Aberrant Cytokine Signaling in Ovarian Cancer

by

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B.Sc., M.Sc.

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Deakin University

October, 2013
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List of Publications

Publications related to Thesis


Kumar J and Ward AC. “Role of the interleukin 6 receptor family in epithelial ovarian cancer and its clinical implications”. BBA Reviews on Cancer – under review (IF: 9.03)

Kumar, J., McCulloch, DR., and Ward AC. “Leptin receptor signaling induces mesenchymal to epithelial transformation in ovarian cancer cells” – under preparation

Other Publications

Conference Participation

- Poster: Cytokine 2012, Geneva.
- Oral presentation: ICSCC 2010, India.
- Attendance: IgV conference, Geelong, Australia.

Awards

- DUPRS Deakin University Postgraduate scholarship (2010-2013).
- Bellberry Medical research scholarship (2010-2011).
Abstract

Ovarian cancer remains a major cause of cancer mortality in women, with relatively poor understanding of disease aetiology at the molecular level and only limited treatment options. This is exacerbated by the asymptomatic nature of the disease, such that women with ovarian cancer typically present with advanced-stage disease, when the cancer has already spread through the peritoneum and sometimes to distant metastatic sites. Therefore, further investigation of the biology of ovarian cancer is an important area of research to underpin improvements in clinical outcomes.

Cytokines are known to play a pivotal role in a diverse range of developmental processes and cellular responses via specific cell surface receptors. The IL-6R family of cytokine receptors exerts a myriad of functions, including in blood and immune cell development, neurogenesis and appetite regulation, mediated via several intracellular signaling pathways, including the so-called ‘JAK-STAT’ pathway. Dysregulation of IL-6R family-JAK-STAT signaling has been shown to contribute to a number of diseases, including cancer. This Thesis describes studies aimed at investigating the role of the IL-6R family in ovarian cancer.

Chapter Three assessed the expression of IL-6R components and their ligands in a panel of ovarian cancer cell lines and patient samples. Broad expression of IL-6R family receptors and their corresponding ligands was observed, including in the same samples, suggesting possible autocrine activation. Two of the IL-6R family receptors were chosen for further study: the Granulocyte colony-stimulating factor receptor (G-CSFR), which was predominantly expressed in high-grade ovarian epithelial tumor samples, and Leptin receptor (OBR), which was expressed in a subset of ovarian cancer cell lines.
G-CSF is a key regulator of both normal and emergency hematopoiesis, and is used clinically to aid hematopoietic recovery following ablative therapies for a variety of solid tumors, including ovarian cancer. Chapter Four demonstrated that stimulation of G-CSFR-positive ovarian epithelial cancer cells with G-CSF led to increased migration and survival, including a resistance to chemotherapy-induced apoptosis. The effects of G-CSF were shown to be mediated via the downstream JAK2/STAT3 pathway.

Leptin is a multifunctional peptide hormone that functions in appetite regulation, bone formation and reproductive function, with signaling via its receptor (OBR) previously implicated in cancer cell migration, proliferation and survival. Studies in Chapter Five showed that stimulation of OBR-expressing ovarian cancer cells with Leptin caused a variety of cellular responses, which included increased migration and survival, of several cell types, mesenchymal to epithelial transition (MET) in a teratocarcinoma cell line. Each of these phenotypes was shown to be mediated by the JAK2/STAT3 pathway.

Collectively, the studies described in this Thesis suggest that signaling via IL-6R family members significantly impacted on ovarian cancer cells. G-CSF was shown to influence migration and survival on G-CSFR-positive cancers, which has implications for its current therapeutic use in ovarian cancer. In contrast, Leptin was demonstrated to exert differential effects on ovarian cancer cells, which also has potential clinical ramifications. These results suggest that assessment of IL-6R family expression represents a potential prognostic tool in determining appropriate therapy for ovarian cancer patients.
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>αFP</td>
<td>Alpha-fetoprotein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>Cis</td>
<td>Cisplatin</td>
</tr>
<tr>
<td>CNTF</td>
<td>Ciliary Neurotrophic Factor</td>
</tr>
<tr>
<td>CRP</td>
<td>C-Reactive protein</td>
</tr>
<tr>
<td>Ctrl</td>
<td>Control</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>dpf</td>
<td>Days post fertilization</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transformation</td>
</tr>
<tr>
<td>EOC</td>
<td>Epithelial ovarian cancer</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating protein</td>
</tr>
<tr>
<td>FTE</td>
<td>Fallopian tube epithelium</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte Colony-Stimulating Factor</td>
</tr>
<tr>
<td>GP130</td>
<td>Glycoprotein 130</td>
</tr>
<tr>
<td>HCG</td>
<td>Human chorionic gonadotropin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Inh</td>
<td>Inhibitor</td>
</tr>
<tr>
<td>J2I</td>
<td>JAK2 inhibitor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia Inhibitory Factor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal to epithelial transformation</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix-metalloproteinase</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cells</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>OB</td>
<td>Obese</td>
</tr>
<tr>
<td>OSE</td>
<td>Ovarian surface epithelium</td>
</tr>
<tr>
<td>OSM</td>
<td>Oncostatin M</td>
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<tr>
<td>Pac</td>
<td>Paclitaxel</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>q</td>
<td>Quantitative</td>
</tr>
<tr>
<td>R</td>
<td>Receptor</td>
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<tr>
<td>RT</td>
<td>Reverse Transcriptase</td>
</tr>
<tr>
<td>s</td>
<td>Soluble</td>
</tr>
<tr>
<td>S3I</td>
<td>STAT3 inhibitor</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signaling</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>WNT</td>
<td>Wingless Type</td>
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Chapter 1 ~ Literature review
1.1 Introduction

Ovarian cancer is the most lethal gynaecological cancer, with approximately 200,000 new cases diagnosed each year globally, and a greater than 60% mortality rate within five years (NBOCC, 2011). This is partly due to the asymptomatic nature of the disease, such that the primary tumor has often already spread at the time of diagnosis (Lengyel, 2010). However, there is also a poor understanding of disease etiology at the molecular level, which continues to hamper targeted therapeutic development.

1.2 Biology of ovarian cancer

1.2.1 Types of ovarian cancer

Ovarian cancer is not a single disease, but rather a spectrum of malignancies exhibiting varied histopathology and biology (Chen et al., 2003). Ovarian cancers are grouped into three major categories based on the cells from which they originate, namely: germ cells, stromal cells and epithelial cells (Soslow, 2008) (Figure 1.1).

1.2.1.1 Germ cell tumors

Germ cell tumors are derived from oocytes within the ovaries, and represent approximately 10%-15% of all ovarian cancers (Auersperg et al., 2001). They tend to occur in women who are in their early 20s. Specific markers such as beta-human chorionic gonadotropin (ß-HCG) and alpha-fetoprotein (αFP) are able to be used to aid in diagnosis (Kobel et al., 2008).
**Figure 1.1: Model of biology of ovarian cancer and its progression**

Diagrammatic representation of types of ovarian cancer and their progression, indicating key phenotypes of cancers, namely survival, proliferation and invasion/migration.
1.2.1.2 Stromal cell tumors

Stromal (or ‘Sex cord’) tumors are formed from cells of the stromal mesenchyme/sex-cord within the ovary. They represent about 5% of all ovarian cancers and more commonly seen in postmenopausal women (Auersperg et al., 2001). These tumors contain immature fibroblasts and connective tissue-forming cells, with granulosas being the most prevalent type (Roth, 2006). These patients usually show symptoms such as vaginal bleeding and elevated blood levels of the tumor markers in particular a peptide hormone inhibin, which prevents the release of follicle stimulating hormone (FSH) (Jamieson and Fuller, 2012). This probably contributes to the generally earlier diagnosis of patients with stromal cell tumors, with concomitant improvement in prognosis for this cohort of patients (Sehouli et al., 2004).

1.2.1.3 Epithelial ovarian cancer

Epithelial ovarian cancer (EOC) is the most common form of this disease, representing about 80% of all cases. EOC consists of distinct histological subtypes: 70% serous histology (cells resembling the lining of the fallopian tube), 15% endometrioid (cells resembling the endometrium), 10% clear cell (cells with clear cytoplasm) and 5% mucinous (cells resembling endocervical endothelium) (Kurian et al., 2005, Marquez et al., 2005, Bell, 2005). Each EOC subtype has identifiable precursor lesions, with some specific genetic alterations identified (Breedlove and Busenhart, 2005, Chien et al., 2007), with most EOC originating from the malignant transformation of the ovarian surface epithelium (OSE) (Dubeau, 2008).
1.2.2 Ovarian cancer progression

There is a growing consensus that ovarian cancer, like many other cancers, is a stem cell disease in which a small population of cells has acquired the ability to both self-renew as well as generate the cell types characteristic of that tumor (Ponnumusamy and Batra, 2008). During the transformation process, ovarian cancer cells acquire a number of properties, such as increased cell proliferation, survival and migration. There are specific changes in cell-cell and cell-extracellular matrix (ECM) interactions, with increased proteolysis and ECM degradation observed. This culminates in the shedding of tumor cells into the peritoneum where they survive in an anchorage-independent manner as cellular aggregates or spheroids, until they attach to a suitable secondary site for further growth (Shield et al., 2007, Shield et al., 2009). Women with ovarian cancer typically present with advanced-stage disease, when the cancer has already spread throughout the peritoneum, and in some cases, to distant metastatic sites. With aggressive surgery supplemented with paclitaxel and platinum-based chemotherapy, most patients initially return to a state of microscopic disease with minimal residual tumor. However, this is usually short-lived with recurrent disease characterised by increased metastatic behaviour and acquisition of drug-resistance to multiple types of chemotherapy, due – at least in part – to increased activity of the ATP-binding cassette (ABC) drug transporters, enhanced DNA repair and reduced apoptosis (Gottesman, 2002). Only ~30% of patients with recurrent disease survive for >5 years (Soslow, 2008).

1.2.3 Therapies in ovarian cancer

Current therapies for ovarian cancer include cytoreductive surgery followed by combination platinum/taxane-based chemotherapy (McGuire et al., 1996). Patients initially respond to chemotherapy but eventually develop resistance thereby
leading to poor survival rate (Ozols, 2003, Agarwal and Kaye, 2003). This exemplifies the need to identify new therapeutic targets to treat ovarian cancer patients. Studies have shown that several factors influence the autocrine production of cytokines and growth factors thereby influencing drug resistance to these patients and activation of numerous signaling pathways could contribute to this resistance (Wang et al., 2010). Therefore, targeting these cytokines and growth factors and their signaling pathway may provide new therapeutic opportunities to treat ovarian cancer patients.

1.3 Growth factors and cytokines

1.3.1 Growth factors in ovarian cancer

Growth factors are proteins that bind to receptor tyrosine kinases on the cell surface to regulate a variety of cellular responses, such as activating cellular proliferation and/or differentiation (Owens et al., 1991). Many different families of growth factor and their receptors exist. Their exact functions vary: for example, bone morphogenic proteins stimulate bone cell differentiation (Overman et al., 2013), while fibroblast growth factors and vascular endothelial growth factors stimulate blood vessel differentiation (angiogenesis) (Hoeben et al., 2004, Yun et al., 2010). Many factors and/or receptors are seen to be over expressed in malignant cells. One of the most widely studied growth factors in the context of ovarian cancer progression is the epidermal growth factor receptor (EGFR) (Langdon and Smyth, 1998, Langdon et al., 2009).

1.3.1.1 Epidermal growth factor receptor

The EGF receptor (EGFR) is a member of the ErbB family of receptors (Zhang et al., 2007). Binding of EGF to its receptor complex leads to the activation
Chapter 1 Literature Review

of numerous downstream signaling pathways that are involved in tumor cell growth and survival (Citri and Yarden, 2006, Linggi and Carpenter, 2006). EGFR expression is generally recognized to have a deleterious impact on the clinical outcome of cancer patients whereby its expression is associated with invasive tumor cells (Condeelis et al., 2005). This has led to the development of targeted therapeutics (Wieduwilt and Moasser, 2008, Mendelsohn and Baselga, 2006, Zhang et al., 2007).

Epithelial ovarian cancer has been shown to express EGFR (Auersperg et al., 2001, Conti et al., 2006, Lafky et al., 2008), with EGF stimulation leading to phenotypic changes in the ovarian cancer cells (Ben-Ami et al., 2006). Studies have also shown that patients expressing EGFR have a poor survival rate compared to those who do not express the receptor (Tingulstad et al., 2003).

EGFR in ovarian cancer correlates with increased tumor growth rate (Siemens and Auersperg, 1988), with activation of genes associated with tumor invasion and metastasis, which can be blocked with specific inhibitors (Barbolina et al., 2009, Cowden Dahl et al., 2008, Hudson et al., 2009). EGFR activation can also result in an epithelial to mesenchymal transition with the disruption of E-cadherin and degradation of extracellular matrix by metalloproteinase enzymes (Huber et al., 2005, Barbolina et al., 2009, Sabbah et al., 2008). Therefore, targeting EGFR in ovarian cancer represents an attractive therapy.

1.3.1.2 Cytokines in ovarian cancer

Cytokines are small polypeptides released from cells to regulate the activities of other cells via interactions with specific cytokine receptors expressed on their surface (Leonard and Lin, 2000). They have a wide range of functions, including the ability to stimulate proliferation, differentiation, survival and activation (Kurian et al., 2005, Obata et al., 1997).
As many as 16 different cytokines have been found to be expressed in normal ovaries, along with many of the corresponding receptors (Burke et al., 1996). Indeed virtually all of the cytokines and their receptors found in ovarian tumors are also found in the normal ovary (Nash et al., 1999). However, there appears to be a shift in the expression of these cytokines and their receptor, with significantly increased expression of certain cytokines and receptors observed in ovarian cancer. While their specific physiological roles remain poorly understood they appear to act – along with growth factors such as EGF – to enhance key phenotypes associated with tumor progression, such as proliferation, survival, migration or chemo-resistance (Shield et al., 2007).

1.4 The IL-6R family

1.4.1 Structure and function

Of particular relevance to ovarian cancer are cytokines that act via members of the so-called interleukin-6 receptor or “IL-6R” family of cytokine receptors (Figure 1.2). The core members of this family employ a ligand-specific receptor α chain in combination with a shared receptor chain, glycoprotein 130 (GP130), and sometimes a third receptor chain. However, also in this family are the GP130-related granulocyte colony-stimulating factor receptor (G-CSFR) and obesity gene receptor (OBR), both of which form homomorphic complexes (Devos et al., 1997, Hiraoka et al., 1994).

Individual IL-6R family members have distinct functions: for example, IL-6R mediates immune, hematopoietic and hepatic responses (Kishimoto, 2005), oncostatin M receptor (OSMR) has a role in the differentiation and survival of neural and hematopoietic cells (Morikawa, 2005), leukemia inhibitory factor receptor
Figure 1.2: The interleukin-6 receptor family

The IL-6R family members, with their constituent receptor chains.
(LIFR) functions in stem cell maintenance, neural and hematopoietic development (Taupin et al., 1998, Kurzrock et al., 1991, Graf et al., 2011). G-CSFR plays a key role in granulocytic development, hematopoietic stem cell mobilization and myeloid cell migration (Lieschke et al., 1994, Liongue et al., 2009a, Liongue et al., 2009b), while OBR, the receptor for the cytokine Leptin, functions in the regulation of energy homeostasis (Cottrell and Mercer, 2012). The expression of these receptors and their ligands is tightly controlled to ensure these processes occur in a non-pathological manner.

1.4.2 Downstream signaling

Within the IL-6R family, the utilization of GP130 or related receptor chains to transduce intracellular signals means that similar downstream pathways are activated in each case (O'Sullivan et al., 2007). These include the mitogen-activated protein kinase (MAPK) including ERK1/2 or p38 or JNK, phosphatidylinositol 3-kinase (PI3K), and Janus kinase-Signal transducer and activator of transcription (JAK/STAT) pathways (Imada and Leonard, 2000, Hong et al., 2007, Heinrich et al., 2003, Heinrich et al., 1998). JAK tyrosine kinases are constitutively associated with IL-6R family members and are activated following ligand binding (Heinrich et al., 2003, Heinrich et al., 1998, Calo et al., 2003). This, in turn, leads to tyrosine phosphorylation of the receptor forming docking sites for signaling molecules, including STATs, which in turn also become activated. While other JAKs and STATs may also be activated, virtually all members of the IL-6R family utilise the JAK1-2/STAT3 signaling pathway as a core mechanism for mediating their downstream effects (Rawlings et al., 2004). Phosphorylation of STAT3 causes it to dimerize and translocate from the cytoplasm to the nucleus where it initiates changes in the transcription of a number of genes, including those affecting growth,
differentiation, and survival (Heinrich et al., 1998, Levy and Lee, 2002, Yu and Jove, 2004). Although the full spectrum of STAT3 target genes is yet to be defined, several have been identified that contribute to specific cell phenotypes (Figure 1.3) (Dijkgraaf et al., 2012). These include pro-proliferative genes Cyclin D1 and c-MYC, the anti-apoptotic genes BCL-2 and Survivin, functional genes such as MMPs, ICAM, Integrins and CRP, as well as the key negative regulator Suppressors of Cytokine Signaling (SOCS) 3 (Kim et al., 2007). The latter acts in concert with tyrosine phosphatases and other regulatory proteins to extinguish signaling from IL-6R family members (Wormald and Hilton, 2004)

1.5 Role of the IL-6R family in ovarian cancer

There is increasing evidence that signaling via the IL-6R family may play a significant role in the progression of ovarian and other cancers.

