role of macrophages is an important goal. The interaction of this protein with other cytokines also needs further attention in particular as the array demonstrated significant increases in IL-6 and IL-8 in both mesothelial cell types. A relevant study demonstrated that chemo-atraction of neutrophils by female reproductive tract cells (but not OSE) was dependent on synergy between IL-8 and granulocyte / monocyte colony stimulating factor (GM-CSF), coded by the gene CSF2 (Shen, L. et al. 2004). In the PCR microarray CSF2 mRNA was significantly elevated in both OSE and PSE by IL-1α treatment suggesting that such a mechanism may be important in the mesothelium also. The demonstration of these inflammation associated genes changing in the OSE may suggest that the OSE participates in the process of ovulation while the delayed up-regulation of 11βHSD1 as well as the rise in collagen synthesising elements such as lysyl oxidase indicates that the OSE may also play a
role in resolution of the injury caused by ovulation. These repair mechanisms are also potential targets for prevention of pelvic adhesions.

The second group of genes highlighted by the microarray are those involved with reproductive endocrinology. Further examination of ER would be of value particularly as its expression has not been examined in human primary mesothelial cells and it may have an important role in the development of endometriosis where ERα appears to predominate (Matsuzaki, S. et al. 2000). The FSH receptor is also a candidate gene of potential interest as gonadotrophins are elevated following the menopause and the effect of high gonadotrophins has been hypothesised to predispose to EOC development (Choi, J.H. et al. 2007).

The final group of genes raised by the microarray are the IgLONs. The discovery of OPCML as a potential tumour suppressor gene in EOC led to interest in this gene and its family of immunoglobulin like proteins (Sellar, G.C. et al. 2003). The PCR array demonstrated a significant rise in OPCML mRNA expression with IL-1α in both OSE and PSE, but no change in the mRNA levels of LSAMP, NEGR1 and HNT. The effect of IL-1α on all of these genes was examined in Chapter 5 and confirms these findings, although there appeared to be a significant effect of IL-1α on HNT mRNA expression by Taqman analysis. There is scope for further exploration of these genes within the female reproductive tract and further functional studies in the OSE by interference with gene expression using transfection techniques. To date, there have been no data published describing IgLON expression in the OSE. The results presented here indicate that OPCML expression is related to inflammation and as such strengthens its position as a player in the origin of some ovarian cancers.

In summary, this thesis examines diverse aspects of EOC origins through establishment of and experimentation on primary cultures of EOC, OSE, and PSE. It has revealed that 11βHSD1 expression may not be so different in normal OSE and malignant EOC tissue while 11βHSD2 is higher in ovarian cancers. The OSE, from
which EOC may derive, resembles the peritoneal surface epithelium, but has higher 11βHSD1 activity. The mRNA signature of both OSE and PSE in response to IL-1α is almost identical in a panel of 48 selected genes. One of these genes, OPCML is a candidate for a tumour suppressor gene in EOC and appears to be physiologically regulated by inflammatory mediators.


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human ovarian surface epithelial model of malignant transformation."


Appendix I

Patient Information Sheets, Consent Forms and GP Letters
Ovulation, Inflammation and The Normal Ovarian Surface Epithelium

Chief Investigator – Dr Scott Fegan
Research Nurses – Catherine Murray, Sharon McPherson
Centre for Reproductive Biology, The Queen’s Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ
Telephone – 0131 242 2483

You are being invited to take part in a research study. Before you decide to take part it is important for you to understand why the research is being done and what it will involve. Please read the following information carefully and ask us if there is anything that is not clear, or if you would like more information.

Background information

There is now evidence that the ovarian surface (OSE) may play an important role in the release of an egg from the ovary – a process known as ovulation. Ovulation is a complicated event which we are only beginning to understand. Our research involves the investigation of how ovulation affects the OSE. This will help us understand not only ovulation but also how more serious conditions affecting the ovary might arise. We want to start off by looking at OSE cells from normal healthy women, which is why we are asking for your help.

Our Project

We are asking for your permission to take a sample of your OSE cells during your operation. The OSE cells are very loosely attached to the outside of the ovary and can be easily collected at the time of operation by gentle scraping of the ovary, rather like when a cervical smear is performed. We are interested in finding out if the OSE cells are related to the cells lining the rest of the abdominal cavity (peritoneum) and may also wish to collect a further sample of these peritoneal cells, again by gentle scraping. The sample collection takes only a few seconds and will not delay your operation or cause any side effects. It will not cause any damage to the ovaries or underlying tissues. There will be no increased pain or
discomfort as a result of your participation and your recovery from the operation will not be affected.

Once the cells are collected they will be taken to the laboratory until enough cells have grown (possibly up to 6-8 weeks). At this stage the effect of hormones involved with ovulation on the cells will be studied. In most cases the cells will not survive beyond this time. However in a few cases we may wish to keep the cells alive for longer by introducing a survival gene called hTERT which prevents the cells from ageing. This means the cells could be used for related experiments in the future. All laboratory work will be carried out at the University of Edinburgh.

What to do if you wish to participate.