1.5.1 IL-6/IL-6R

IL-6 is a potent, pleiotropic cytokine. It acts via a receptor complex consisting of the ligand-binding IL-6Ra chain and the signal-transducing GP130 chain, both of which are widely expressed (Heinrich et al., 2003, Heinrich et al., 1998). There are two forms of IL-6Ra: a membrane-bound form with low affinity for IL-6 that forms a complex with GP130 after ligand binding, and a soluble form (sIL-6R) that binds with IL-6 extracellularly and then associates with GP130 molecules on target cells (Scheller et al., 2006, Imada and Leonard, 2000). IL-6R signaling is involved in hepatic acute phase response and immune responses and is also known to contribute to follicle development in normal ovaries (Machelon et al., 1994, Lidor et al., 1993). In addition, IL-6R signaling plays numerous roles in the inflammatory response (Hong et al., 2007, Nilsson et al., 2005).
Figure 1.3: IL-6R family signaling in ovarian cancer.

Signaling from the IL-6R family of receptors (autocrine or paracrine) via JAK1-2/STAT3 and its impact on expression of target genes involved in the key malignant phenotypes of proliferation, survival and migration/invasion, as well as negative feedback.
IL-6 has been implicated in a wide range of cancers, such as multiple myeloma (Fulciniti et al., 2009, Shi et al., 2008, Lauta, 2001), endometrial cancer (Bellone et al., 2005), lung cancer (Songur et al., 2004), colorectal cancer (Belluco et al., 2000), renal cell carcinoma (Altundag et al., 2005, Negrier et al., 2004), cervical cancer (Wei et al., 2003) and breast cancer (Garcia-Tunon et al., 2005, Salgado et al., 2003). Overexpression of both IL-6 and its receptors (IL-6R and sIL-6R) in cancer patients has been associated with poor clinical outcomes (Trikha et al., 2003, Hong et al., 2007, Chen et al., 1999, Kishimoto, 2005). IL-6 is also known to directly promote tumor cell attachment, proliferation and migration through the activation of several downstream signaling pathways such as the JAK/STAT and MAPK cascades (Kamimura et al., 2003, Heinrich et al., 1998, Steelman et al., 2004). Other studies have shown that the high expression of IL-6 and its receptors in breast tumor increases BCL-2 expression, thus altering the balance of proliferation and apoptosis toward neoplastic cell proliferation (Garcia-Tunon et al., 2005). The unchecked production of IL-6 can also lead to chronic inflammation, which is implicated in many types of cancer (Ayre, 1956, Dalgleish and O'Byrne, 2002, Farrow and Evers, 2002, Nelson et al., 2004, Schwartsburd, 2003).

Several preclinical studies have explored the role of IL-6 in ovarian cancer. IL-6 can be produced directly by EOCs, or through secondary inflammation (Watson et al., 1990, Wu et al., 1992). Increased levels of IL-6 are associated with poor prognosis (Berek et al., 1991, Stone et al., 2012) and is associated with subtypes with poorer outcomes (Anglesio et al., 2011). IL-6 can act in a number of ways to augment ovarian cancer progression. Through its actions on endothelial cells it can stimulate angiogenesis (Nilsson et al., 2005, Coward et al., 2011) and blunt the potency of chemotherapeutic agents (Lo et al., 2011), as well as act on macrophages.
to polarise them toward the immunosuppressive M2 phenotype (Duluc et al., 2007). However, it can also act directly on ovarian cancer cells to enhance migration, invasion and attachment (Obata et al., 1997, Colomiere et al., 2009, Wang et al., 2012), mediate anchorage-independent growth (Wang et al., 2012) and enhance chemoresistance by increased expression of multi drug resistance pumps and anti-apoptotic genes (Duan et al., 2006, Wang et al., 2010).

Studies have shown that there is an increased expression of IL-6 and different IL-6R isoforms in ovarian tumors, and that both tumor and host cells contribute to soluble IL-6R expression in the tumor environment (Robinson-Smith et al., 2007, Rath et al., 2010, Wang et al., 2012, Colomiere et al., 2009). Increased levels of the soluble form of the receptor are also associated with malignant ovarian cancer, generated either via alternate splicing or increased expression of sheddases that can act on the full-length receptor. This can mediate increased proliferation and other effects in tumor cells, as well as adjacent stromal cells or peritoneal cells (Rath et al., 2010). One study showed that an ovarian cancer cell line positive for IL-6R expression showed an approximately 50% increased rate of tumor formation in mice compared with cells lacking IL-6R expression. The tumors which developed also showed elevated expression of IL-6R (Rath et al., 2010). IL-6 is also known to be constitutively secreted from the ovarian cancer microenvironment and high plasma levels are known to be correlated with poor prognosis (Lutgendorf et al., 2008, Scambia et al., 1995). Production of IL-6 has been shown to be increased in the EOC cell lines SKOV3 and OVCA433 after stimulation with EGF (Colomiere et al., 2009), and in advanced-stage epithelial ovarian cancer via the NFκB pathway (Alberti et al., 2012), indicating cross-talk of other pathways with cytokine signaling. There have also been a number of studies that have reported an increased production
of IL-6 in paclitaxel resistant ovarian cancer cells and also in the serum and ascites of patients (Penson et al., 2000, Duan et al., 1999, Scambia et al., 1995, Berek et al., 1991). These studies suggest that targeting IL-6/IL-6R could provide an effective therapeutic target in ovarian cancer.

1.5.2 G-CSF/G-CSFR

G-CSF is a 25 kD secreted glycoprotein that binds to a single-chain receptor, G-CSFR, closely related to GP130, but which forms a homodimeric complex (Liongue et al., 2009b). The G-CSFR is expressed on a range of hematopoietic cells, including mature neutrophilic granulocytes, myeloid progenitors, hematopoietic stem cells, monocytes and lymphocytes, as well non-hematopoietic tissues, including cardiomyocytes, neuronal precursors, endothelial cells and placental tissue (Touw and van de Geijn, 2007). Its principal role is in the mobilization of hematopoietic stem cells and production of neutrophils during “emergency hematopoiesis” (Panopoulos and Watowich, 2008). These properties have seen G-CSF commonly used in the clinic to increase the production of neutrophils in patients with chemotherapy induced neutropenia (Nakamae-Akahori et al., 2006, Roberts, 2005).

The G-CSFR has previously been implicated in a diverse range of malignancies. For example, a significant proportion of invasive bladder carcinomas have been shown to express both G-CSF and G-CSFR, with subsequent autocrine signaling contributing to their proliferation and survival in vitro (possibly via STAT3), as well as the size of their induced tumors in vivo (Chakraborty et al., 2004). G-CSF was also shown to stimulate the proliferation and migration of tumor cells derived from patients with head and neck squamous cell carcinoma, which could be blocked with neutralizing antibodies. Interestingly, while G-CSFR-positive
tumors grew at a slower rate, increased invasion and angiogenesis, coupled with decreased necrosis, was observed (Gutschalk et al., 2006). Functional G-CSFR complexes have also been identified on other solid tumors (Westphal et al., 2002).

The role of G-CSF/G-CSFR in ovarian cancer has remained controversial. Broad expression of the G-CSFR has been reported in ovarian cancer cells (Brandstetter et al., 1998, Ninci et al., 2000, Savarese et al., 2001, Brandstetter et al., 2001). There was often co-expression of G-CSF in these cells, or in neighbouring stromal tissue, meaning both autocrine and paracrine activation is possible. The clinical significance of G-CSF expression remains unclear, with one study showing it was an adverse prognostic factor if part of a paracrine loop (Savarese et al., 2001), but another suggesting it did not correlate with prognosis (Munstedt et al., 2010). G-CSF was able to support proliferation in certain primary ovarian cancer cells and cell lines (Spinner et al., 1995, Brandstetter et al., 1998), but in others it inhibited proliferation (Spinner et al., 1995) or had no effect (Savarese et al., 2001, Brandstetter et al., 2001). G-CSF was found to enhance EGF-mediated mitogenesis in the G-CSFR-positive cell line OVCAR-3 (Savarese et al., 2001), suggesting cross-talk with growth factor receptor signaling was possible.

1.5.3 Leptin/OBR

Leptin, a 16kDa protein, signals via OBR (Tartaglia, 1997). Like G-CSFR, OBR does not form oligomers with GP130 but signals directly as a homodimer (Nakashima et al., 1997), although it initiates similar signaling pathways to other IL-6R receptors, namely the JAK/STAT, MAPK and PI3K pathways (Ghilardi et al., 1996, Villanueva and Myers, 2008). OBR signaling has a wide variety of roles in regulating energy metabolism, appetite regulation, bone formation and angiogenesis (Bouloumie et al., 1998, Huang and Li, 2000, Sierra-Honigmann et al., 1998).
However, it also has important roles in cancer cell proliferation, invasion and metastasis, including in breast, prostate, hepato and endometrial cancers (Somasundar et al., 2004, Saxena et al., 2007a, Saxena et al., 2007b, Sharma et al., 2006). In the case of breast cancer, there have been extensive studies on Leptin-induced Epithelial-to-Mesenchymal transition via the Wingless Type 1 (WNT1) signaling pathway (Yan et al., 2012), leading to increased cell proliferation (Yin et al., 2004, Hu et al., 2002). Other studies have reported proliferative and anti-apoptotic effects of Leptin in prostate and breast cancers via STAT3 activation (Wazir et al., 2012, Hoda et al., 2012).

The role of Leptin/OBR signaling in ovarian cancer remains less clear. Both Leptin and its receptor are expressed in normal ovary (Loffler et al., 2001), but OBR overexpression represents a poor prognostic factor for epithelial ovarian cancer (Uddin et al., 2009). OBR expression was found in three ovarian cancer cell lines, SKOV-3, BG-1 and OVCAR-3, but only the BG-1 cell line showed an increased rate of proliferation in response to Leptin (Choi et al., 2005). However, another study showed Leptin could stimulate cell proliferation and inhibit apoptosis in the OVCAR-3 cell line (Ptak et al., 2013). Recent studies have confirmed that Leptin can stimulate cell growth and inhibit apoptosis in OVCAR-3 cell line through increased Cyclin D1 and Mcl-1 expression, in this case via the activation of the MEK/ERK1/2 and PI3K/Akt signaling pathways (Chen et al., 2013). Interestingly, while the short forms of OBR are expressed in all EOCs, the expression of the long forms of the receptor is more variable (Choi et al., 2005), which may explain some of the variations in responses to Leptin. Studies on the ovarian cancer lines OVCAR-3 and A2780 have also shown that Leptin is involved in the activation of the estrogen
receptor pathway thereby indirectly promoting tumor cell growth (Choi et al., 2011). However, these studies are yet to be confirmed in vivo.

1.5.4 OSM/OSMR and LIF/LIFR

Oncostatin M (OSM) and Leukemia Inhibitory Factor (LIF) share many biological properties (Gomez-Lechon, 1999) due to the fact that they share receptor subunits, including the GP130 signal transducing subunit (Gearing et al., 1992). Upon binding of either ligand, the receptor complex activates similar signaling cascades, especially JAK2/STAT3 (Stahl et al., 1994, Stahl et al., 1995). LIFRα has been identified on a wide range of hematopoietic cells and cell lines originating from bone marrow, thymus, spleen, liver, placental tissue, and peritoneum (Hilton et al., 1988). OSMRα is also expressed on many cell types, including endothelial cells, hepatic cells, lung cells and bone marrow (Gomez-Lechon, 1999).

Both LIFR and OSMR have been shown to be frequently expressed in tumors, where they can activate downstream signaling pathways upon ligand stimulation (Savarese et al., 2002). OSM was shown to suppress the growth of some but not all breast and lung carcinoma cell lines (Douglas et al., 1997, Ganapathi et al., 1996), but to increase proliferation in prostate carcinoma cell lines (Mori et al., 1999). The functional significance of LIFR in human solid tumor cell lines is also not fully understood. LIF and LIFR has been found to be expressed in breast, kidney and prostate cancer cell lines, and demonstrated to induce tumor growth in these cell lines either in an autocrine or paracrine manner (Dhingra et al., 1998, Kellokumpu-Lehtinen et al., 1996). Autocrine activation of LIFR signaling has also been shown in ovarian cancer. However, this did not alter cell growth, but did cause a modest increase in the rate of apoptosis in these cells (Savarese et al., 2002).
1.6 JAK/STAT/SOCS pathway

Cytokine receptors mediate several of their effects via activation of JAK/STAT proteins, particularly JAK2 and STAT3 in the core of the IL-6R family (Shimoda et al., 1997, Ward et al., 1999b, Dror et al., 2000, de Koning et al., 2000, Ilaria et al., 1999).

1.6.1 JAK2/STAT3 in cancer

Constitutive activation of both JAK2 and STAT3 has been reported in several disease states, including cancer. Thus, activating mutations in JAK2 contribute to various myeloproliferative disorders (Staerk et al., 2007), while increased activity contributes to both renal cell carcinoma (Wu et al., 2007) and lung cancer (Gao et al., 2007). Much more is known about the STAT3 (Bromberg, 2002), which has been shown to play a key role in a variety of malignancies, including lung (Gao et al., 2007) and breast (Sansone et al., 2007) cancers, with constitutively-activated mutant forms of STAT3 sufficient to transform fibroblasts in vitro (Bromberg et al., 1999). Though it’s a transcription factor functions, STAT3 can directly influence gene expression via specific binding sites in the promoters of responsive genes (Bromberg, 2001). Indeed, STAT3 ‘gene signatures’ have been elucidated (Alvarez et al., 2005). Many of the genes known to be regulated by STAT3 function in the broad categories of proliferation (eg. c-myc (Grivennikov and Karin, 2008) and Cyclin D1 (Leslie et al., 2006)), adhesion (eg. ICAM-1 (Yang et al., 2005b), GlyCAM-1 (Hou et al., 2003), E-cadherin (Sultan et al., 2005) and integrins (Panopoulos et al., 2002)) and survival (eg. HIF (Sansone et al., 2007)). Furthermore, STAT3 is also known to contribute to migration/invasion (Silver et al., 2004, Poehlmann et al., 2005). The activation of the JAK2/STAT3 pathway is augmented in some cancer is the epigenetic silencing of the negative regulator, SOCS3 (Isomoto
et al., 2007). Current understanding of the JAK2/STAT3 pathway in ovarian cancer is limited however, although one report has described a positive correlation between STAT3 activation and proliferation (Rosen et al., 2006).

The discovery of constitutively active STAT3 in head and neck cancer and multiple myeloma, provided the link between STAT3 activity and its transformation capabilities in vivo (Song and Grandis, 2000). High STAT3 activity has subsequently been detected in leukaemias, lymphomas, breast cancer, melanoma, ovarian cancer, lung cancer, cervix cancer, pancreatic cancer, and prostate cancer (Sandur et al., 2006). Some of these studies have directly demonstrated the contribution of STAT3 in oncogenesis (Yu and Jove, 2004, Turkson et al., 1998, Bromberg et al., 1998). Definitive evidence for a causative role of STAT3 in cancer was provided following the development of a constitutively active form of STAT3 (STAT3C), which was found to be sufficient for malignant transformation (Liddle et al., 2006).

STAT3 has been identified as playing a key role in cancer progression with inappropriate activation of kinases, in particular tyrosine kinases often via aberrant cytokine or growth factor receptor signaling which leads to the persistent activation of STAT3 in numerous forms of cancer (Gibbs, 2000). STAT3 activity has been shown to play a critical role in tumor cell growth, angiogenesis, resistance apoptosis, and evasion of the host’s immune surveillance (Yu and Jove, 2004). The involvement of STAT3 in these critical processes has made it a popular target for anti-cancer drugs (Yu and Jove, 2004, Lewis et al., 2008, Frank, 2007). Inhibition of STAT3 by a variety of means has been shown to exert anti-cancer effects (Frank, 2007).
1.7 Hypothesis and Aims

This Project is based on the Hypothesis that aberrant activation of the JAK2/STAT3 pathway via IL-6R family members contributes to the malignant phenotypes of ovarian cancer, which may provide new therapeutic opportunities for this disease.

This hypothesis was investigated by pursuing the following aims:

(i) To evaluate the expression/activation of IL-6R family signaling components in normal and neoplastic ovarian specimens.

(ii) To investigate the functional role of specific IL-6R family members in mediating key ovarian cancer phenotypes.

(iii) To understand the importance of the JAK2/STAT3 in mediating the effects of these receptors.
Chapter 2 ~ Materials & Methods
2. Materials & Methods

2.1 Materials

The following companies supplied reagents and equipment used in this work:

Abcam, Cambridge, MA, USA
Agilent Technologies, Forest Hill, Victoria, Australia
Ajax Chemicals, Sydney, NSW, Australia
Ambion, Austin, TX, USA
American Type Tissue Culture, Cryosite, NSW, Australia
Amersham Biosciences, Piscataway, NJ, USA
Amresco, Solo, OH, USA
Applied Biosystems, Foster City, CA, USA
Astral Scientific, Gymea, NSW, Australia
Australian Science Supply, Glen Waverley, Victoria, Australia
Axygen Scientific, Union City, CA, USA
Barwon Health Pharmacy Services, Geelong, Victoria, Australia
BD Biosciences, North Ryde, NSW, Australia
Becton Dickinson Labware, Franklin Lakes, NJ, USA
Berthold Technologies, Bundoora, Victoria, Australia
BioBest, Wentworthville, NSW, Australia
BioCore Pty. Ltd, Alexandria, NSW, Australia
Biocorp, Quebec, Montreal, Canada
Bioline, Alexandria, NSW, Australia
Bio-Rad, Regents Park, NSW, Australia

Cell Signaling Technologies Inc., Danvers, MA, USA

Dako LSAB, Cambellfield, Victoria, Australia

Eppendorf, Hamburg, Germany

Fluka BioChemika, Castle Hill, NSW, Australia

Gilson, Guelph, ON, Canada

Greiner Bio-one GmbH, Frickenhausen, Germany

Hospira, Melbourne, Victoria, Australia

Integrated Sciences, Willoughby, NSW, Australia

Interpath, West Heidelberg, Victoria, Australia

Invitrogen, Mount Waverley, Victoria, Australia

Leica Microsystems GmbH, Wetzlar, Germany

Life Research Pty. Ltd., Scoresby, Victoria, Australia

Life Technologies, Rockville, MD, USA

MatTek Corporation, MA, USA

Merck Millipore, Kilsyth, Victoria, Australia

Merck/Calbiochem, San Diego, CA, USA

Millennium Science Pty. Ltd., Mulgrave, Victoria, Australia

Millipore, Billerica, MA, USA

Nalgen Nunc International, Roskilde, MA, USA

Olympus, Tokyo, Japan

Pacific Lab Products, Blackburn, Victoria, Australia

Perkin Elmer, Glen Waverly, Victoria, Australia
Materials and Methods

Pfizer, Perth, Australia

Promega, Annandale, NSW, Australia

Proscience, Nunawading, Victoria, Australia

RnDSystems, Minneapolis, MN, USA

Sigma-Aldrich, Castle Hill, NSW, Australia

Stratagene GmbH, Heidelberg, Germany

Thermo Fisher, Scoresby, Victoria, Australia

Thermoline Scientific, Smithfield, NSW, Australia

Victorian Bio Bank, Melbourne, Victoria, Australia

Whatman, Middlesex, United Kingdom
2.1.1 Oligonucleotides

Standard oligonucleotides used for reverse transcription-polymerase chain reaction (RT-PCR), quantitative real time-polymerase chain reaction (qRT-PCR) and sequencing were designed using the NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and Primer3 (http://frodo.wi.mit.edu/primer3/) design tools and synthesized by Sigma-Aldrich (Table 2.1.)

Table 2.1: List of oligonucleotides used in this study

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</tbody>
</table>

**Materials and Methods**
2.1.2 Antibodies, cytokines, growth factors and inhibitors

For Western blotting, the following primary antibodies were used: rabbit anti-phospho STAT3 (pTyr 705) (cat # 9131), rabbit anti-total STAT3 (cat # 9130), rabbit anti-E-Cadherin, rabbit anti-N-Cadherin, rabbit anti-Vimentin, rabbit anti-Slug, rabbit anti-Snail (cat # 9782), rabbit anti-BCL2 (cat # 2872), rabbit anti-pNFkB p65 (pSer 536) (cat # 3039), rabbit anti-NFkB p65 (cat # 6956), rabbit anti-pERK (pTyr 204) (cat # 4373), rabbit anti-total-ERK (cat # 4372), rabbit anti-phospho AKT (pSer 473) (cat # 4058), rabbit anti-total-AKT (cat # 4057), rabbit anti-ERCC1 (Cell Signaling) (cat # 3885), rabbit anti-CRP (cat # ab13426), rabbit anti-MDR1 (cat # ab170904), and rabbit anti-GST-pi (Abcam) (cat # ab135535) at a dilution of 1:1000 as well as mouse anti-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (Millipore) (cat # CB1001) at 1:10000 dilution. Secondary antibodies for Western blot were: IRDye 800CW goat anti-mouse IgG (LiCor) and IRDye 680 goat anti-rabbit IgG (LiCor) as appropriate at 1:10000. For flow cytometry mouse anti-G-CSFR conjugated with PE or rabbit anti-OBR (Abcam) followed by anti-rabbit Alexa Fluoro conjugated with FITC (Invitrogen) was used. For immunohistochemistry, mouse anti-G-CSFR (AbCam) or rabbit anti-OBR (Abcam) or rabbit anti-phospho STAT3 (pTyr 705) or rabbit anti-total STAT3 and developed using appropriate secondary antibodies and the Peroxidase Universal Kit (Dako) after elimination of non-specific peroxidase activity.
The following recombinant human cytokines and growth factors were used at 10 ng/ml final concentration: Epidermal Growth Factor, Interleukin 6, Granulocyte Colony-Stimulating Factor (Cell Signaling) and Leptin (Sigma Aldrich).

Where appropriate the following specific inhibitors were used: AG490 (Saito et al., 2006) (JAK inhibitor, Sigma Aldrich), WP1066 (Verstovsek et al., 2008) (JAK2 inhibitor, Bio Core), LLL12 (Lin et al., 2010a) (STAT3 inhibitor, Bio Core).

2.1.3 Media and serum

The following media were used to propagate cell lines: Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen), McCoy’s 5a (Invitrogen), RPMI-1640 (Invitrogen), Eagle’s Minimum Essential Medium (BME) (Invitrogen), Medium 199 (Invitrogen), MCDB 105 (Sigma). These were supplemented with Fetal Bovine Serum (FBS) (Thermofisher), 2 mM L-Glutamine (Invitrogen), and 1% Pen/Strep (Invitrogen).

2.1.4 Cell lines

The cell lines used in this study along with their respective media requirements are described in Table 2.2.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Description</th>
<th>Source</th>
<th>Media</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>CAOV3</td>
<td>Ovarian adenocarcinoma</td>
<td>ATCC cat no #HTB-75</td>
<td>DMEM+10% FBS</td>
<td>(Karlan et al., 1994)</td>
</tr>
<tr>
<td>ES-2</td>
<td>Ovarian clear cell carcinoma</td>
<td>ATCC cat no #CRL-1978</td>
<td>McCoy’s 5a +10% FBS</td>
<td>(Lau et al., 1991)</td>
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<td>HEY</td>
<td>Ovarian adenocarcinoma</td>
<td>Gift from N. Ahmed, Royal’s Women Hospital Melbourne</td>
<td>DMEM+10%FBS</td>
<td>(Shield et al., 2007)</td>
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<tr>
<td>NIH: OVCAR3</td>
<td>Ovarian epithelial adenocarcinoma</td>
<td>ATCC cat no #HTB-161</td>
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<td>(Hamilton et al., 1983)</td>
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<tr>
<td>OV90</td>
<td>Ovarian serous adenocarcinoma</td>
<td>ATCC cat no #CRL-11732</td>
<td>1:1 mixture of MCDB 105 medium/ Medium</td>
<td>(Ahmed et al., 2002, Lounis et al., 1998)</td>
</tr>
</tbody>
</table>
Materials and Methods

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Tumor Type</th>
<th>Source</th>
<th>Culture Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVCA429</td>
<td>Ovarian adenocarcinoma</td>
<td>Gift from N. Ahmed, Royal’s Women Hospital Melbourne</td>
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<td>OVCA433</td>
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<td>SKOV3</td>
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<td>McCoy’s 5a +10% FBS</td>
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<td>TOV 21G</td>
<td>Ovarian adenocarcinoma</td>
<td>ATCC cat no #CRL-11730</td>
<td>1:1 mixture of MCDB 105 medium/ Medium 199 +15% FBS</td>
</tr>
</tbody>
</table>

2.1.5 Chemotherapeutic agents

Cells were treated with Paclitaxel (Hospira) (1 µg/ml for 5 days) or Cisplatin (Pfizer) (5 µg/ml for 5 days) for cytokine expression studies or at 12.5 µg/ml in combination with cytokines to induce apoptosis.

2.1.6 Clinical Samples

Ovarian epithelial tumours of different pathological grades were collected from patients diagnosed with high-grade ovarian carcinoma, after obtaining written informed consent under protocols approved by the Human Research and Ethics Committee (HREC #09/09) of The Royal Women’s Hospital, Melbourne, Australia, or via the Victorian Cancer Biobank and approved by the Deakin University Human Research Ethics Committee (DUHREC#2010-104). The histopathological diagnosis and tumour grades were determined by staff pathologists as part of the clinical diagnosis. Normal ovaries, needed for control comparisons were obtained from
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patients undergoing surgery as a result of suspicious ultrasound images or a family history of ovarian cancer. Specimens were snap frozen under liquid nitrogen and subsequently stored at -80°C for expression analysis or preserved in Tissue-TekR embedding medium and frozen for immunohistochemical studies.

2.2 Cell methods

2.2.1 Cell line maintenance

Cells were retrieved from liquid nitrogen and slowly thawed in approximately 1 ml of the relevant medium (Table 2.2). The cells were then centrifuged at 100 g for 5 min, the supernatant removed and the cell pellet resuspended in fresh medium. Cells were then transferred to a culture flask, which was maintained at 37°C and 5% (v/v) CO₂ in an air-jacketed CO₂ incubator.

2.2.2 Subculturing

Cells were sub-cultured to about 80% confluence. The culture medium was removed and the cells were washed twice with Phosphate-Buffered Saline-EDTA (1×) (Ameresco) before trypsinization. The cells were trypsinized with 0.25% (1×) Trypsin–EDTA (Invitrogen) for 3-5 min at 37°C. Once cells were detached fresh medium was added to wash the cells, which were centrifuged at 100 g for 5 min. The cell pellet was resuspended in fresh medium, cells were counted using a hemocytometer and a fixed number of cells were seeded into an appropriate flask or plate.

2.2.3 Cell cryopreservation

Prior to cryopreservation a single cell suspension was prepared using Trypsin as described in Section 2.2.2, and then transferred to cryovials, which were
centrifuged at 100 g for 5 min. The supernatant was removed and approximately 300 µl of freezing mixture (containing relevant media and DMSO in the ratio 9:1) was used to gently resuspend the pellet. Cryovials were sealed and stored in a Mister Frosty™ (Thermo Scientific) overnight at -80°C, before being transferred to liquid Nitrogen for longer term storage.

2.2.4 Fluorescent labeling of cells

Cells were labeled with either CM-Dil (red fluorescence) or DiO (green fluorescence) (Vybrant, Invitrogen) as described (Pruvot et al., 2011). Confluent cells were trypsinized, washed with 67% (v/v) Dulbecco's Phosphate-Buffered Saline (GIBCO, Invitrogen), transferred to 1.5 ml Eppendorf tubes and centrifuged 5 min at 100 g. The cell pellet was re-suspended in DPBS containing either CM-Dil (4 ng/µl final concentration) or DiO (200 µM final concentration). Cells stained with CM-Dil were incubated for 4 min at 37°C and then 15 min at 4°C. Cells stained with DiO were incubated for 20 min at 37°C. Following incubation, cells were centrifuged for 5 min at 100 g and the cell pellet re-suspended in 100% (v/v) Fetal Bovine Serum, centrifuged again and washed twice with DPBS prior to injection.

2.2.5 Migration assay

Confluent cells in a 6-well plate were serum starved for 24 h prior to wounding of the monolayer with a sterile 200 µl pipette tip. Where required, appropriate growth factors, cytokines and/or inhibitors were added 1 h prior to wounding. Three representative fields were marked and imaged immediately after and 15 h after wounding as described (Lim et al., 2007, Chan et al., 2008). Cell migration across the wound was analyzed using Cell Profiler® software from Broad Institute (Carpenter et al., 2006).
2.2.6 Chemotaxis assay

Chemotaxis assays were performed with a two-chamber transwell (Corning) as described previously (Zhao et al., 2011), using a polycarbonate filter insert with 3 μm pores. Cells were trypsinized and suspended in serum-free medium containing 0.1% (v/v) FBS at a concentration of $1 \times 10^5$ cells/insert. The cells were placed in the upper chamber and media was placed in the lower chamber. After 4 h at 37°C, the cells in the upper chamber were wiped off with a cotton swab. The cells on the lower surface of the filter were fixed with 4% (v/v) Paraformaldehyde for 1 min at room temperature, permeabilized with 100% methanol, washed with PBS and stained with Giemsa solution. Migration was quantitated by selecting ten random fields and counting the number of migrated cells.

2.2.7 Growth assay

Growth rates were assessed by manual cell counting as described (Ouellet et al., 2008). On day 0, $1 \times 10^5$ cells were seeded onto 60 mm petri dishes. On day 1, 2, 3, 4, cells were trypsinized, resuspended in medium and counted using a hemocytometer. Each experiment was performed in triplicate for each harvest and repeated thrice.

2.2.8 Apoptosis assay

Cells were grown to confluence and treated with 0.1% Sodium Azide (Huang et al., 2008) or cisplatin (12.5 μM) or paclitaxel (1 μM) (Das et al., 2003) to induce apoptosis and in combination with appropriate growth factors, cytokines and/or inhibitors, before analysis using FITC Annexin V Apoptosis Detection Kit I (BD Phamingen™), according to the manufacturer’s instructions (van Engeland et al., 1996).
2.2.9 Viability assay

Cells were treated with appropriate growth factors, cytokines and/or inhibitors, and the viability was determined using a Trypan Blue cell exclusion assay (GIBCO, Invitrogen) and manual counting using a hemocytometer, as described (Dugas et al., 2010).

2.3 DNA/RNA methods

2.3.1 Isolation of total RNA

Approximately 2×10⁶ were harvested in 1 ml Trizol reagent (Invitrogen), and the phases separated by the addition of 200 μl chloroform. After a brief incubation at room temperature, the Trizol-chloroform mix was centrifuged at 12000 g for 10 min at 4°C. RNA was precipitated with the addition of 500 μl isopropanol and incubated at room temperature (RT) for 10 min, followed by centrifugation at 12000 g for 10 min at 4°C. The RNA pellet was washed with 75% (v/v) ethanol and centrifuged at 7500 g for 10 min at 4°C. RNA was subsequently resuspended in (20-50 μl) sterile nuclease-free water (Ambion).

2.3.2 Nucleic acid concentration and purity

DNA/RNA concentration and purity were determined by UV spectrophotometry at 260 nm and 280 nm wavelengths using a NanoVue™ (General Electric) following the manufacturer’s instructions. RNA was considered pure with an A260/A280 ratio of >1.8, and DNA with an A260/280 ratio of >1.6.

2.3.3 cDNA synthesis

cDNA was synthesized from 0.5 μg of total RNA using an iScript cDNA Synthesis Kit (BioRad), following the manufacturer’s recommendations. Negative
controls were prepared by substituting iScript reverse-transcriptase enzyme mixture with an equivalent volume of water (no RT-control).

2.3.4 Polymerase chain reaction (RT-PCR)

PCR reactions were performed on a My-Cycler thermal cycler (Bio-Rad) using 25 μL reaction volumes containing 500 ng cDNA template, 5.5 μL sterile nuclease free water (Ambion), 12.5 μL PCR GoTaq Green Master Mix containing Taq DNA polymerase, dNTPs, MgCl₂ and reaction buffers at optimal concentrations (Promega) and 10 μM each of gene specific forward and reverse primer (Table 2.1). Amplification conditions were typically 94°C for 2 min, followed by 94°C for 1 min, 58°C for 1.5 min and 72°C for 1 min for a total of 30 cycles, with a final incubation of 72°C for 10 min, unless otherwise specified. Control reactions were performed using the no RT-control to confirm the absence of contaminating genomic DNA, and without template to ensure that amplicon products were not the result of contamination or primer-dimer effects. Parallel amplification of the β-ACTIN (ACTNB) gene was used as a loading control.