If you wish to participate we will ask you a few questions about your health. All information about you will be kept confidential. Your name or address will not appear on any sample container. It is up to you to decide whether you take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive. We will seek your permission to contact your GP and let them know of your participation.

You will not benefit directly by participating but your involvement in this study will add to our knowledge of ovarian biology. Your gynaecologist is independent of this study and will not benefit from your involvement. You will not be able to find out the results of experiments on your own sample but we hope to publish the results of the project in scientific journals at a later date. Posters that have been presented at scientific meetings may be available for viewing at the Simpson Centre for Reproductive Health.

Cover for negligence is in place by virtue of the code of employment for approved patient research. No special arrangements will be made to cover non-negligent actions but normal channels for dealing with complaints are in place.

This study has been approved by the local ethics committee.

If you wish further information from a doctor who is not involved in this study in any way you can contact Dr C P West, Simpson Centre for Reproductive Health, Royal Infirmary of Edinburgh, 51 Little France Crescent, Edinburgh EH16 4SA. Tel 0131 242 2525.

Thank you for reading this information sheet.
CONSENT FORM
Version 3 – 7th July 2005

Study Title Ovulation, Inflammation and the Normal Ovarian Surface
Epithelium

Name of Researcher:
Dr K S Fegan
University of Edinburgh Cancer Research Unit
Western General Hospital
Crewe Road South
Edinburgh
EH4 2XR
Tel: 0131 777 3500
Or: 0131 242 2483 (Research Nurses)

1. I confirm that I have read and understand the information sheet for the above study and
have had the opportunity to ask questions.
2. I understand that my participation is voluntary and that I am free to withdraw at any time,
without giving any reason, and without my medical care or legal rights being affected.
3. I agree to a letter being sent to my General Practitioner about my participation in this
study.
4. I agree to the provision of any clinically significant information to my General Practitioner.
5. I understand that my medical notes may be looked at by the researchers involved in the
study or from regulatory authorities where it is relevant to my taking part in research.
I give permission for these individuals to have access to my records.
6. I agree that the sample I have given and the information gathered from me may be stored
by Dr K S Fegan and Professor S G Hillier at the Centre for Reproductive Biology and
Cancer Research Unit, University of Edinburgh for possible use in future projects, as
described in the attached information sheet. I understand that some of these projects
may be carried out by researchers other than Dr Fegan who ran the first project.
7. I understand that this is non-therapeutic research from which I cannot expect to derive any
benefit.
8. I agree to take part in the above study.

__________________________________  ______________________  ______________________
Name of Patient  Date  Signature

__________________________________  ______________________  ______________________
Name of Person taking consent (if different from researcher)  Date  Signature

__________________________________  ______________________  ______________________
Researcher  Date  Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes
Dear Dr

Re:

Your patient has agreed we may contact you to inform you that she has consented to taking part in a research study entitled “Ovulation, Inflammation and the Normal Ovarian Surface Epithelium”. Ethical approval has been granted by the local ethics committee. The study involved obtaining a sample of ovarian surface epithelial (OSE) cells at the time of her scheduled hysterectomy / laparoscopy on the / / .

The ovarian surface epithelium, or OSE is intimately involved in ovulation, which can be considered a naturally occurring inflammatory process. However, inflammation itself can cause cell damage and damage to DNA, which may predispose to cancer. The aim of this study is to investigate the role of inflammation in the development of ovarian cancer.

Please contact me if you would like any further information.

With kind regards,

Yours sincerely,

Dr K Scott Fegan MRCOG
Cancer Research UK Clinical Research Fellow
You are being invited to take part in a research study. Before you decide to take part it is important for you to understand why the research is being done and what it will involve. Please read the following information carefully and ask us if there is anything that is not clear, or if you would like more information.

**What is the Research About?**

You are undergoing an operation because your doctor has discovered a problem with one or both of your ovaries. It is uncertain how ovarian disease arises but by examining the ovaries after your operation we hope to identify some clues as to how it occurs. We are hoping to grow some of the cells from the ovary and the fluid. Then we can look at the genetic material (DNA) in the ovary and fluid and compare it with that in your blood. This may help us in the future to identify women with ovarian disease before it becomes serious and help to find ways of preventing it.

**What will I be asked to give?**

Firstly, we are asking for your permission to take an extra sample of blood when you are in hospital. This will be a similar amount to that taken for routine tests before your operation (about an egg-cupful). Your surgeon will carry out your operation as planned. In addition to removal of the diseased tissue, the surgeon will take samples of fluid from your pelvis to check for the presence of abnormal cells. This fluid and tissue is sent to the pathologist for detailed examination.
We are asking for your permission to take samples from:

1. the cells covering both the normal and diseased ovaries by gently scraping the ovaries at the beginning of the operation
2. the fluid from inside your tummy
3. the normal and diseased ovaries
4. the fatty tissue inside the tummy

These will be used for research in our laboratory. This will not affect the length of your operation or delay your recovery and will not affect the quality of the samples received by the pathologist. Participating carries no additional risk to your health. We are also asking for your permission to keep and store any samples you donate for related experiments in the future. All work will be done at the University of Edinburgh.

How Can I Take Part?