2.3.5 Agarose gel electrophoresis

Electrophoretic analysis of DNA was performed using 1-3% (w/v) agarose (Amresco) gels in 1×TAE buffer (4.8% (w/v) Tris-HCl, 1.1% (v/v) Glacial acetic acid, 3.7% (w/v) 0.5 M EDTA; pH8.0) with the addition of SYBR® Safe (1:10000) (Invitrogen) per 50 ml. Electrophoresis was undertaken at 110 V for 30-60 min in a Bio-Rad mini-tank apparatus, followed by imaging under ultra violet (UV) illumination using a Chemidoc XRS Molecular Imager System (Bio-Rad) using Quantity One software (Bio-Rad).
2.3.6 Quantitative real time polymerase chain reaction (qRT-PCR)

Gene expression was quantified by qRT-PCR on the Agilent Stratagene MX3000P. Reactions (25 μL) contained 3.125 μL nuclease-free water, 3.125 μL cDNA template (1:5 dilution), 12.5 μL iQ SYBR Green Supermix containing Reaction buffer, dNTPs, 50 U/ml iTaq DNA polymerase, 6 mM MgCl₂, 20 nM SYBR® Green I (Bio-Rad) and 3.125 μL each of forward and reverse primer (2.4 μM) (Table 2.1). Typical PCR conditions consisted of 95°C for 30 sec, followed by 45 cycles of 95°C for 10 sec, 60°C for 30 sec, 72°C for 20 sec, then 95°C for 1 min, 55°C for 1 min, and finally incremental increases (0.5°C) from 55°C to 95°C to establish the melting curve for each sample. Appropriate control reactions were performed to ensure products were not the result of DNA contamination or due to primer-dimer formation. Data retrieved from these assays were analysed using the Livak method (Livak and Schmittgen, 2001).

2.4 Protein methods

2.4.1 Immunohistochemistry

Paraffin embedded primary tumor samples were analyzed by immunohistochemistry, as previously described (Colomiere et al., 2009, McCulloch et al., 2009). Briefly, tissue sections were blocked (1:50 human serum) and incubated with primary antibodies, washed and developed using appropriate secondary antibodies and the Peroxidase Universal Kit (Dako) after elimination of non-specific peroxidase activity. Slides were counterstained with 1% (w/v) hematoxylin to highlight cell nuclei. Optical image capture and analysis was performed using AxioVision and K5400 Zeiss software image analysis on a Zeiss Axioskop2 Microscope. Ten fields from each section were analysed in a “blind” fashion and the
percent tissue staining determined, which was arbitrarily scored as follows: 0 (≤10%), 1 (≥11-25%), 2 (≥26-50%), 3 (≥51-75%), 4(≥76-90%) and 5 (≥91-100%). Control slides were processed in the absence of primary antibody, secondary antibody and chromogenic substrate, or with isotype control IgG to ensure specificity.

2.4.2 Protein isolation

Approximately 2×10^6 cells were harvested using a cell scraper and suspended in 0.5 ml of RIPA Lysis Buffer, containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% (v/v) NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin (Sigma) and phosphatase inhibitor cocktail (5mg/ml). After 5 min on ice, cells were centrifuged at 14,000 g for 15 min at 4°C, with the supernatant containing extracted proteins collected and stored at -80°C.

2.4.3 Protein concentration determination

Total protein content was determined using a BCA Protein Determination Kit (Pierce) following the manufacturer’s instructions, using bovine serum albumin as the standard and reading the absorbance at a wavelength of 562 nm using a PerkinElmer VICTOR X Multilabel Plate Reader.

2.4.4 Western blotting

Protein lysates were denatured by heating at 100°C for about 3 min and cooling immediately on ice for 5 min before separation on a 10% Tris-HCl Polyacrylamide gel (1.5 ml 1.5 M Tris Buffer, 2.5 ml 30% (v/v) acrylamide mix, 3.475 ml water, 25 µl 10% (v/v) APS and 15 µl TEMED), and electroblotted to a polyvinylidene difluoride (PVDF) membrane in 20% (v/v) methanol, 25 mM Tris
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base, 192 mM glycine, pH 8.0. The PVDF membranes were blocked with 5% (w/v) BSA in 20 mM Tris base, pH 7.6, containing 200 mM NaCl, and 0.05% (v/v) Tween-20, and then incubated with primary antibodies at 1 μg/ml overnight at 4°C. Membranes were then washed and incubated with a secondary antibody IRDye 800CW Goat anti-Mouse IgG and IRDye 680 Goat anti-Rabbit IgG (1 μg/ml) (LI-COR Biosciences) and antibody binding detected using an Odyssey Imager.

2.4.5 Flow cytometry

Cell lines were grown to confluence prior to harvest for fluorescence activated cell sorter (FACS) analysis. Cell monolayers were washed twice with phosphate buffered saline (PBS), detached with 0.25% (w/v) trypsin-EDTA solution, collected by centrifugation and washed a further two times with PBS. Approximately 1×10^6 cells were fixed using 4% (w/v) PFA and subsequently blocked for 30 min in PBS containing 10% (v/v) goat serum. After incubation, the cells were washed twice with PBS, incubated with appropriate antibody at a concentration of 1 μg/μl for 1 h at room temperature, followed by three washes in PBS. The cells were then incubated with appropriate secondary antibody for 30 min at room temperature. The sample were washed and resuspended with PBS and were analysed on FACS Canto (Becton Dickinson) using FACS DIVA software, in comparison to appropriate controls.

2.4.6 Enzyme – Linked immunosorbent assay (ELISA)

ELISA was performed using a Human G-CSF Quantikine ELISA Kit (RnDSystems, Minneapolis, USA), on conditioned media, following the manufacturer’s instruction (Robinson et al., 2009). The absorbance was read using a Fusion-Alpha-HT plate reader.
2.4.7 Electrophoretic mobility shift assay (EMSA)

Cells treated with/without cytokines and inhibitors were washed with 1 ml cold PBS/0.1 mM Na$_3$VO$_4$ before resuspending in 200 μl hypotonic buffer (200 mM HEPES; pH 8, 1 mM Na$_3$VO$_4$, 20 mM NaF, 1 mM EDTA, 0.2 mM DTT, 1×protease inhibitor cocktail). Cells were lysed by the addition of 4 μl 10% (v/v) Tween-20 followed by vortexing for 10 sec. Nuclei were pelleted by centrifugation at 13000 rpm for 30 sec at 4°C and then resuspended in 20 μl hypertonic buffer (100 mM HEPES pH 8, 420 mM NaCl, 20% (v/v) glycerol, 1 mM Na$_3$VO$_4$, 20 mM NaF, 1 mM EDTA, 1 mM DTT, 1×protease inhibitor cocktail) before rocking for 30 min at 4°C to extract nuclear proteins. Samples were then centrifuged at 13000 rpm for 20 min at 4°C and the supernatant transferred to a fresh tube for storage at –80°C. Nuclear extracts were thawed on ice and an aliquot incubated with 1 μg dI-dC (Sigma) and approximately 200 cpm $^{32}$P-labelled STAT (m67) probe in 1×Lamb binding buffer (10 mM HEPES; pH 7.6-7.8, 17 mM NaCl, 3 mM NaMoO$_4$, 0.85 mM DTT, 5% (v/v) glycerol, 0.15 mM MgCl$_2$) at room temperature for 20-30 min. Samples were subsequently loaded onto a 4% (w/v) (29:1 acryl:bis-acryl) acrylamide ½×TBE vertical gel and run at 80 V for 3 h before drying and being subjected to autoradiography as described (Ward et al., 1999a)

2.5 Animal studies

2.5.1 Zebrafish maintenance

Wild-type zebrafish were commercially sourced and reared using standard practices (Kimmel et al., 1995) at the Deakin University Aquarium Facility. Water temperature was maintained at 28.5°C, pH 7.0. An automatic cycle of 14 h of light and 10 h of dark simulated the day/night cycle. Adult zebrafish were fed daily with a
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A mixture of brine shrimp and flake food, with additional protein supplements for laying pairs. Embryos were collected after spawning and allowed to develop synchronously in egg water (2.5% (w/v) Na$_2$HPO$_4$; pH 6.0–6.3) within a Petri dish at 28°C.

2.5.2 Xenotransplantation

Xenotransplantation into zebrafish embryos was performed essentially as described (Marques et al., 2009, Latifi et al., 2011). Fluorescently labelled cells were suspended in Daineau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO$_4$, 0.6 mM CaCl$_2$, 5.0 mM HEPES; pH 9.0), and ~100 cells injected into the yolk sac of 2 day post fertilization (dpf) dechorionated and anesthetized with 0.1 mg/ml of benzocaine as published (Liongue et al., 2009a). Embryos were incubated at 35°C, and xenotransplanted cells visualized using a confocal microscope (Olympus FluoView 1000, Australia) at 24 and 48 hours post injection (hpi).

2.6. Statistical Analyses

All statistical analyses were performed using GraphPad Prism 4. Data were expressed as mean ± S.E.M. The statistical correlation of data between groups was analyzed by one-way analysis of variance (ANOVA) and a two-tailed Student’s t test, where $p<0.05$ was considered significant.
Chapter 3 ~ Expression of IL-6R family members in ovarian cancer
3.1 Introduction

The etiology of ovarian cancer remains poorly understood, although it has been postulated that the repetitive breaking and repair of the ovarian surface epithelium in the course of the ovulation process has a tumor promoting influence (Auersperg et al., 2001). This repair process is governed by a variety of growth factors and cytokines acting in a paracrine and/or autocrine fashion (Kwintkiewicz and Giudice, 2009).

The IL-6R family of cytokine receptors are members of the class I hematopoietin cytokine receptors. These consist of a ligand specific receptor chain, and often a separate, shared signal-transducing chain, such as GP130 (Kishimoto et al., 1995, Nakashima and Taga, 1998, Taga, 1997). The IL-6R family signals through various intracellular signaling cascades, including the JAK/STAT pathway, with JAK1/2 and STAT3 being preferentially utilized.

A number of studies have implicated perturbation of the cytokine receptor/JAK/STAT pathway in a range of solid tumors by promoting tumor cell proliferation and survival, angiogenesis and immune evasion (O'Sullivan et al., 2007, You et al., 2012, Doucette et al., 2012, Sansone and Bromberg, 2012). Both tumor cells and immune cells are known to secrete cytokines and growth factors to enable both autocrine and paracrine signaling that underpins these key cancer phenotypes (Punnonen et al., 1998, Zeimet et al., 1998, Zebrowski et al., 1999). Therefore, the expression of functional IL-6 receptors on tumors cells suggests a potential role in tumorigenesis (Lu et al., 2006, Chalaris et al., 2011).
Studies have suggested a role for aberrant cytokine receptor signaling in ovarian cancer (Tsai-Turton et al., 2009). For example, several studies have shown autocrine activation of the IL-6 receptor mediates a number of malignant phenotypes in ovarian cancer cells (Colomiere et al., 2009, Guo et al., 2010). In light of this, the aim of this Chapter was to comprehensively evaluate the expression of members of the IL-6 receptor family and their ligands in ovarian cancer.
3.2 Results

3.2.1 Expression of IL-6 family receptors and their ligands in ovarian cancer cell lines

3.2.1.1 Basal level expression

The expression of the genes encoding components of the IL-6R family and their respective ligands was determined using semi-quantitative PCR on a panel of ovarian cancer cell lines, with $\beta$-ACTIN used as a control for input RNA. In addition, expression of EGF and EGFR was examined as EGF/EGFR signaling is a known mediator of ovarian cancer pathology. As previously described (Kohler et al., 1989, Kohler et al., 1992, Psyrri et al., 2005, Colomiere et al., 2009), EGFR was expressed in all cell lines examined, with lower expression of EGF.

For the IL-6R family chains, the common signaling chain GP130 was almost universally expressed. Other individual chains were broadly expressed in unique patterns, which was irrespective of the ovarian cancer subtype. IL-6R$\alpha$, LIFR$\alpha$ and OSMR$\alpha$ were found to be widely expressed. Most of the cell lines also expressed IL-11R$\alpha$, IL-27R$\alpha$ and IL-31R$\alpha$, although often at low levels, with the OVCAR3 cell line not expressing IL-27R$\alpha$, and both CAOV3 and OV90 not expressing IL-31R$\alpha$. G-CSFR$\alpha$, OBR$\alpha$ and CNTFR$\alpha$ were found to be expressed in a subset of cell lines. In terms of ligands, all cell lines expressed IL-6, LIF, CNTF and IL-11 to some extent. G-CSF, LEPTIN, OSM and IL-31 were variably expressed, while IL-27 was only expressed by a single cell line (Figure 3.1).
Figure 3.1: Basal expression of IL-6R family members and their ligands in ovarian cancer

RNA derived from a panel of ovarian cancer cell lines was analyzed with primers specific for genes encoding components of IL-6R family members along with their ligands using semi-quantitative RT-PCR, as shown. The relative expression of each was scored on a 0-5 scale where 0 being no expression and 5 being high expression, as indicated in the key.
### Chapter 3 IL-6R family gene expression

**Ovarian cancer sub-types**

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<td>TOV21G</td>
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<tr>
<td>OVCAR5</td>
<td>HEY</td>
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- **GP130**
- **IL-6R**
- **IL-6**
- **LIFRα**
- **LIF**
- **OSMRα**
- **OSM**
- **IL-31Rα**
- **IL-31**
- **IL-11Rα**
- **IL-11**
- **G-CSFRα**
- **G-CSF**
- **OB-Rα**
- **LEPTIN**
- **CNTFRα**
- **CNTF**
- **IL-27Rα**
- **IL-27**
- **EGFR**
- **EGF**

**KEY:**

- 5
- 4
- 3
- 2
- 1
- +/-
- 0
Based on the expression pattern of the individual receptor chains, it was possible to deduce which cell lines expressed particular receptor complexes. Most expressed IL-6R (IL-6Rα+GP130), LIFR (LIFRα+GP130) and OSMR (OSMRα+GP130), while many expressed IL-11R (IL-11Rα+GP130) and IL-31R (IL-31Rα+OSMRα+GP130). However, IL-27R (IL-27Rα+IL-27Rβ+GP130) and CNTFR (CNTFRα+LIFR+GP130), along with the homodimeric G-CSFR and OBR, were expressed by just a subset of cell lines. The broad presence of these complexes and their ligands also suggested autocrine activation was possible for multiple receptors in each cell line.

### 3.2.1.2 Expression following cytokine or growth factor stimulation

Studies have previously demonstrated that stimulation with cytokines and growth factors could potentially impact on ligand/receptor expression (Colomiere et al., 2009, Wang et al., 2008). Therefore, the various cell lines were also treated with IL-6 or EGF and reanalyzed for the expression pattern of the IL-6R family components and their ligands. In comparison to unstimulated cells, the overall expression patterns when stimulated with IL-6 or EGF did not markedly change, although the relative levels of individual gene expression did (Figure 3.2). A group of cell lines showed a generally decreased expression in response to IL-6 and EGF (ES2, OVCA433), others showed generally increased expression (CAOV3, SKOV3, OVCA429), and in others there was no effect with IL-6 but varied expression with EGF (OV90, HEY) or when IL-6 but not EGF caused increased expression (PA1).
Figure 3.2: Alteration in expression of IL-6R family members and their ligands in ovarian cancer by EGF and IL-6

A panel of ovarian cancer cell lines was treated with either EGF (10 ng/ml) or IL-6 (10 ng/ml) for 1h in comparison with untreated cells, and analysed for the expression of IL-6R family components and ligands as described in Figure 3.1.
### Chapter 3 IL-6R family gene expression

#### Ovarian cancer sub-types

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<tr>
<th>Clear Cell</th>
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<table>
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<td></td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>LIF</td>
<td></td>
</tr>
<tr>
<td>OSMRα</td>
<td></td>
</tr>
<tr>
<td>OSM</td>
<td></td>
</tr>
<tr>
<td>IL-31Rα</td>
<td></td>
</tr>
<tr>
<td>IL-31</td>
<td></td>
</tr>
<tr>
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<tr>
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<tr>
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<td>EGFR</td>
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<td>EGF</td>
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</table>

**KEY:**

- 5: High expression
- 4: Moderate expression
- 3: Low expression
- 2: Very low expression
- 1: Negligible expression
- +/-: Variable expression
- 0: No expression
3.2.2 Expression of selected IL-6R family members in ovarian cancer

Having established broad expression of IL-6R family members in ovarian cancer cell lines, a sub-set of these receptors were analysed in patient samples. This included two archetypal receptors using a ligand-specific chain and GP130, IL-6R and LIFR, one heterotrimeric receptor, CNTFR, that is a complex of CNTFRα, LIFRα and GP130, and two homomeric receptors, G-CSFR and OBR. This involved RT-PCR analysis of the transcripts encoding GP130, IL-6Rα, LIFRα, CNTFα, G-CSFR, OBR, as well as those encoding their ligands: IL-6, LIF, CNTF, G-CSF and Leptin (Table 3.1). IL-6Rα, G-CSFR and CNTFRα chains were not expressed in the benign mucinous cancer type, whereas the LIFRα chain (1/4) and OBR chain (2/4) were expressed in a few samples of this type. In contrast, the benign serous type expressed IL-6Rα (3/4), LIFRα (4/4) and OBR (4/4), but not G-CSFR or CNTFRα. There was, however, strong expression of all receptor chains in the Grade 3 serous cancer type. Importantly, the common signal transducing chain GP130 was universally expressed. These results suggests the presence of the following receptor complex: in benign mucinous type LIFR (1/4) and OBR (2/4); in benign serous type: IL-6R (3/4), LIFR (4/4), OBR (4/4); and in Grade 3 serous type: IL-6R, LIFR, OBR, CNTFR, G-CSFR.