If you wish to take part we will speak to you when you come into hospital and ask you a few questions about your health. All information about you will be kept confidential. Your name or address will not appear on any sample container. It is up to you to decide whether you take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. We will seek your permission to contact your GP and let them know of your participation.

Can I Change My Mind?

If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

You will not benefit directly by participating but your involvement in this study may help women with diseases of the ovary in the future. Your gynaecologist is not involved in this study and will not benefit if you take part. You will not be able to find out the results of experiments on your own sample, but we hope to publish the results of the project in scientific journals at a later date.

Normal channels for dealing with complaints are in place and your legal rights will not be affected by your participation.

This study has been approved by the local ethics committee.

If you wish further information from a doctor who is not involved in this study in any way you can contact Dr C P West, Simpson Centre for Reproductive Health, Royal Infirmary of Edinburgh, 51 Little France Crescent, Edinburgh EH16 4SA. Tel 0131 242 2698.

Thank you for reading this information sheet.
CONSENT FORM

Study Title: Inflammation in the Normal and Diseased Ovary

Name of Researcher:
Dr K Scott Fegan
University of Edinburgh Cancer Research Unit
Western General Hospital
Crewe Road South
Edinburgh
EH4 2XR
Tel: 0131 777 3500 Or : 0131 242 2483 (Research Nurses)

1. I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected.

3. I agree to a letter being sent to my General Practitioner about my participation in this study.

4. I agree to the provision of any clinically significant information to my General Practitioner.

5. I understand that my medical notes may be looked at by the researchers involved in the study or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

6. I agree that the samples I have given and the information gathered from me may be stored at the Centre for Reproductive Biology and Cancer Research Centre, University of Edinburgh for possible use in future projects, as described in the attached information sheet. I understand that some of these projects may be carried out by researchers other than Dr Fegan who ran the first project.

7. I understand that this is non-therapeutic research from which I cannot expect to derive any benefit.

8. I understand that the research may include genetic research aimed at understanding the genetic background of ovarian disease but that these investigations are unlikely to have any implications for me personally.

9. I agree to take part in the above study.

________________________ ____________________ ________________
Name of Patient Date Signature

________________________ ____________________ ________________
Name of Person taking consent Date Signature
(if different from researcher)

________________________ ____________________ ________________
Researcher Date Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes
Thank you for agreeing to participate in this research.
Dear Dr

Re:

Your patient has agreed we may contact you to inform you that she has consented to taking part in a research study entitled “Inflammation in the Normal and Diseased Ovary”. Ethical approval has been granted by the local ethics committee. The study involves obtaining a pre-operative blood sample and samples of ovarian tissue and peritoneal fluid at the time of her scheduled laparotomy on / / . Follow-up for research purposes will not be required.

While it appears that the ovarian surface epithelium is the likely origin of ovarian cancer the factors involved in oncogenesis are unexplained. We wish to compare the expression of inflammatory mediators and tumour suppressor genes in benign, borderline and malignant ovarian tumours.

Please contact me if you would like any further information.

With kind regards,

Yours sincerely,

Dr K Scott Fegan MRCOG
Clinical Research Fellow
Cancer Research UK
Anti-inflammatory steroid signalling in the human peritoneum

K S Fegan, M T Rae¹, H O D Critchley¹ and S G Hillier¹

University of Edinburgh Cancer Research Centre, Crewe Road South, Edinburgh EH4 2XR, UK
¹Queen’s Medical Research Institute, Centre for Reproductive Biology, University of Edinburgh, 47 Little France Crescent, Edinburgh EH16 4TJ, UK

Abstract

Peritoneal surface epithelial (PSE) cells participate in adhesion formation following inflammatory injury yet adjacent ovarian SE (OSE) cells regenerate without scarification after ovulation. OSE cells show inflammation-associated expression of 11βhydroxysteroid dehydrogenase type 1 (11βHSD1) enzyme, enabling intracrine generation of anti-inflammatory cortisol to minimise tissue damage. We asked if human PSE cells show an 11βHSD1 response to pro-/anti-inflammatory stimulation and if so, how the 11-oxoreductase activity generated compares with OSE. PSE collected from premenopausal women undergoing surgery for benign gynaecological conditions were used to establish primary PSE cell cultures that were treated for 48 h with interleukin-1α (IL-1α) with/without anti-inflammatory steroid (cortisol or progesterone). mRNA levels corresponding to the genes of interest (11βHSD1, 11βHSD2, cyclooxygenase-2, COX-2) were measured by quantitative RT-PCR. IL-1α (0.5 ng/ml) stimulated 11βHSD1 and COX-2 mRNA levels in PSE cells but 11βHSD2 was unaffected. Cortisol (1 μM), not progesterone (1 μM), increased 11βHSD1 mRNA and synergistically enhanced IL-1α action. Cortisol suppressed IL-1α-stimulated COX-2 more effectively than progesterone. PSE cells had a significantly lower basal 11-oxoreductase enzyme activity than OSE cells; IL-1α did not significantly increase the 11-oxoreductase activity in PSE cells but did so in OSE cells. We conclude that PSE cells respond to IL-1α and anti-inflammatory steroids in qualitatively similar ways as OSE. However, the enzymatic activity of 11βHSD1 is lower in PSE and less responsive to IL-1α. This could help explain why peritoneal healing often leads to adhesion formation, whereas postovulatory ovarian healing is scar-free.

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Introduction

An emerging theme in inflammation research is that inflammatory stimuli regulate genes that localise and limit potentially damaging cellular responses. When this machinery fails, pathological sequelae ensue. One such ‘anti-inflammatory’ gene is HSD11B1, encoding 11βhydroxysteroid dehydrogenase type 1 (11βHSD1), a steroidogenic enzyme that metabolises cortisone to cortisol through its 11-oxoreductase enzymatic activity. The activity of 11βHSD1 is offset by 11βHSD2, the encoded product of HSD11B2 that back converts cortisol to cortisone. Since cortisol – but not cortisone – binds glucocorticoid receptor (GR) and activates downstream anti-inflammatory signalling, relative levels of 11βHSD1 and 11βHSD2 potentially set local inflammatory tone (Draper & Stewart 2005). Differentially expressed 11βHSD1 and 11βHSD2 in a naturally inflamed tissue was first observed in human ovary approaching ovulation. Ovulation bears vascular, haemodynamic and biochemical hallmarks of inflammation, and granulosa cells from periovulatory follicles selectively express 11βHSD1 mRNA over 11βHSD2 mRNA (Tetsuka et al. 1997). From work on renal mesangial cells it emerged that inflammatory cytokines such as interleukin-1α (IL-1α) and tumour necrosis factor α directly stimulate 11βHSD1 and suppress 11βHSD2 activities in vitro (Escher et al. 1997). This is now known to hold for several other cell types that naturally respond to inflammatory signals, including ovarian granulosa (Tetsuka et al. 1999) and surface epithelial cells (Yong et al. 2002), bronchial epithelial cells (Feinstein & Schleimer 1999), preadipocytes (Tomlinson et al. 2001), osteoblasts (Cooper et al. 2001), aortic smooth muscle cells (Cai et al. 2001), peritonal macrophages (Gilmour et al. 2006), trophoblast (Li et al. 2006) and fetal membranes (Sun & Myatt 2003).

At the ovarian level, cortisol generated through intracrine 11βHSD signalling potentially contributes to the natural injury-repair process associated with ovulation. In particular, the ovarian surface epithelium (OSE), which is contiguous with the peritoneal SE (PSE), undergoes serial inflammation-associated injury and repair with each follicular rupture (Rae & Hillier 2005). We have previously shown that OSE cells express 11βHSD1 mRNA and 11-oxoreductase enzyme activity catalysed by the encoded 11βHSD1 protein (Yong et al. 2002). We have also shown that IL-1α-induced experimental ‘inflammation’ of OSE cells measured as an increased expression of cyclooxygenase-2 (COX-2) is suppressed by cortisol and to a lesser extent progesterone (Rae et al. 2004). Both cortisol and
progesterone are anti-inflammatory steroids that act via nuclear GR and progesterone (PR) receptors in target cells to repress pro-inflammatory transcription factors such as nuclear factor κ B and activating protein-1 (van der Burg & van der Saag 1996, Rhen & Cidlowski 2005). Since micromolar concentrations of cortisol and progesterone accumulate in follicular fluid at ovulation (Andersen 1991, Andersen & Hornnes 1994), either or both might participate in postovulatory ovarian healing and act on adjacent PSE.

The mesothelial cells lining the peritoneal surface are also potential glucocorticoid and progesterone targets and are inevitably exposed to high concentrations of follicular steroids following follicular rupture. Interestingly, when postovulatory OSE repairs it does so rapidly without scarification or involving i.p. adhesions. On the other hand, inflammatory injury to the PSE frequently leads to adhesion formation with adverse clinical sequelae including pain, bowel obstruction and, in the female, infertility. Thus, despite their common embryological origin, the anti-inflammatory machineries of PSE and OSE cells likely differ. Here, we describe a simple method to collect and culture human PSE cells, based on the one we have previously used to study human OSE cells (Hillier et al. 1998, Auersperg et al. 2001). Using this culture system, we define interactions between an inflammatory cytokine (IL-1β) and two anti-inflammatory steroids (cortisol and progesterone) on 11βHSD1, 11βHSD2 and COX-2 mRNA expression in PSE cells in vitro. We also compare basal and cytokine-responsive 11-oxoreductase activities of PSE and OSE cells. Our results define properties of PSE cells that differ from OSE, which could bear on the particular way in which the peritoneum responds to inflammation and becomes involved in gynaecological disease states in vivo.

### Materials and Methods

#### Patients

Samples of PSE were obtained at the time of surgery from premenopausal women undergoing surgery for benign gynaecological conditions. OSE cells for comparative purposes were also obtained from a subset of patients. All participants gave informed consent and the Local Research Ethics Committee approved the study. Relevant clinical details of the patients who donated cells are shown in Table 1. Patients with endometriosis or overt signs of peritoneal pathology were excluded from study.