The expression of the respective ligands was found to be irrespective of cancer type. The strong co-expression of ligands and their receptor complexes in the Grade 3 serous type indicates a strong possibility of autocrine activation in these cells.
Table 3.1: Summary of the gene expression profile of selected IL-6R family components and their ligands in ovarian cancer samples.
Chapter 3 IL-6R family gene expression

<table>
<thead>
<tr>
<th>Grades</th>
<th>Genes</th>
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<th>Benign (Serous)</th>
<th>Grade 3 (Serous)</th>
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<td>3/4</td>
<td>3/4</td>
</tr>
<tr>
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<td>0/4</td>
<td>3/4</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>LIFRα</td>
<td>1/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>LIF</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>G-CSFR</td>
<td>0/4</td>
<td>0/4</td>
<td>3/4</td>
</tr>
<tr>
<td></td>
<td>G-CSF</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>OBR</td>
<td>2/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>Leptin</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>CNTFRα</td>
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<td>0/4</td>
<td>3/4</td>
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<tr>
<td></td>
<td>CNTF</td>
<td>4/4</td>
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<tr>
<td></td>
<td>GP130</td>
<td>4/4</td>
<td>4/4</td>
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</tr>
</tbody>
</table>
3.2.3 Expression of G-CSFR and OBR components in ovarian cancer

The potential involvement of IL-6/IL-6R was not unexpected given other studies. However, the other results were intriguing. Of particular interest was the expression of G-CSFR on high grade tumors, since G-CSF is used to restore neutrophils after chemotherapy, and of OBR, given the correlation between Leptin levels in obese individuals and cancer incidence. Therefore, the focus of my research narrowed to analysing these two cytokine receptor complexes and their ligands, with IL-6/IL-6R serving as a positive control. The expression patterns of these ligands/receptor sets were analysed using Real-Time PCR on 17 Grade 3 serous patient samples (Figure 3.3A-C). The majority of samples showed statistically-significant elevation in expression levels of both G-CSF/G-CSFR and Leptin/OBR compared to normal samples.

In the case of G-CSFR, the availability of a suitable antibody meant that this study could be extended to primary tissue sections. Immunohistochemistry with α-G-CSFR confirmed the absence of G-CSFR expression on normal ovaries (Figure 3.4A), occasional weak staining on benign tumors (Figure 3.4B), but significant scattered epithelial staining within a cohort of tumors (Figure 3.4C), along with some vessel-associated staining. In total, ~60% (22/36) of tumor samples tested showed significant G-CSFR staining (score ≥ 2), compared with 0% (0/11) in normal controls and 20% (2/10) in benign tumors. Parallel staining with anti-pSTAT3 revealed an overall low level epithelial staining on normal ovary and benign tumors (Figure 3.4D, E), but significant staining of vessels (Figure 3.4E). In contrast, a cohort of Grade 1-3 tumors again exhibited strong scattered pSTAT3 epithelial staining (Figure 3.4F). Importantly, the majority of samples with a significant G-
CSFR staining score were also positive for pSTAT3 staining (Figure 3.4G, H), consistent with active signaling in these cells.
RNA derived from Grade 3 ovarian carcinoma samples was analyzed by qRT-PCR with primers specific for *G-CSFR* and *G-CSF* (A), *IL-6R* and *IL-6* (B) and *OBR* and *LEPTIN* (C). These were normalized relative to *β-ACTIN*, and compared to the expression observed in normal tissues. Tumor samples (shaded in dark blue) with statistically significant differences in expression and tumor samples (shaded in light blue) with non-statistically significant difference in expression compared to standard deviation of normal controls (red normal bar) are indicated (*p*<0.05).

Figure 3.3: Expression of selected IL-6R components in primary ovarian cancer
Chapter 3 IL-6R family gene expression

A

Relative G-CSFR expression (log₂ fold)

B

Relative IL-6R expression (log₂ fold)

C

Relative OB-R expression (log₂ fold)

Relative G-CSF expression (log₂ fold)

Relative IL-6 expression (log₂ fold)

Relative OB-R expression (log₂ fold)

Relative LEPTIN expression (log₂ fold)

Normal Grade 3

Normal Grade 3

Normal Grade 3

Normal Grade 3
Figure 3.4: Detection of G-CSFR and phospho-STAT3 in primary ovarian cancer

Immunohistochemical staining of normal ovary (A, D), benign tumor (B, E), or Grade 3 tumor (C, F) samples with α-G-CSFR (A-C) or α-pSTAT3 (D-F), as indicated. Arrows indicate scattered epithelial staining with both antibodies in Grade 3 tumors, and arrowheads indicate vessel-associated staining. Immunohistochemical staining with α-G-CSFR or α-pSTAT3 was scored on a scale of 0-5, and represented as a scatter-plot for normal ovary, benign and pooled tumor groups, with the level of statistical significance indicated (ns: not significant; *: p<0.05).
Chapter 3 IL-6R family gene expression
3.3 Discussion

The results presented in this Chapter revealed broad expression of multiple members of the IL-6R family and their ligands in ovarian cancer samples. The expression patterns could be categorised into three groups. Firstly, those in which both the ligand and their receptor component were expressed, namely IL-6/IL-6Rα+GP130, LIF/LIFRα+GP130, OSM/OSMRα+GP130, IL-31/IL-31Rα+OSMRα+GP130 and IL-11/IL-11Rα+GP130. Secondly, those where the ligand was widely expressed but the receptor chain showed specific expression, such as G-CSF/G-CSFR, Leptin/OBR and CNTF/CNTFRα+LIFR+GP130. Finally, those with broad expression of the receptor but with little expression of the ligand, such as CNTFRα+LIFR+GP130 and IL-27Rα+IL-27Rβ+GP130. Treatment with IL-6 or EGF altered the expression of the cytokines and their receptor chains in subtle ways. However, expression of the shared GP130 chain was similar, suggesting signaling was generally maintained. This is the first time the entire set of IL-6R family is analysed in ovarian cancer which adds further information to this field.

Having identified the expression of genes encoding multiple IL-6R family members in ovarian cancers, the key question to be answered was: what role do these receptors play? IL-6/IL-6R has been widely studied in ovarian cancer and has been shown to enhance migration and invasion (Wang et al., 2007b, Obata et al., 1997). However, functional analysis of the other receptor complexes was still required. The observation in the case of G-CSFR of corresponding increased pSTAT3 levels suggest signaling is occurring, but the consequences of this signaling are unclear. Importantly, the identification of ovarian cancer cell lines expressing G-CSFR and OBR complexes provides the key reagents to commence functional studies on these receptors.
Chapter 4 ~ Functional role of G-CSFR in ovarian cancer
4.1 Introduction

The vast majority of ovarian cancers arise from malignant transformation of either the ovarian surface epithelium (OSE) (Hudson et al., 2008) or the secretory cells of Fallopian tube epithelium (FTE) (Karst and Drapkin, 2010, Colombo et al., 2010). The process by which it occurs remains poorly understood; however, it has been postulated the clonal expansion of OSE and/or FTE secretory cell forms a neoplastic precursor lesion, which ultimately gives rise to ovarian tumors (Colombo et al., 2010). One key event in the progression of the disease is the spread of these neoplastic cells into the peritoneum where they survive as cellular aggregates or spheroids, prior to attachment to a suitable secondary site for further growth, invasion and metastasis (Shield et al., 2009, Burleson et al., 2006). Tumor cells are known to secrete cytokines and growth factors (Zeimet et al., 1998, Punnonen et al., 1998, Zebrowski et al., 1999), which results in autocrine and paracrine loops that are able to support the anchorage-independent growth of ovarian cellular aggregates and stimulate invasion (Shield et al., 2007, Ahmed et al., 2005).

G-CSF principally regulates the proliferation and maturation of granulocytes (Solaroglu et al., 2006). It acts by binding to its receptor complex, G-CSFR, which leads to the activation of many signaling pathways, including the JAK2/STAT3 pathway (Ward, 2007). G-CSF and G-CSFR are known to be expressed in many tumors, and have been shown to induce proliferation and invasion in several cases, including glioma and skin carcinoma cells in vitro (Mueller and Fusenig, 1999, Guo et al., 2010, Mueller et al., 1999). However, studies examining G-CSF/G-CSFR in ovarian cancer – and the phenotypic changes induced – have not been definitive.
Studies from the previous Chapter identified expression of G-CSFR on a subset of high-grade ovarian cancers. This is significant since its ligand G-CSF, has been employed to aid haematopoietic recovery following chemotherapy for a range of malignancies, including ovarian cancer (Bohlius et al., 2003, Ray-Coquard et al., 2007). Therefore, the potential exists for deleterious effects from the use of G-CSF as part of ovarian cancer therapy in those cases where G-CSFR is expressed. Therefore, the Aim of this Chapter was to investigate the functional consequences of G-CSFR activation on ovarian cancer cells.
4.2 Results

4.2.1 Expression of G-CSFR and its ligand in ovarian cancer cell lines

Based on the results of Chapter Three, five ovarian cancer cell lines were chosen for further study. This included three cell lines (HEY, TOV21G, OVCA429) positive for G-CSFR expression, two of which (HEY, OVCA429) were also positive for G-CSF expression, and two cell lines (OVCA433, SKOV3) negative for G-CSFR, with OVCA433 positive for G-CSF. All cell lines were positive for IL-6R and the majority also for IL-6, consistent with previous reports (Watson et al., 1990).

FACS analysis was used to confirm that the G-CSFR expression observed by RT-PCR led to detectable cell-surface expression of the encoded G-CSFR (Fig. 4.1A). Strong staining was observed with α-G-CSFR antibodies in the three cell lines that were positive for G-CSFR expression by RT-PCR (HEY, TOV21G, OVCA429), but not the two RT-PCR negative lines tested (SKOV3, OVCA433). Conditioned media were analyzed using a G-CSF-specific ELISA, which confirmed robust production of G-CSF, particularly in the HEY, OVCA429 and OVCA433 cells lines (Fig. 4.1B), despite the latter not expressing G-CSFR, confirming the two genes are not co-ordinately regulated.

4.2.2 G-CSF stimulates ovarian cancer cell migration

Having established the cell-surface expression of G-CSFR in these ovarian cancer cell lines, the functional significance of this was investigated with regard to key cancer phenotypes. Migration was examined using a wound healing assay (Jones et al., 2008), which was performed either without cytokines or in the presence of G-CSF or IL-6 as a control (Fig. 4.2A).
Figure 4.1: Expression of G-CSF/G-CSFR in ovarian cancer

A-B: Expression of G-CSF/G-CSFR in a panel of ovarian cancer cell lines. Cells were subjected to FACS analysis using α-G-CSFR-PE (red line) or an isotype control (black line), or conditioned media analysed for G-CSF by ELISA (B), which has a standard limit of detection as 20pg/ml.
Chapter 4 G-CSFR in ovarian cancer

A

OVCA429

HEY

TOV21G

OVCA433

SKOV3

B

G-CSF levels (pg/ml)

HEY

TOV21G

OVCA429

OVCA433

SKOV3

detection limit
**Figure 4.2: Phenotypic effects of G-CSF on G-CSFR-positive ovarian cancer cells**

A. Wound healing assay. Indicated cells were incubated in a medium lacking cytokines (Ctrl) or containing G-CSF (10 ng/ml) or IL-6 (10 ng/ml). Images are representative of single independent experiments performed in triplicate. Graphs represent the mean percentage area of wound closure from three independent experiments (n=3; *p<0.05).

B. Transwell migration assay. Indicated cells were analysed for migration in a Transwell chamber in the presence and absence of G-CSF and IL-6. Mean number of cells migrated through the chamber was counted from 5 different areas from three independent experiments (n=3; *p<0.05).

C. Proliferation assay. Indicated cells were incubated over a period of 5 days in the presence and absence of G-CSF (thick dotted line) and IL-6 (thin dotted line). The graph represents the mean cell count of three independent experiments (n=3; *p<0.05).

D. Apoptosis assay. Indicated cells were treated with sodium azide alone (Ctrl) or in the presence of G-CSF and IL-6. Graph shows the mean percentage of apoptotic cells (n=3; *p<0.05).
Chapter 4 G-CSFR in ovarian cancer

A

OVARCA429  HEY  TOV21G  OVARCA433  SKOV3

Wound closure (%)

Ctrl  G-CSF  IL-6

0h  15h  0h  15h  0h  15h  0h  15h  0h  15h

B

OVARCA429  HEY  TOV21G  OVARCA433  SKOV3

Cell no.

Ctrl  G-CSF  IL-6

150  150  150  150  150

C

OVARCA429  HEY  TOV21G  OVARCA433  SKOV3

Cell no. (x10^5)

0  1  2  3  0  1  2  3  0  1  2  3  0  1  2  3

D

OVARCA429  HEY  TOV21G  OVARCA433

Apoptotic cells (%)

Ctrl  G-CSF  IL-6

0  15  30  0  15  30  0  15  30  0  15  30
Migration was increased upon G-CSF stimulation in the G-CSFR-positive cell lines but not in the G-CSFR-negative cell lines. In contrast, IL-6 enhanced migration in each of the cell lines tested, all of which were IL-6R-positive.

To determine whether the migration observed was part of a chemotactic response, a Transwell assay was used to measure directional cell migration. Addition of G-CSF again increased migration in all G-CSFR-positive cell lines (Fig. 4.2A, B). However, no significant difference in migration was observed whether the G-CSF was placed in the upper or lower chamber, suggesting G-CSF-stimulated increased cell migration was not chemotactic in nature.

4.2.3 G-CSF does not affect proliferation but protects against apoptosis

Cytokines are known to stimulate proliferation and survival in responsive cells. Therefore, these parameters were examined in ovarian cancer cell lines in the presence or absence of cytokines. IL-6 elicited a significant increase in proliferation in all cell lines, but no enhancement in proliferation was observed with G-CSF (Fig. 4.2C). Possible effects of G-CSF on survival were then investigated. However, the cell lines tested showed very low levels of apoptosis, even in low serum, and so apoptosis was induced by the addition of sodium azide (0.5%) in order to evaluate potential anti-apoptotic responses. G-CSF was able to provide variable but statistically significant protection against apoptosis in all G-CSFR-positive cell lines (Fig. 4.2D). IL-6 also generally enhanced survival in this setting.
4.2.4 Downstream pathways utilized by G-CSFR

Granulocyte colony-stimulating factor is known to activate many different signaling pathways, but principal amongst these is the JAK2/STAT3 pathway (Ward, 2007). To investigate whether this was functionally important in ovarian cancer cells, lysates were prepared from cell lines stimulated with G-CSF, or IL-6 as a control, and analyzed for STAT3 activation using phospho-STAT3-specific antibodies. Stimulation of the G-CSFR-positive cells OVCA429, HEY and TOV21G with G-CSF led to robust phosphorylation of STAT3, whereas the G-CSFR-negative OVCA433 and SKOV3 did not show STAT3 phosphorylation upon G-CSF stimulation (Fig. 4.3A). In contrast, all cell lines showed IL-6-induced STAT3 phosphorylation (Fig. 4.3A). Antibodies to detect total levels of STAT3 and GAPDH confirmed equivalent loading in each case. To verify the integrity of the JAK2/STAT3 pathway in these cells, specific inhibitors for JAK2 and STAT3 were used in combination with cytokine stimulation. Both inhibitors effectively blocked STAT3 phosphorylation by both cytokines (Fig. 4.3B). The potential activation of ERK and AKT was also examined using phospho-specific antibodies (Fig. 4.3C). G-CSF induced strong activation of ERK, but not AKT, in these cells, despite robust activation of AKT by IL-6. The addition of a JAK2 inhibitor was able to block the G-CSF-mediated ERK activation (Fig. 4.3D), indicating it lay downstream of JAK2. To confirm that signaling via the JAK2/STAT3 pathway also contributed to the G-CSF-induced phenotypes, responsive cell lines were re-analyzed in combination with the specific inhibitors. Both inhibitors were able to suppress G-CSF-mediated migration (Fig. 4.3E).
Figure 4.3: G-CSFR signals via JAK2/STAT3 to mediate its effects on ovarian cancer cells

A. Activation of STAT3 by G-CSF. Lysates from either untreated (Ctrl) cells or those treated with G-CSF or IL-6 were immunoprecipitated with α-STAT3 and analyzed by Western blot with α-phospho-STAT3 and α-STAT3 antibodies. The input lysate was analysed using α-GAPDH to confirm equivalent amounts of protein was examined in each case. Experiments were performed thrice and blots are representative of one experiment.