#### Collection and culture of PSE cells

The method for PSE collection and culture was similar to that previously described for OSE (Hillier et al. 1998). The culture medium was Medium 199:MCDB 105 (1:1, v/v) supplemented with fetal calf serum (15% v/v), penicillin (50 IU/ml), streptomycin (50 μg/ml) and t-glutamine (2 mmol/l), all from Sigma–Aldrich Company Ltd. Briefly, the peritoneum was gently brushed using a sterile Aylesbury spatula or cytobrush (Cook Ireland Ltd, Limerick, Ireland) and then rinsed into prewarmed culture medium. Two sites were chosen for sampling PSE cells: the anterior abdominal wall, which has no contact with the OSE or pelvic organs, and the uterine fundus. The purpose of using these two sites was to determine whether the Müllerian source of PSE behaves in a manner similar to cells sourced from the non-Müllerian abdominal wall. The medium used to collect cells was transferred to 75 cm² plastic tissue culture flasks (Corning BV Life Sciences, Schiphol-Rijk,

#### Table 1 Relevant clinical details of patients donating peritoneal surface epithelial (PSE) cells from which cultures were established and measurements obtained

<table>
<thead>
<tr>
<th>Age (year)</th>
<th>Parity</th>
<th>Contraception</th>
<th>Day of cycle</th>
<th>Surgery</th>
<th>Indication for surgery</th>
<th>Measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>38</td>
<td>1 + 2</td>
<td>DMPA</td>
<td>NA</td>
<td>Lap Ster</td>
<td>Unwanted fertility</td>
<td>mRNA</td>
</tr>
<tr>
<td>40</td>
<td>3 + 0</td>
<td>Fem Ster</td>
<td>2</td>
<td>TAH</td>
<td>HMB</td>
<td>mRNA</td>
</tr>
<tr>
<td>42</td>
<td>0 + 1</td>
<td>None</td>
<td>?</td>
<td>TAH</td>
<td>HMB, dysmen</td>
<td>mRNA</td>
</tr>
<tr>
<td>50</td>
<td>2 + 0</td>
<td>None</td>
<td>11</td>
<td>Diag Lap</td>
<td>Pelvic pain, dyspareunia</td>
<td>mRNA</td>
</tr>
<tr>
<td>33</td>
<td>0 + 0</td>
<td>COCP</td>
<td>NA</td>
<td>Diag Lap</td>
<td>Pelvic pain, dyspareunia</td>
<td>mRNA</td>
</tr>
<tr>
<td>31</td>
<td>0 + 0</td>
<td>COCP</td>
<td>NA</td>
<td>Diag Lap</td>
<td>Pelvic pain, dyspareunia</td>
<td>mRNA</td>
</tr>
<tr>
<td>39</td>
<td>1 + 0</td>
<td>None</td>
<td>5</td>
<td>TAH</td>
<td>HMB, dysmen</td>
<td>mRNA</td>
</tr>
<tr>
<td>34</td>
<td>2 + 0</td>
<td>Fem Ster</td>
<td>27</td>
<td>LAVH</td>
<td>HMB, dysmen</td>
<td>mRNA</td>
</tr>
<tr>
<td>47</td>
<td>2 + 0</td>
<td>LNG-IUS</td>
<td>NA</td>
<td>TAH&amp;BSO</td>
<td>Irreg bleeding, fibroids</td>
<td>mRNA</td>
</tr>
<tr>
<td>48</td>
<td>3 + 0</td>
<td>Fem Ster</td>
<td>2</td>
<td>TAH</td>
<td>HMB, fibroids</td>
<td>mRNA</td>
</tr>
<tr>
<td>43</td>
<td>2 + 0</td>
<td>Barrier</td>
<td>?</td>
<td>Lap Ster</td>
<td>Unwanted fertility</td>
<td>11-oxo-reductase</td>
</tr>
<tr>
<td>29</td>
<td>3 + 0</td>
<td>None</td>
<td>13</td>
<td>Diag Lap</td>
<td>Dyspareunia</td>
<td>11-oxo-reductase</td>
</tr>
<tr>
<td>22</td>
<td>1 + 0</td>
<td>DMPA</td>
<td>NA</td>
<td>Lap ovarian cystectomy</td>
<td>Benign ovarian cyst</td>
<td>11-oxo-reductase</td>
</tr>
<tr>
<td>28</td>
<td>0 + 0</td>
<td>COCP</td>
<td>NA</td>
<td>Diag Lap</td>
<td>Pelvic pain, dyspareunia</td>
<td>11-oxo-reductase</td>
</tr>
</tbody>
</table>

Parity is defined in x+y format indicating number of pregnancies over 24 weeks (x) + number of pregnancies failing to reach 24 weeks (y). NA, not applicable; ?, uncertain; DMPA, depo-medroxyprogesterone acetate; Fem Ster, female sterilisation; COCP, combined oral contraceptive pill; LNG-IUS, levonorgestrel intra-uterine system; Lap Ster, laparoscopic sterilisation; TAH, total abdominal hysterectomy; BSO, bilateral salpingo-oophorectomy; Diag Lap, diagnostic laparoscopy; HMB, heavy menstrual bleeding; dysmen, dysmenorrhoea.
The Netherlands) precoated with donor calf serum. Each flask was examined by phase-contrast microscopy to verify that sufficient cellular material had been obtained. Cultures were then established by incubating the flasks at 37 °C in a humidified atmosphere of 5% CO2 and 95% air for up to 42 days. The flasks were inspected regularly, with medium renewal every 7 days.