B. G-CSF-induced STAT3 activation is blocked by specific inhibitors of the JAK2/STAT3 pathway. Cell lysates were prepared from cells either untreated or treated with G-CSF or IL-6 with or without specific inhibitors for STAT3 (S3I) or JAK2 (J2I), as indicated, and analysed for STAT3 phosphorylation as per panel A.

C-D. G-CSF-induced ERK activation and its inhibition by a JAK2 inhibitor. Cell lysates were prepared from untreated (Ctrl) cells or those treated with G-CSF or IL-6, and analysed by Western blot with anti-phospho-ERK, anti-ERK, anti-phospho-AKT, anti-AKT and anti-GAPDH (C). This analysis was repeated for OVCA429 cells in the presence of JAK2 inhibitor (J2I) (D).

E. Migration is blocked by specific inhibitors of the JAK2/STAT3 pathway. Wound closure in OVCA429 cells by G-CSF and IL-6, with or without specific inhibitors for STAT3 or JAK2, as indicated (n=3; *p<0.05, **p<0.005, compared to control untreated cells).
Chapter 4 G-CSFR in ovarian cancer

A

<table>
<thead>
<tr>
<th></th>
<th>OVCA429</th>
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<th>TOV21G</th>
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<tr>
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4.2.5 G-CSF stimulates STAT3-dependant transcription

It was important to determine whether the effects of G-CSF on STAT3 phosphorylation translated into active STAT3-mediated transcription, and so this was examined at several levels. Nuclear extracts were analyzed by EMSA using a STAT3 binding site. G-CSF induced strong binding to this probe, which was also blocked by both JAK2 and STAT3 inhibitors (Fig. 4.4A). The expression of two STAT3-responsive genes, *CRP* and *BCL-2*, was then examined by RT-PCR. This revealed strong induction of both genes by G-CSF in the G-CSFR-positive cell lines, OVCA429, HEY and TOV21G, but not in the G-CSFR-negative cell lines, OVCA433 and SKOV3 (Fig. 4.4B). This was confirmed by both qRT-PCR (Fig. 4.4C) and Western blot analysis (Fig. 4.4D). The addition of JAK2 or STAT3 inhibitors effectively blocked the induction of BCL2 (Fig. 4.4E), confirming it as a STAT3 target.

4.2.6 Interaction between chemotherapy agents and the G-CSF/G-CSFR pathway

Both cisplatin and paclitaxel are common and effective chemotherapy treatments for ovarian cancer patients (Bookman, 2012, Neijt et al., 2000). Hence, it was relevant to investigate the status of G-CSF and G-CSFR expression in response to these chemotherapeutic agents in ovarian cancer cells. Addition of cisplatin resulted in increased expression of G-CSFR (~2-4 fold) in the four cell lines tested and of G-CSF (~2-5 fold) in three of these lines, as determined by real-time PCR (Fig. 4.5A). This was further confirmed by ELISA on cell supernatants, which identified increased levels of secreted G-CSF in two of the cell lines, albeit to a modest extent (Fig. 4.5B), and by FACS analysis, which showed increased levels of cell-surface G-CSFR on the four cell lines identified by qRT-PCR, but not in SKOV3 (Fig. 4.5C).
Figure 4.4: Induction of STAT3 transcriptional activity by G-CSF

A. Induction of STAT3 DNA binding by G-CSF. Nuclear extracts were prepared from OVCA429 cells either untreated (Ctrl) or stimulated by G-CSF or IL-6 alone (No Inh) or in combination with inhibitors for STAT3 (S3I) or JAK2 (J2I), as indicated.

B-E. Expression of STAT3 responsive genes by G-CSF. Cells either untreated or treated with G-CSF (grey bar) or IL-6 (black bar) were analyzed by semi-quantitative RT-PCR (B), with the fold change determined by subsequent Real-Time RT-PCR (C, *p<0.05 compared to the control). Expression of the encoded proteins in these cells was determined by Western blot analysis as indicated (D). For BCL2, this analysis was repeated in the presence of inhibitors for JAK2 (J2I) and STAT3 (S3I) (E).
Chapter 4 G-CSFR in ovarian cancer
Addition of paclitaxel led to a similar induction of G-CSF and G-CSFR (Fig. 4.5D-E). The transcription factor NFκB is known to be activated by chemotherapeutic agents (Lagunas and Melendez-Zajgla, 2008) and to regulate genes encoding cytokines (Lawrence, 2009), including G-CSF (Dunn et al., 1994). Treatment with either cisplatin or paclitaxel induced NFκB activation (Fig. 4.5F), suggesting that it may be involved in the induction of G-CSF, and potentially G-CSFR.

Cisplatin was found to enhance wound closure, to a similar extent of that induced by G-CSF (Fig. 4.6A). This was blocked by inhibitors of the JAK2/STAT3 pathway (Fig. 4.6A), which were effective in this setting (Fig. 4.6B). Both cisplatin and paclitaxel were also able to induce significant apoptosis in these cell lines (Fig. 4.6C-D, respectively). This could be ameliorated by treatment with G-CSF (or IL-6), but not in the presence of STAT3 or JAK2 inhibitors, consistent with robust activation of STAT3 by G-CSF in combination with either agent (Fig. 4.7A). In this context, ERK was also activated by G-CSF, but not AKT (Fig. 4.7B). Finally, G-CSF was able to induce MDR1, GST-pi and ERCC1 to various extents in all three cell lines tested, including in the presence of cisplatin or paclitaxel (Fig. 4.7C), suggesting these proteins likely contribute to the G-CSF-induced chemoresistance phenotype observed.
Figure 4.5: Interaction between chemotherapeutic agents and G-CSF/G-CSFR

A-E. The effects of the chemotherapeutic agents cisplatin and paclitaxel on the expression of G-CSF/G-CSFR. The impact of 5 day chemotherapy treatment on the indicated ovarian cancer cell lines was determined by RT-PCR for G-CSFR and G-CSF in response to cisplatin (A) or paclitaxel (D), analysis of cell supernatants using ELISA for G-CSF in response to cisplatin (B), or FACS analysis for G-CSFR expression in response to cisplatin (C) or paclitaxel (E) (red line: untreated, blue line: chemotherapy agent) or isotype control (black line).

F. Chemotherapeutic agents activate NFκB. Cell lysates were prepared from OVCA433 cells treated with cisplatin (Cis) or paclitaxel (Pac) as indicated, and subjected to Western blot analysis with anti-phospho-NFκB, anti-NFκB or anti-GAPDH.
Chapter 4 G-CSFR in ovarian cancer

A

Cisplatin

OVCA429 | HEY | TOV21G | OVCA433

Relative level (fold)

G-CSFR | G-CSF

B

G-CSF levels (ng/ml)

OVCA429 | HEY | TOV21G | OVCA433

C

Cisplatin

OVCA429 | HEY | TOV21G | OVCA433 | SKOV3

D

Paclitaxel

OVCA429 | HEY | TOV21G | OVCA433 | SKOV3

Relative level (fold)

G-CSFR | G-CSF

E

Paclitaxel

OVCA429 | HEY | TOV21G | OVCA433 | SKOV3

F

p-NFκB

NFκB

GAPDH

OVCA433
Figure 4.6: Effects of chemotherapeutic agents on cell survival and migration

A. Wound healing assay. Indicated cells were treated with cisplatin alone, or with G-CSF and IL-6 treatment, in the presence or absence of inhibitors for STAT3 (S3I) and JAK2 (J2I). Graph shows the percentage wound closure, expressed as mean ± S.E.M. (*p<0.05 compared to control, #p<0.005 compared to cisplatin)

B. Induction of STAT3 activity. Cell lysates were prepared from OVCA429, HEY and TOV21G cells treated with cisplatin (Cis) as indicated, and subjected to Western blot analysis with anti-phospho-STAT3, anti-STAT3 and anti-GAPDH.

C-D. Apoptosis assay. Indicated cells were treated with cisplatin (C) or paclitaxel (D), or with G-CSF and IL-6 treatment, in the presence or absence of inhibitors for STAT3 and JAK2. Graph shows the percentage of apoptotic cells, expressed as mean ± S.E.M. (*p<0.005 compared to chemotherapeutic agent). For TOV21G cells, cisplatin was increased to 20 μg/ml in order to achieve significant levels of apoptosis.
Chapter 4 G-CSFR in ovarian cancer

A

OVCA429

Cisplatin

G-CSF IL-6

HEY

Cisplatin

G-CSF IL-6

TOV21G

Cisplatin

G-CSF IL-6

B

C

OVCA429 Cisplatin

G-CSF IL-6

HEY Cisplatin

G-CSF IL-6

TOV21G Cisplatin

G-CSF IL-6

D

OVCA429 Paclitaxel

G-CSF IL-6

HEY Paclitaxel

G-CSF IL-6

TOV21G Paclitaxel

G-CSF IL-6
Figure 4.7: Effects of chemotherapeutic agents on downstream pathways

Cell lysates were prepared from OVCA429, HEY and TOV21G cells treated with cisplatin (Cis) or paclitaxel (Pac) as indicated, and subjected to Western blot analysis with anti-phospho-STAT3, anti-STAT3 and anti-GAPDH (A), anti-phospho-ERK, anti-ERK, anti-phospho-AKT, anti-AKT and anti-GAPDH (B), or anti-MDR1, anti-GST-pi, anti-ERCC1 and anti-GAPDH (C).
Chapter 4 G-CSFR in ovarian cancer

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4.3 Discussion

Signaling through the G-CSFR has several important functions, including effective mobilization of hematopoietic progenitor cells and neutrophilic granulocytes from the bone marrow (Christopher and Link, 2007), as well as stimulation of myelopoiesis (Liongue et al., 2009a, Touw and van de Geijn, 2007). On the basis of these properties, G-CSF is used widely to restore neutrophil numbers following chemotherapy, including for ovarian cancer (Ray-Coquard et al., 2007). Therefore, it is of strong clinical interest if G-CSF can exert additional effects on ovarian cancer cells. In this Chapter, the expression of G-CSFR on a panel of ovarian cancer cell lines was confirmed and the effects of G-CSF treatment on proliferation, migration and survival determined.

The G-CSFR has previously been implicated in a diverse range of malignancies. In the case of hematological cancers, its pathogenic effects are mediated via the expression of hyperactive truncated forms, generated through either somatic mutation or disrupted splicing (Liongue et al., 2009b). In solid tumors, mis-expression of both the G-CSFR and its ligand has been implicated. Thus, a significant proportion of invasive bladder carcinoma cells have been shown to express both G-CSF and G-CSFR, with subsequent autocrine signaling contributing to their proliferation and survival in vitro (possibly via STAT3), as well as the size of their induced tumors in vivo (Chakraborty et al., 2004). Both G-CSF and G-CSFR have also been found to be expressed in a series of Ewing’s Sarcoma patient samples and cell lines, with in vivo tumor growth significantly increased by G-CSF treatment (Morales-Arias et al., 2007). Similarly, dysplastic and squamous cell carcinomas (SCCs) have been shown to exhibit higher expression of G-CSF and G-CSFR than
normal controls (Hirai et al., 2001). G-CSF has also been demonstrated to stimulate the migration of tumor cells derived from patients with head and neck squamous cell carcinoma, with G-CSFR-positive tumors showing increased invasion (Gutschalk et al., 2006).

The role of G-CSF/G-CSFR signaling in ovarian cancer has remained controversial. Previous reports have shown that G-CSFR is expressed on primary ovarian carcinomas (Brandstetter et al., 1998, Brandstetter et al., 2001, Ninci et al., 2000, Savarese et al., 2001). G-CSF is often co-expressed in the cancer cells or surrounding stroma, with the potential for both autocrine and paracrine activation (Savarese et al., 2001). However, the importance of G-CSF expression is ambiguous, with one study suggesting that it does not represent an adverse prognostic factor in ovarian cancer (Munstedt et al., 2010), but another showing that overall survival was worse if present as part of a paracrine loop (Savarese et al., 2001). G-CSF has also been demonstrated to stimulate the proliferation of a subset of primary ovarian cells and cells lines (Spinner et al., 1995, Brandstetter et al., 1998, Connor et al., 1994), although in other ovarian cancer cells lines increased proliferation was only observed in synergy with EGF (Savarese et al., 2001), and in others there was either no effect (Savarese et al., 2001, Brandstetter et al., 2001), or indeed inhibition (Spinner et al., 1995). Consistent with these studies, no effect of the G-CSF/G-CSFR pathway on ovarian cancer cell proliferation was observed. In contrast, an alternate role for G-CSF/G-CSFR signaling was identified with respect to ovarian cancer cell migration and survival – including in response to chemotherapy agents. This has not been reported previously, but has a parallel in a recent studying that autocrine IL-6R can confer chemoresistance, including to cisplatin, in ovarian cancer cells (Wang et al., 2010). Interestingly, G-CSF/G-CSFR signaling can directly enhance the motility of
human neutrophils (Nakamae-Akahori et al., 2006) and is essential for the directional migration of myeloid cells during embryonic development (Liongue et al., 2009b), and also plays a key role in myeloid cell survival (Ward, 2007, Eyles et al., 2006). The results presented in this Chapter suggest that these downstream functions can be ‘hijacked’ by ovarian cancer cells.

G-CSFR signaling involves several distinct downstream intracellular signaling cascades, including the JAK2/STAT3 pathway, which ultimately lead to transcriptional changes that impact on survival, proliferation, differentiation and migration (Liongue et al., 2009b). The effects of G-CSF/G-CSFR in augmenting both ovarian cancer cell migration and survival were also shown to be mediated via the JAK2/STAT3 pathway. It has previously been shown that G-CSF-mediated STAT3 activation contributes to myeloid cell survival (Ward et al., 1999a), while STAT3 activation has recently been shown to contribute to both migration (Yoon et al., 2013) and chemoresistance (Zhou et al., 2010) of solid tumors. Published work has also shown that chemoresistant ovarian tumors express higher levels of STAT3 in their non-adherent population (Latifi et al., 2012), while cisplatin treatment was able to generate cells with mesenchymal properties (Latifi et al., 2011). Treatment with either cisplatin or paclitaxel was able to enhance expression of both G-CSF and G-CSFR, potentially via NFκB activation, which may augment STAT3 activation and possibly contribute to chemoresistance. This is consistent with studies demonstrating that NFκB can synergise with STAT3 (Yoon et al., 2013), and that NFκB inhibition can increase the efficacy of paclitaxel (Mabuchi et al., 2004). Finally, three well established contributors to chemoresistance, the P-glycoprotein pump MDR1 (Goda et al., 2009), the DNA repair component ERCC1 (Kirschner and Melton, 2010), and the detoxifying enzyme GST-pi (Vasieva, 2011) were all induced
by G-CSF, providing several potential mechanisms for mediating the effects observed. However, cause and effect relationships need to be firmly established using additional approaches.
Chapter 5 ~ Functional role of OBR in ovarian cancer
5.1 Introduction

Leptin is a 16 kDa protein product of the *obese* gene (Tartaglia, 1997), which exerts its action through a specific receptor variously called the Obese Receptor (OBR), or Leptin receptor, encoded by the *db* gene (Tartaglia et al., 1995, Cioffi et al., 1996). OBR is a member of the IL-6R family that forms homodimers of a single receptor chain. However, there are six isoforms of the OBR chain, which are products of alternative mRNA splicing (Lee et al., 1996, Bacart et al., 2010). These share a common extracellular domain, which is required for ligand binding (Zhang et al., 1994, Ghilardi et al., 1996, Fong et al., 1998), but differ in their intracellular domains. The “long” Leptin receptor chain is the only one capable of inducing intracellular signaling through association with JAK2 (Janus kinase 2) (Baumann et al., 1996, White et al., 1997, Ghilardi and Skoda, 1997, Bjorbaek et al., 1997), which activates STAT3, MAPK’s, and MTA1/WNT1. This chain typically complexes with one of the five “short” receptor chains.

OBR has a wide variety of biological roles including regulation of energy metabolism, appetite, bone formation and angiogenesis (Bouloumie et al., 1998, Huang and Li, 2000, Sierra-Honigmann et al., 1998). High levels of leptin expression are observed in obese individuals (Matsuda et al., 1995), and these individuals have an increased risk of diabetes, fatty liver disease and cancer (Rose-John, 2001). However, Leptin is also known to have an important role in cancer cell proliferation, invasion and metastasis (Somasundar et al., 2004) in several cancer types, including breast, hepato, and endometrial cancers (Choi et al., 2005, Saxena et al., 2007a, Saxena et al., 2007b, Sharma et al., 2006).

In breast cancer, the effects of Leptin have been shown to be mediated through the activation of specific genes by STAT3 to produce different phenotypes:
cyclin D1 and c-Myc for proliferation, integrin β6 for migration and VEGF and its receptor for angiogenesis (Saxena et al., 2007b, Saxena et al., 2007a, Saxena et al., 2008, Gonzalez and Leavis, 2001, Gonzalez et al., 2001). Leptin has also been shown to induce epithelial to mesenchymal transition (EMT) of breast cancer cells via the AKT/GSK3 and MTA1/WNT1 signaling (Yan et al., 2012), with EMT an early step during tumor metastasis.

The results of Chapter Three suggested that Leptin/OBR pathway might also be important in ovarian cancer, supporting an earlier study showing that Leptin induced proliferation in epithelial ovarian cancer cells (Choi et al., 2005). The aim of this Chapter was to examine the functional consequences of the OBR expression on ovarian cancer cells.
5.2 Results

5.2.1 Expression of OBR and its ligand in ovarian cancer cell lines

The OBR expression was further extended to the different isoforms of the gene in three ovarian cancers cell lines of different types, namely PA1 (teratocarcinoma), HEY (adenocarcinoma) and ES2 (clear cell carcinoma) (Fig 5.1A). This revealed strong expression of the OBR long form in PA1 and HEY cell line, with no expression in the ES2 cell line. PA1 and HEY also expressed the OBR short isoform 1 and 3 (S1 and S3), but not the OBR short isoform 2 (S2). ES2 showed very low expression of the OBR short isoform 1 (S1) but not the other two short isoforms. However, all the cell lines showed strong expression of the ligand, Leptin. Leptin treatment resulted in increased expression of the OBR long receptor form in HEY cells but no change in gene expression in the other cell lines. FACS analysis was used to confirm cell-surface expression of OBR (Fig. 5.1B), with strong staining observed in PA1 and HEY, but not in the ES2 cell line.

5.2.2 Role of OBR signaling in ovarian cancer

The functional significance of OBR expression in ovarian cancer cells was investigated with regard to key cancer phenotypes. Migration was examined using a wound-healing assay (Jones et al, 2008), which was performed either without cytokines or in the presence of Leptin (Fig. 5.2A). Migration of cells was found to be increased upon Leptin stimulation in the cell lines expressing the OBR long isoforms (PA1 and HEY) but not in those expressing an OBR short isoform (ES2). Whether the migration observed was part of a chemotactic response was explored using a Transwell assay to measure directional cell migration. Addition of Leptin again increased migration only in the OBR long-positive cell lines (Fig. 5.2B, C).
Figure 5.1: Expression of Leptin/OBR in ovarian cancer

A-B: Expression of Leptin/OBR in a panel of ovarian cancer cell lines. Cells were analysed by RT-PCR for expression of the indicated genes (A), subjected to FACS analysis using $\alpha$-OBR-FITC (red line) or an isotype control (black line) (B).
Chapter 5 OBR in ovarian cancer

A

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<tr>
<th>OBR</th>
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B

- PA1
- HEY
- ES2

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<th>OBR</th>
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<td>10^3</td>
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Figure 5.2: Phenotypic effects of Leptin on OBR long -positive ovarian cancer cells

A. Wound healing assay. PA1, HEY and ES2 cells were incubated in a medium lacking cytokines (Ctrl) or containing Leptin (100 ng/ml). Images are representative of single independent experiments performed in triplicate. Graphs represent the mean percentage area of wound closure from three independent experiments (B).

C. Transwell migration assay. PA1, HEY, ES2 cells were analysed for migration in a transwell chamber in the presence and absence of Leptin. Mean number of cells migrated through the chamber was counted from 5 different areas from three independent experiments.

D. Proliferation assay. Cell counts for the indicated cell lines over a period 3 days in the absence and presence of Leptin. The graph represents the mean of three independent experiments (*p<0.005).

E. Apoptosis assay. Graph shows the mean percentage of three independent experiments of apoptotic cells pre- and post- Leptin treatment (*p<0.05).
Addition of Leptin again increased migration only in the OBR long-positive cell lines (Fig. 5.2B, C).
However, no significant difference in migration was observed whether the Leptin was placed in the upper or lower chamber, suggesting Leptin-stimulation of cell migration was not chemotactic in nature.

Cytokines are also known to stimulate proliferation and survival in responsive cells, and so these parameters were assessed in response to Leptin stimulation. Leptin elicited a significant increase in proliferation in OBR long-positive cell lines (PA1, HEY), but not in the negative line (ES2) (Fig. 5.2D). With respect to survival, the cell lines tested showed very low levels of apoptosis, even in low serum, and so apoptosis was induced by the addition of sodium azide (0.5%) in order to evaluate anti-apoptotic responses. Leptin provided variable but statistically significant protection against apoptosis in the OBR long-positive cell lines (PA1, HEY) but not in the negative line (ES2) (Fig. 5.2E).

5.2.3 OBR mediates its effect in ovarian cancer cells via the JAK2/STAT3 pathway

Leptin is known to activate a number of different signaling pathways, including the JAK2/STAT3 pathway. To investigate whether the OBR expressed on the ovarian cancer cells was functional, cell lines were analyzed for activation of STAT3 following Leptin. Leptin stimulation of PA1 and HEY, which both expressed the long form of OBR, led to a robust phosphorylation of STAT3, but not of the ES2 cell line, which only expressed the short receptor isoforms. Levels of total STAT3 and GAPDH were used to confirm equivalent loading in each case (Fig 5.3A). This experiment was repeated in the presence of specific JAK2 and STAT3 inhibitors alongside a DMSO (vehicle) control. Both inhibitors effectively blocked STAT3 phosphorylation by Leptin in PA1 and HEY cell lines, confirming a functional JAK2-STAT3 pathway (Fig. 5.3B).
Figure 5.3: Pathways activated by Leptin/OBR in ovarian cancer

A. Activation of STAT3 by Leptin. Cell lysates from the indicated lines either untreated (-) or treated with Leptin analysed by Western blot with α-phospho-STAT3 and α-STAT3. The input lysate was analysed using α-GAPDH to confirm equivalent lysate was used in each case. Experiments were performed thrice and blots are representative of one experiment.

B. Leptin-induced STAT3 activation is blocked by specific inhibitors of the JAK2/STAT3 pathway. Cell lysates were prepared from cells either untreated or treated with Leptin with or without specific inhibitors for STAT3 or JAK2, as indicated, and analysed for STAT3 phosphorylation as per panel A.

C-D. Expression of STAT3 responsive genes by Leptin. Cells either untreated or treated with Leptin were analysed by semi-quantitative RT-PCR (C), with the fold change determined by subsequent Real-Time RT-PCR (D) (*p<0.05).
Chapter 5 OBR in ovarian cancer

A

PA1 | LEPTIN | HEY | LEPTIN | ES2 | LEPTIN

pSTAT3 | STAT3 | GAPDH

B

DMSO | LEPTIN | S3 | J2 | S3 | J2 | NO INH

PA1

pSTAT3 | STAT3 | GAPDH

HEY

pSTAT3 | STAT3 | GAPDH

ES2

pSTAT3 | STAT3 | GAPDH

C

PA1 | LEPTIN | LEPTIN | LEPTIN

ICAM1 | CCND1 | BCL2 | MMP2 | MMP9

D

PA1

Relative fold increase

c-MYC | ICAM1 | CCND1 | BCL2 | MMP2 | MMP9

HEY

Relative fold increase

c-MYC | ICAM1 | CCND1 | BCL2 | MMP2 | MMP9
Figure 5.4: Effects of Leptin/OBR on cell survival and migration are mediated via the JAK2/STAT3 pathway

A. Wound healing assay. Percentage of wound closure for cells untreated or with Leptin treatment, in the presence or absence of inhibitors for JAK2 (J2I) and STAT3 (S3I) (mean ± S.E.M; *p<0.05; **p<0.05 compared to Leptin treated).

B. Proliferation assay. Cell counts for the indicated cell lines over a period of 3 days in the presence or absence of Leptin and also in the presence or absence of inhibitors of JAK2 (J2I) shown in red dotted line and STAT3 (S3I) shown in purple dotted line. The graph represents the mean of three independent experiments.

C. Apoptosis assay. Percentage of apoptotic cells when treated with sodium azide alone, or with Leptin treatment, in the presence or absence of inhibitors for STAT3 and JAK2 represented as mean ± S.E.M (n=3; *p<0.05 compared to untreated; **p<0.05 compared to Leptin treated).
Since OBR was signaling via the JAK2/STAT3 pathway the expression of STAT3 responsive genes following Leptin stimulation was examined using semi-quantitative RT-PCR. The genes encoding the following proteins were chosen based on their involvement downstream Leptin: ICAM1, MMP2 and MMP9 for migration, BCL2 for survival and c-MYC and CCND1 for proliferation. Leptin treatment increased the expression of each of these genes in the cell lines expressing the long OBR form (PA1 and HEY), but produced no change in the negative line (ES2), with β-ACTIN was used as a control (Fig 5.3C). This was quantified by subsequent Real-Time RT-PCR (Fig 5.3D).

To confirm that the signaling of the JAK2-STAT3 pathway also contributed to the Leptin-induced phenotypes, responsive cell lines were re-analysed in combination with specific inhibitors. Both the JAK2 and STAT3 inhibitors were able to suppress Leptin-mediated migration (Fig 5.4A), proliferation (Fig 5.4B) and survival (Fig 5.4C).

5.2.4 OBR-induced Mesenchymal to Epithelial changes in PA1 teratocarcinoma cells

Distinct morphological changes were observed in PA1 cells following long term Leptin treatment (2 days) but not in HEY or ES2 cells (Fig 5.5A). These Leptin-treated PA1 cells became tightly packed, cobblestone-like, with little intercellular space; this represents an epithelial phenotype, clearly distinct from their initial fibroblast-like mesenchymal appearance. This observation suggested that Leptin was inducing a mesenchymal to epithelial transformation (MET). To confirm this expression, specific epithelial and mesenchymal markers was examined with or without Leptin. Long term treatment of PA1 cells with Leptin for 2 days, resulted in
a significant decrease in the expression of the mesenchymal markers, N-cadherin and vimentin, and an increase in the expression of the epithelial marker, E-cadherin by Real Time PCR (Fig 5.5B). There was also an increase in the expression of the pro-epithelial transcription factor SNAIL and a decrease in the expression of the pro-mesenchymal transcription factor SLUG. These long term Leptin-induced changes in expression were confirmed at the protein level, this time in the presence and absence of JAK2 and STAT3 inhibitors. Western blot analysis confirmed long term Leptin-induced increased expression of epithelial proteins at the expense of mesenchymal markers relative to GAPDH (Fig 5.5C). Treatment with inhibitors to JAK2 and STAT3 prevented these changes suggesting the involvement of the JAK2/STAT3 pathway downstream of OBR in mediating these alterations. Long term Leptin treatment also suppressed short term Leptin-mediated migration of PA1 (Fig. 5.4D,E), confirming MET transformation.
Figure 5.5: Induction of MET by Leptin

A. PA1 and HEY cells were treated with Leptin (100 ng/ml) for 2 days or untreated. Morphological changes associated with MET are shown in phase-contrast images are shown by arrow marks.

B-C. Expression of MET – responsive genes by Leptin. PA1 cells either untreated or treated with Leptin for 2 days with the fold change determined by subsequent Real-Time RT-PCR with line representing the control (B). Leptin-induced MET activation was blocked by specific inhibitors of the JAK2/STAT3 pathway. Cell lysates were prepared from cells either untreated or treated with Leptin for 2 days with or without specific inhibitors for STAT3 or JAK2, as indicated, and analyzed for change in expression of MET markers and the expression determined using Western blot analysis (C) as per panel B (*p<0.05 compared to control).

D-E. Analysis of PA1 cells following long term treatment with Leptin for migration (D) and proliferation (E).
Chapter 5 OBR in ovarian cancer

A

Untreated

Leptin 48h

B

Relative expression (Fold change)

PA1

ECAD
NCAD
VIMENTIN
SNAIL
SLUG

4
2
0
-2
-4

24h
48h

C

PA1

E-CADHERIN
N-CADHERIN
VIMENTIN
SNAIL
SLUG
GAPDH

Leptin

D

% Wound Closure

PA1

100
50
0

Ctrl Leptin

E

Cell Number

PA1

300
150
0

Ctrl Leptin
5.3 Discussion

Obesity is considered an important etiological factor for cancer that is associated with increased incidence of many common types of cancers (Calle et al., 2003). However, the mechanism by which obesity contributes to cancer remains less clear. Elevated levels of Leptin are observed in obese individuals (Sauter et al., 2004) while epidemiological and in vitro studies have found a correlation between obese individuals expressing high Leptin levels and risk of breast or prostate cancers (Falk et al., 2006, Stattin et al., 2001). Other studies have shown that increased Leptin levels leads to an increased incidence of prostate, breast and endometrial cancer (Yin et al., 2004, Somasundar et al., 2004, Garofalo and Surmacz, 2006, Giovannucci et al., 2003, Mor et al., 2005).

Leptin signaling via OBR has a wide variety of roles in regulating energy metabolism, appetite regulation, bone formation and angiogenesis (Bouloumie et al., 1998, Huang and Li, 2000, Sierra-Honigmann et al., 1998). The long OBR isoform achieves this by initiating downstream signal cascades, including the JAK2/STAT3 pathway. Numerous studies have shown that Leptin signaling can induce cancer cell adhesion, proliferation, survival and migration (Carino et al., 2008, Chen et al., 2007, Li et al., 2012b, Tanaka and Umesaki, 2008, Yuan et al., 2013, Somasundar et al., 2004, Sharma et al., 2006, Saxena et al., 2007a, Saxena et al., 2008). However, its role in ovarian cancer has not been well understood.

One study has demonstrated that Leptin/OBR signaling could induce proliferation of ovarian cancer cells via the MAPK pathway (Choi et al., 2005). Consistent with that study, results from this Chapter also identified a role for OBR/Leptin in inducing proliferation, although mediated via the JAK2/STAT3 pathway. Importantly, the MAPK pathway typically lies downstream of JAK2 and
so is likely blocked with the JAK2 inhibitors used in this study, and may still play a role. However, since Leptin-induced proliferation was also abrogated with a STAT3 inhibitor, it appears that alternate pathways to MAPK also exist.

Leptin was previously shown to induce cancer cells migration via the MAP kinase in prostate cancer (Frankenberry et al., 2004), via the AKT pathway in endothelial cells (Goetze et al., 2002), via the JAK/STAT, PI3K/AKT, and MAPK pathways in hepato cellular carcinoma cells (Saxena et al., 2007a), and via the NF-κB pathway in glioma cells (Yeh et al., 2009). This Chapter also identified OBR/Leptin induced migration signaling pathway in ovarian cancer cells via the JAK2/STAT3. In addition, a novel role in the survival of ovarian cancer cells, also mediated via JAK2/STAT3, was identified. This suggests that inhibitors targeting these molecules may be useful for the management of clinical ovarian cancer.

Studies in breast cancer have shown Leptin is able to induce another phenotypic change, epithelial to the mesenchymal transition (EMT), involving AKT/GSK3 and MTA1/WNT1 pathways (Yan et al., 2012). However, the results in this Chapter showed Leptin could instead induce a mesenchymal to epithelial transition (MET) of ovarian teratocarcinoma-derived PA1 cells. MET is a process that converts motile mesenchymal cells to polarized epithelial cells, a physiological example of cell plasticity that occurs for example during kidney development (Bryant and Mostov, 2008). MET is associated with an up-regulation of epithelial markers, such as E-cadherin and SNAIL and a down regulation of mesenchymal markers such as N-cadherin, Vimentin and SLUG, which was also seen in Leptin treated PA1 cells, confirming that MET was occurring. MET has been shown to decrease cancer cell proliferation, migration and chemo-resistance, acting as anti-tumorigenic process in melanoma cells under hypoxic conditions (Moen et al., 2009,
Na et al., 2009). These data suggest that Leptin has the potential to act as an anti-tumorigenic agent in these cases through induction of MET change. Whether this is restricted to teratocarcinoma cells remains to be determined. Leptin-induced MET was also blocked by JAK2/STAT3 inhibitors, indicating the JAK2/STAT3 pathway was also utilized to mediate this process. JAK2/STAT3 inhibitors would potentially be counter-indicated in such cases.
Chapter 6 ~ General discussion
6.1 Overview

Ovarian cancer and its molecular etiology remain poorly understood. Previous research has suggested growth factors and cytokines might play a role, with several studies identifying expression of cytokine receptors (and their ligands) in ovarian cancer (Burke et al., 1996, Ninci et al., 2000, Choi et al., 2005). However, few studies have investigated either the functional consequences, nor the downstream signaling pathways responsible.

The work described in this Thesis has provided a comprehensive analysis of the expression of the IL-6R family in a panel of ovarian cancer cell lines, including the possible involvement of autocrine loops, and confirmed expression of key ligand/receptor pairs in relevant clinical samples. It has also further shown that both G-CSFR and OBR complexes signal via the downstream JAK2/STAT3 pathway in ovarian cancer cells to facilitate a number of key cancer phenotypes. These results have provided new insights into ovarian cancer that have therapeutic implications.