**Experimental treatment of PSE cultures**

Confluent cell monolayers were washed twice with PBS prior to incubation in 1X Trypsin/EDTA solution (Invitrogen) for 5 min at 37 °C. Dissociated cells were then aspirated and sedimented by centrifugation for 5 min at 800 g. The resulting cell pellet was resuspended in 2 ml culture medium. Cell number and viability were estimated using a haemocytometer and vital staining with Trypan Blue (Sigma–Aldrich). To provide cell monolayers for immunohistochemical assessment (see below), PSE cells were seeded into eight-well chamber slides (VWR, Batavia, IL, USA) at a density of $5 \times 10^5$ cells/0.5 ml medium and incubated at 37 °C overnight. For experiments involving hormone treatment and subsequent analysis of effect, cells were distributed into six-well plates (Corning) at a density of $4.5 \times 10^5$ cells/0.5 ml medium (for mRNA analysis) or 12-well plates at a density of $2 \times 10^5$ cells/0.5 ml medium (for 11-oxoreductase analysis). The medium was then aspirated and replaced with serum-free culture medium, containing 0.01% BSA (Sigma–Aldrich). IL-1α (R&D Systems Europe Ltd, Abingdon, Oxon, UK) was diluted in serum-free culture medium, containing 0.01% Tween 20 (Sigma–Aldrich). IL-1α (R&D Systems Europe Ltd, Abingdon, Oxon, UK) was diluted in serum-free culture medium, containing 0.01% BSA (Sigma–Aldrich). IL-1α was added to culture medium at a final concentration of 1 µg/ml, this being the dose established by incubating the flasks at 37 °C in a humidified atmosphere of 5% CO2 and 95% air for up to 42 days. The flasks were inspected regularly, with medium renewal every 7 days.

**Immunohistochemistry**

Following methanol fixation and triplicate 3-min washes in PBS, cell monolayers were sequentially blocked with avidin (Vector, Peterborough, UK), biotin (Vector) and non-immune serum (horse, Vector). Primary monoclonal antibodies for human GR (Santa Cruz Biotechnology Inc., Heidelberg, Germany), cytokeratin or PR (Dako Corp., Glostrup, Denmark), diluted 1/100, 1/1000 and 1/50 respectively were applied for 1 h at 37 °C. After three washes with PBS + 0.01% Tween 20 (Sigma–Aldrich; 3 min each), secondary antibody (horse-antimouse) diluted in non-immune horse serum was applied for 1 h at room temperature. Sequential washes with PBS + 0.01% Tween 20 (3×3 min) were then performed before incubating with Vector-ABC Elite reagents and visualisation via chromagen (diaminobenzidine) staining. Negative controls consisted of non-immune mouse IgG2 substituted for primary antiserum.

**Statistical analysis**

Quantitative RT-PCR measurements were normalised to the control (no treatment) value for each mRNA measured. Data from multiple patients (see figure legends) were grouped and expressed as mean ± S.E.M. Statistical differences due to treatment were determined by repeated measures ANOVA of raw data reflecting the mean C, difference between 18S rRNA and target mRNA. The mean 11-oxoreductase enzymatic activity expressed as pmol/culture per h was compared between treated and untreated samples of the same cell type using paired Student’s t-tests, and between OSE and...
PSE samples using unpaired Student’s *t*-tests. Treatment effects were considered significant at *P* < 0.05.

**Results**

**PSE cell morphology**

The morphology of PSE cells in culture and their responses to experimental treatment *in vitro* were unaffected by the site within the abdominal cavity from which they had been obtained (data not shown). Sheets of PSE cells collected into culture medium usually attached to the serum-coated flask surface within 48 h. Further incubation allowed multiple epithelial cell colonies to become established, eventually producing confluent monolayers. The ‘cobblestone’ morphology (Fig. 1a) and the mode of cytokeratin expression (Fig. 1b) shown by cultured PSE cells was similar to that previously described for OSE cells cultured in the same way (Hillier et al. 1998). Flasks that became contaminated by cells showing a fibroblastic phenotype were excluded from experimentation. Overall, out of the 29 patient PSE specimens collected, 21 (72.4%) produced cultures suitable for further study.

**PSE cell steroid receptor status**

PSE cell monolayers contained GR and PR mRNA levels similar to those present in OSE cells cultured under identical conditions (data not shown). Immunostaining with antisera to GR (Fig. 1c) or PR (Fig. 1d) revealed the presence of both the steroid receptor proteins in PSE cells, with a predominantly nuclear location.

**PSE cell responses to IL-1α and cortisol**

To determine influences of IL-1α and cortisol on inflammation-associated gene expression in PSE cells, we assessed 11βHSD1, 11βHSD2 and COX-2 mRNA levels, reasoning that the 11βHSD isoform expression profile defines the potential for intracrine generation of anti-inflammatory cortisol, while COX-2 provides a referent inflammatory readout.

The treatment of PSE cell cultures from five patients with IL-1α significantly increased the expression of 11βHSD1 mRNA relative to non-treated control values (mean fold induction, 9.75, *P* < 0.01; Fig. 2a). PSE cells sampled from the anterior abdominal wall and the uterine serosal surface responded similarly to IL-1α (average fold induction, 10.4 and 9.0 respectively). Cortisol alone also up-regulated 11βHSD1 mRNA expression 12.6-fold (*P* < 0.001). In combination, cortisol and IL-1α synergised to increase 11βHSD1 expression 67.9-fold relative to the control (*P* < 0.001).

Neither IL-1α nor cortisol, alone or in combination, significantly affected 11βHSD2 mRNA expression (Fig. 2b).

The exposure to IL-1α stimulated COX-2 mRNA expression on average was 10.6-fold (*P* < 0.001; Fig. 2c).

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Figure 1  Morphology and immunohistochemical properties of cultured human PSE cells. (A) Phase-contrast microscopy of PSE cell monolayer on day 12 of culture following collection. (B) Cytokeratin immunohistochemistry using antiserum against a panel of low MW cytokeratins confirms epithelial phenotype of cultured PSE cells and purity of cell culture. (C) Glucocorticoid receptor (GR) and (D) progesterone receptor (PR) immunohistochemistry detect each receptor protein in PSE cell nuclei. Insets in B–D show matched non-immune control staining. Bars represent 100 μm.
Again, there was no significant difference in the magnitude of response in relation to the site of cell sampling from within the same patient (data not shown). Cortisol alone had no significant effect on COX-2 mRNA expression. However, in combination with IL-1α, cortisol significantly suppressed the IL-1α-induced rise in COX-2 mRNA to near control levels ($P<0.001$).

Antioxidative actions of cortisol and progesterone compared

PSE cell cultures from a further six patients were used to test the relative anti-inflammatory effects of cortisol and progesterone on inflammation-associated gene expression. Stimulation of 11βHSD1 mRNA by IL-1α alone (average 7.25-fold, $P<0.001$) was confirmed in PSE cell cultures from this second series of patients (Fig. 3a). Cortisol alone was also stimulatory (average 10-fold, $P<0.001$). However, progesterone was inactive (Fig. 3a). Once again, cortisol augmented IL-1α-stimulated 11βHSD1 mRNA expression (to an average fold induction, 28.3; $P<0.001$). Co-treatment with progesterone and IL-1α also consistently increased 11βHSD1 mRNA expression beyond that caused by IL-1α alone (average 11.8-fold) but this effect was not statistically significant.

11βHSD2 mRNA expression was unaffected by any of the treatments applied (Fig. 3b). Stimulation of COX-2 mRNA by IL-1α was strongly suppressed ($P<0.001$) by the presence of cortisol (average fold induction, 4.5) and to a lesser extent ($P>0.05$) progesterone (average fold induction, 12.8; Fig. 3c).

11-Oxoreductase enzymatic activity in PSE cells

The finding that IL-1α stimulates 11βHSD1 mRNA expression in PSE cells predicts the up-regulation of 11-oxoreductase activity, as demonstrated previously in OSE cells (Yong et al. 2002). We therefore compared...
We show that the cells lining the human peritoneum are able to up-regulate 11βHSD1 gene expression in response to the inflammatory cytokine IL-1β in vitro. Furthermore, cortisol – the product of 11-oxoreductase activity catalysed by 11βHSD1 – is anti-inflammatory in PSE cells. Basal and IL-1β-stimulated 11-oxoreductase enzymatic activity in PSE cells is, however, substantially weaker than that of closely related OSE cells. Thus, we provide mechanistic insight on anti-inflammatory signalling in human peritoneum and establish differences between OSE and PSE cells that could help explain the relative susceptibility of PSE to adhesion formation following inflammation-associated injury in vivo.

Human PSE cells have previously been obtained from resected omentum (Stylianou et al. 1990, Pronk et al. 1993) or peritoneum (Witz et al. 1998) and cultured with varying success. Here, we swept up cells directly from peritoneal sites in situ, avoiding enzymatic tissue digestion to obtain pure mesothelial cell cultures. This atraumatic technique of PSE collection allows single-cell-thick sheets of cells to be cultured with the low likelihood of contamination by other cell types. Morphology of the PSE monolayer in vitro closely resembles the classic ‘cobblestone’ appearance of OSE cultured under similar conditions. The mesothelial origin of the PSE cultures we studied was also confirmed by the pattern of low-molecular weight cytokeratin expression they showed, which is also similar to OSE (Auersperg et al. 2001) and increases confidence that the PSE cultures were not significantly contaminated with any other cell type.

PSE and OSE cells not only look alike but also share a similar biochemical signature in response to inflammation. Along with inflammation-associated COX-2 gene expression in response to IL-1β they show up-regulation of 11βHSD1 mRNA without any change in 11βHSD2. Whether any functional link exists between COX-2 and 11βHSD1 mRNA expression in IL-1β-stimulated PSE cells remains to be established. However, this is suggested by the finding that prostaglandin synthesis is required for the stimulation of 11βHSD1 enzyme activity by IL-1β in human granulosa–luteum cell cultures (Jonas et al. 2006). PSE also has in common with OSE the feed-forward response of 11βHSD1 when stimulated by IL-1β in the presence of cortisol (Rae et al. 2004). This predicts the capacity to mount a compensatory increase in intracellular levels of cortisol in response to inflammatory stimulation in vivo.

Owing to the clinical basis of this study, insufficient patient material was available to delineate the post-receptor signalling mechanisms involved in the actions of IL-1β and anti-inflammatory steroids in PSE cells. However, we did document similar levels of mRNA for PR, and GR, and clearly detected nuclear PR and GR proteins. In other experimental systems, nuclear GR and PR receptors transduce positive and negative regulation of genes that impact signalling via cell-surface receptors for pro-inflammatory cytokines (Rosen & Milner 2005) and it is reasonable to suppose that these mechanisms operate in PSE cells too. Other caveats to our results include the extent to which pro-/anti-inflammatory properties of PSE and OSE cells propagated in vitro might deviate from norm in terms of functionality in vivo. Comparisons of 11-oxoreductase activities in PSE and OSE cell cultures from individual patients also proved problematic owing to a variable adaptation of individual cell biopsies to culture. However, we consistently observed a restricted capacity of PSE cells to undertake 11-oxoreductase activity relative to OSE cells. Moreover, for the single patient from whom both PSE and OSE cell cultures were available for direct comparison, the result obtained (embedded within the data set in Fig. 4) faithfully mirrored the aggregate outcome.

The physiological significance of these results is that cytokine-responsive 11βHSD1 could provide a mechanism throughout the peritoneal surface to localise and limit...
inflammation-associated injury and promote rapid healing of traumatised tissue through pre-receptor amplification of cortisol formation. However, 11βHSD1 enzyme activity in PSE is lower than that in OSE, indicating that the ovary is better protected by anti-inflammatory mechanisms than the peritoneum. The higher 11-oxoreductase tone of OSE, if reflected in higher local cortisol levels in vivo, might be sufficient to minimise fibrosis and thereby reduce the likelihood of postovulatory adhesions. This is borne out by the fact that the ovaries remain mobile and free within the pelvis of most women. On the other hand, the peritoneum is a more stable environment, less prone to natural injury and with a lower 11-oxoreductase tone than the ovarian surface. The sporadic injuries that do occur to PSE tend to be iatrogenic — e.g., during surgery — or involve microbial infection and are inherently more traumatic. This indicates that downstream pro-inflammatory sequelae likely override any compensatory anti-inflammatory benefit arising from 11βHSD1 signalling, permitting fibrosis and adhesion formation to proceed.

Progesterone, present at high concentrations in follicular fluid at the time of ovulation (Andersen & Hornnes 1994), is a candidate anti-inflammatory agent throughout the pelvic cavity. However, progesterone was considerably less potent than cortisol as an anti-inflammatory agent in cultured PSE, matching our previous finding for OSE (Rae et al. 2004). Nevertheless, 1 μM progesterone reduced IL-1α-stimulated COX-2 mRNA expression 37%, on average, in 6/6 cases (Fig. 3). Although this effect was statistically non-significant, it may yet be biologically important since progesterone levels in follicular fluid can attain levels up to 30 times higher than those tested here. Accordingly, we do not rule out the possibility that progesterone exerts a physiologically significant action on OSE and/or PSE cells during ovulation in vivo. The responsiveness of PSE cells to progesterone also has broader clinical relevance. First, it raises the possibility that the peritoneum may be affected by menstrual fluctuations in ovarian progesterone secretion. Secondly, it is relevant to endometriosis where withdrawal of progesterone prior to menstruation coincides with the most severe symptoms. Thirdly, exogenous progesterin is a mainstay treatment for endometriosis.

Finally, our results may have a bearing on the development of epithelial ovarian cancer (EOC). The OSE is widely regarded as the major source of most ovarian cancers (Fleming et al. 2006) and EOC frequently involves other peritoneal structures, hence its overall poor outcome (Freedman et al. 2004). However, the natural history of EOC is not well defined. This is partly due to the fact that patients most commonly present with advanced stage disease with widespread peritoneal and omental involvement. This in turn makes curative treatment difficult leading to poor survival outcomes of 30% at 5 years. If the disease could be contained within the ovary at FIGO (International Federation of Gynaecology and Obstetrics) stage I, an improvement in prognosis would be expected to follow. Therefore, manipulation of the tumour–peritoneum interaction is a potential therapeutic strategy. Progesterone has been suggested as a potential chemopreventative agent for EOC and lack of progesterone may have pathophysiological roles for both endometriosis and ovarian cancer (Ho 2003, Ness 2003).

In summary, we demonstrate a simple method for the collection and culture of human PSE cells. Using this culture system, we find that PSE cells possess an anti-inflammatory machinery similar to that previously described for OSE cells, including a capacity to up-regulate 11βHSD1 in response to treatment with IL-1α. However, enzymatic activity of 11-oxoreductase is lower in PSE than in OSE. Although the cause for this difference remains unknown, our data provide a possible explanation for the observation that peritoneal healing is often accompanied by adhesion formation, while ovulation-associated ovarian healing is scarsless.

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