6.2 Expression of IL-6R family members

Based on previous studies implicating individual IL-6R family members in ovarian cancer cells, Chapter Three investigated the expression of all IL-6R family components and their ligands in a panel of ovarian cancer cell lines, with confirmation studies for a subset of these in relevant clinical samples. This revealed broad expression of several members of the IL-6R family, as well as their ligands, in ovarian cancer cells.

Expression of the IL-6R complex was observed in the majority of ovarian cancer cells lines irrespective of their types, with the co-expression of IL-6 in most of these lines, consistent with previous studies on the expression status of IL-6/IL-6R in
ovarian cancer (Shinriki et al., 2009, Rath et al., 2010, Colomiere et al., 2009). The majority of ovarian cancer cell lines also expressed OBR, OSMR and LIFR while three of five adenocarcinoma ovarian cancer cell line expressed G-CSFR, in line with other studies (Savarese et al., 2002, Choi et al., 2005, Ninci et al., 2000), with LIF, G-CSF and Leptin being expressed more widely than OSM. Variable expression of other members of the IL-6R family, namely CNTFR, IL-27R, IL-31R, IL-11R was also observed, with a similar result for their respective ligands, except for IL-27 which was not expressed in any of the lines. These represent the first analysis of these four cytokine/receptor pairs in ovarian cancer.

Previous studies suggested that cytokine receptor expression levels could be altered in response to cytokines and growth factors (Colomiere et al., 2009, Xu et al., 2010, Zeineldin et al., 2010). Therefore, the expression of IL-6R family members and their ligands was also analysed following the addition of an exogenous cytokine, namely IL-6, and growth factor, EGF. The overall pattern of expression was largely similar to that of the unstimulated cells, although individual receptors/ligands were altered, particularly in some cell lines.

The expression of cytokine receptors on tumor cells has the potential to impact on the migration, proliferation and survival of these cells. Therefore, it was important to verify the expression data in primary ovarian cancer samples. These were analysed for expression of selective cytokine/receptor pairs, namely IL-6/IL-6R, LIF/LIFR, G-CSF/G-CSFR, Leptin/OBR and CNTF/CNTFR. This confirmed the robust expression of these cytokines/receptor pairs in Grade 3 ovarian cancer samples compared to benign and normal samples. Finally, the expression of G-CSFR was also confirmed on tissue sections due to the availability of a suitable antibody against α-G-CSFR. STAT3 phosphorylation was observed in tissue sections
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expressing G-CSFR, providing preliminary evidence that active signaling was occurring in these cells.

6.3 Functional role of G-CSFR in ovarian cancer

Chapter Four focused on the functional consequences of G-CSFR expression in ovarian cancer cells. Aberrant G-CSFR signaling has been implicated in a range of other malignancies including small-cell lung carcinoma (Avalos et al., 1990), astrocytoma (Kikuchi et al., 1996) and primary bladder cancer (Lindemann et al., 1988, Dong et al., 1993, Tachibana et al., 1995). Moreover, G-CSF is used clinically following ablative therapies for a variety of solid tumors, including ovarian cancer (Vial and Descotes, 1995, Nieboer et al., 2001), providing additional clinical relevance.

Stimulation of G-CSFR-positive ovarian cancer cells with G-CSF resulted in increased migration and survival, including against chemotherapy-induced apoptosis indicating the G-CSFR complexes were functional and augmented key cancer phenotypes. G-CSFR utilizes several signaling pathways in hematopoietic cells, including the RAS/MAPK, PI3K/AKT and JAK1-2/STAT3 modules (Carvalho et al., 2011, Avalos, 1996). The phenotypic effects of G-CSFR activation in ovarian cancer cells was shown to be mediated by signaling through the downstream JAK2/STAT3 pathway. This is consistent with the role of G-CSFR-mediated activation of STAT3 in hematopoietic cells, especially with regard to increased survival (Liongue et al., 2009b).

G-CSF is commonly used in the context of ovarian cancer to restore neutrophils ablated by standard chemotherapy (Yamada et al., 2001). However, if the tumor expresses G-CSFR, then clearly G-CSF treatment could actually contribute to
tumor development by increasing migration and/or survival, irrespective of endogenous G-CSF expression. This is consistent with G-CSFR expression status having poor prognosis in oral, colon and pharyngal cancer (Tsuzuki et al., 1998, Yang et al., 2005a). Importantly, aberrant G-CSFR signaling was actually found to be enhanced by both cisplatin and paclitaxel in a number of ovarian cancer cell lines, underlying the need for caution with regard to G-CSF therapy.

Indeed, the results of Chapter Four suggest that it is prudent to re-evaluate G-CSF therapy in ovarian cancer, particularly in patients bearing G-CSFR-positive tumors. Therefore, screening ovarian tumors for G-CSFR expression – by histology, FACS or Next Gen sequencing approaches – could be beneficial prior to administration of G-CSF, potentially indicating alternative therapies for patients with G-CSFR-positive tumors. Of relevance, co-administration of granulocyte-macrophage colony-stimulating factor and recombinant interferon gamma 1b in concert with carboplatin treatment in women with recurrent ovarian cancer was shown to be efficacious and elicited a favourable hematological profile (Schmeler et al., 2009), providing a potential alternative option in this regard.

6.4 Role of OBR in ovarian cancer

Chapter Five examined the role of OBR in ovarian cancer. Epidemiological data have shown a positive correlation between obesity and cancer growth, with obese individuals having a poorer prognosis (Dieudonne et al., 2002, Reeves et al., 2007, Renehan et al., 2008). The biological mechanisms underlying the relationship between obesity and cancer are not well understood. However, obesity is associated with high levels of Leptin (Flier, 1998), which has also been shown to stimulate
proliferation on a range different of cancers (Pai et al., 2005, Choi et al., 2005, Hu et al., 2002).

Chapter Five demonstrated various OBR isoforms were expressed in cell lines representing different types of ovarian cancer. However, the long isoform of OBR that is capable of intracellular signaling was only expressed on the adenocarcinoma and teratocarcinoma subtypes. In these OBR positive cancer cell lines, lines short-term treatment with Leptin increased migration and proliferation, and also protected the cells from undergoing apoptosis. Use of specific inhibitors confirmed that each of these phenotypes was mediated by JAK2/STAT3 pathway. One previous study has shown that OBR signalled via ERK1/2 to induce proliferation of ovarian cancer cells (Choi et al., 2005). This Chapter demonstrated that signaling via the JAK2/STAT3 pathway also contributes to OBR-stimulated proliferation, but also to the other phenotypes.

The teratocarcinoma cell line was also found to undergo a mesenchymal to epithelial (MET) transition in response to long-term Leptin treatment, confirmed by altered expression of specific markers. This transition was also JAK2/STAT3 dependent. MET is a process that can allow cancer cells to stabilise at a distant metastatic site by regaining important phenotypic properties (Yang and Weinberg, 2008). However, studies have also shown that tumors that have undergone MET showed decreased migration and proliferation, as well as increased apoptosis, resulting in increased susceptibility to chemotherapeutic agents (Gunasinghe et al., 2012, Chaffer et al., 2007). Therefore, there is a possibility that Leptin treatment in this context may in-fact be beneficial.
Chapter Five has added to our understanding of the role of OBR in ovarian cancer, potentially throwing some light on the link between obesity and cancer. This Chapter also suggested that Leptin mediated OBR signaling via the JAK2/STAT3 pathway could potentially be pro-tumorigenic or an anti-tumorigenic in ovarian cancer depending on the cell context.

6.5 Future directions

The results presented in this Thesis suggest that further investigation into aberrant cytokine receptor signaling in cancer is warranted.

6.5.1 Further analysis of G-CSFR and OBR

To delineate a functional role for G-CSFR and OBR in key cancer phenotypes in vivo studies in mice would be informative. Xenografts of genetically manipulated (enforced cytokine receptor expression or cytokine receptor knockdown) ovarian cancer cells could be orthotopically transplanted into the ovaries of SCID mice, which mimics the peritoneal invasion of ovarian carcinoma observed in patients (Steinkamp et al., 2013). The mice could then be sacrificed, with the ovaries and surrounding organs evaluated for relative extent of spread and size of the xenografts. These mice could also be treated with rh G-CSF and rh Leptin as appropriate to delineate the effects of increase ligand levels on cancer development. Such experiments could be extended to include mice in which endogenous G-CSF and Leptin genes were knocked-out to distinguish between systemic and local effects. Alternatively, patient derived xenografts with different levels of receptor expression could be assayed in a similar manner.

Ultimately, clinical trials are necessary to definitively assess the roles of G-CSFR and OBR in ovarian cancer etiology. In particular, analysis of the tumor G-
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CSFR status of patients receiving G-CSF as a part of their chemotherapeutic regime and its association with prognosis is urgently required. Similar analyses combining OBR status, Leptin levels, obesity indicators and clinical outcomes in ovarian cancer would also be informative.

6.5.2 Role of other IL-6R family members in ovarian cancer

Chapter Three demonstrated that multiple members of the IL-6R family were expressed in ovarian cancer. Therefore, it would also be of interest to extend the functional studies to other family members that also have relevance to ovarian cancer. Of particular priority would be a detailed study on the role of IL-23R and IL-11R.

Interleukin-23 receptor (IL-23R) is complex of interleukin-12 receptor β1 (IL12Rβ1) and IL-23Rα (Parham et al., 2002), which is known to exert pro-inflammatory function (Vignali and Kuchroo, 2012). IL-23R has been studied in range of cancers, where it has been shown to induce cancer cell proliferation (So et al., 2013, Fukuda et al., 2010, Langowski et al., 2006). In lung cancer cells these proliferative responses have been shown to be mediated by the JAK2/STAT3 pathway, with JAK2 inhibitors able to block these effects (So et al., 2013). However, other studies have shown that high concentrations of IL-23 can inhibit lung cancer cell growth, suggesting that IL-23 may have potential anti-tumor properties (Baird et al., 2013). Similarly, IL-23 has been found to be overexpressed in ovarian cancer, with a positive correlation between expression and overall survival (Wolf et al., 2010). Moreover, polymorphisms in the IL-23R gene have been associated with late stage ovarian cancer in Chinese, suggesting a role in disease susceptibility (Zhang et
al., 2010). Therefore, a detailed study of the role of IL-23/IL-23R expression in ovarian cancer is required to understand its precise role.

The receptor complex for IL-11 consists of a heterodimer of IL-11Rα and GP130 that also signals via the JAK2/STAT3 pathway (Dimitriadis et al., 2006, Wang et al., 2007a). IL-11R signaling supports the growth of multiple hematopoietic progenitors as well as exerting metabolic and cytoprotective effects on cells outside the hematopoietic system (Nandurkar et al., 1998). IL-11 has been shown to regulate adhesion and migration of chondrosarcoma (Li et al., 2012a) and endometrial cancer via STAT3 (Lay et al., 2012), and was recently demonstrated to be the dominant cytokine in the etiology of gastrointestinal cancer (Putoczki et al., 2013). A previous study has shown that IL-11R is expressed on the majority of ovarian cancer cells, with expression of its ligand much less common (Campbell et al., 2001), consistent with the data in Chapter Three. However, the function of IL-11/IL-11R signaling in ovarian cancer remains uncertain. This is of clinical relevance, since IL-11 is being developed as a treatment for chemotherapy-induced thrombocytopenia (Wu et al., 2012).

6.5.3 Role of other cytokine receptors in ovarian cancer

There exist a large number of cytokine receptors, of which the IL-6R family members represent just a small component. Several of the other cytokine receptors share similar pathways and cellular roles to the IL-6R family, and have also been implicated in cancer, meaning that they are also of potential interest. Two examples are the erythropoietin receptor (EPOR) and the IL-4R.

EPOR is a homodimeric receptor that activates JAK2/STAT5 to promote erythroid progenitor survival, proliferation, and differentiation (Wojchowski et al.,
As a consequence, erythropoietin (EPO) is used to increase red blood cell counts in cancer patients following chemotherapy (Filip et al., 1999). However, recent studies have reported reduced survival in patients receiving both EPO and chemotherapy, potentially related to EPO-induced cancer progression (Miyake et al., 2013). EPO has also been shown to induce growth in prostate cancer cells via STAT5 (Feldman et al., 2006). Both EPO and EPOR have been identified in the female reproductive tract, including the ovary (Yasuda et al., 1998, Yasuda et al., 2001a). EPOR is frequently expressed on ovarian tumors and cell lines (McBroom et al., 2005, Jeong et al., 2008). Long-term EPO treatment of patients expressing EPOR-positive ovarian tumors led to increased resistance to paclitaxel or cisplatin (Solar et al., 2008), while targeting the EPOR resulted better shrinkage of the tumors (Yasuda et al., 2001b). However, the exact mechanism through which EPOR signaling mediates these effects remains unclear. Further studies in ovarian cancer cells could provide important insights to inform clinical practice.

There exist two alternate receptors for IL-4, one a heterodimer of IL-4Rα and the γc chain, and the other a heterodimer of IL-4Rα and IL-13Rα1 (LaPorte et al., 2008). These activate several signaling molecules, notably including JAK1 and JAK3, which in turn activate STAT6 (Ihle, 1995, Pernis et al., 1995). IL-4R signaling is known to modulate proliferation of B-cells (Nelms et al., 1999). Previous studies have shown IL-4R is expressed on solid tumors including lung and breast carcinoma (Obiri et al., 1994). Both in vitro and in vivo experiments on cancer cells have shown that IL-4 can function as an immune-modulating anti-tumor agent (Toi et al., 1992, Topp et al., 1995a, Topp et al., 1995b). However, IL-4 has also been shown to play a major role in tumor progression on human cancer cells expressing IL-4R (Li et al., 2009, Burt et al., 2012). Ovarian tumors have been shown to express
IL-4R, which switches on multiple signaling pathways upon ligand binding (Kioi et al., 2005). However, the role of IL-4R signaling in ovarian cancer is not well understood, and studies examining potential anti-tumor versus pro-tumor functions are worthwhile.

6.5.4 Role of IL-6R family in other cancers

A number of studies have described a role for altered signaling from various IL-6R family members as a contributing factor to a range of malignancies, including solid tumors. For example, LIF acts as a growth promoting factor in pancreatic carcinoma (Kamohara et al., 2007), both IL-6 and OSM are able to stimulate proliferation of prostate cancer cells (Godoy-Tundidor et al., 2005), while G-CSF has similar roles in leukemia (Katayama et al., 1998) and squamous cell carcinoma (Hirai et al., 2001). Autocrine activation appears to play a key role, as described for IL-6/IL-6R in a variety of tumors (Grivennikov and Karin, 2008), while high expression of Leptin is observed in prostate cancer patients (Stattin et al., 2001). However, there has been no systematic study and the roles of individual receptors in many of these cancers. Hence, exploring the role of IL-6R family members in these cases is warranted. Indeed the recent clinical development of JAK2 and STAT3 inhibitors (Lin et al., 2010b, So et al., 2013), enabling the targeting of multiple IL-6R family members in essentially any cancer setting, provides even more impetus for these studies.

6.6 Therapeutic implications

The results presented in this Thesis have clear implications for the clinic, especially with regard to the use of cytokines as a part of therapy, and the potential therapeutic targeting of cytokine receptor pathway components.
6.6.1 Cytokine therapy

The standard treatment for ovarian cancer patients remains maximal surgical cytoreduction followed by platinum and taxane based chemotherapeutic regimes with cytokines often used as supportive agents (Fanning et al., 2000). Of particular relevance is IL-6 which is used as part of photodynamic therapy (Wei et al., 2007), while G-CSF has been extensively employed to aid hematopoietic recovery following chemotherapy for a range of cancers, including those of an ovarian nature (Bohlius et al., 2003, Ray-Coquard et al., 2007). However, it is clear from previous studies that aberrant signaling from cytokine receptors, such as IL-6R (Matsuo et al., 2003, Rath et al., 2010, Wang et al., 2012) and G-CSFR (this study) can contribute to ovarian cancer etiology. Therefore, pre-screening patients for the expression of these receptors would be highly recommended prior to administration of the respective cytokine, potentially indicating alternative approaches for these patients.

6.6.2 Pathway specific inhibitors

Multiple molecular targets are currently being explored as alternative treatments, including growth factor receptors and their downstream signal transduction pathways, as well as cell cycle regulators (Wright et al., 2006, Posadas et al., 2007, Bhattacharya et al., 2008). Given that aberrant activation of IL-6R family members appears to contribute to ovarian cancer progression via the JAK1/2/STAT3 pathway (Catlett-Falcone et al., 1999, Grivennikov et al., 2009, Hodge et al., 2005, Hong et al., 2007), specific therapeutics targeting this pathway are also likely to be applicable to ovarian cancer.
6.6.1 Cytokine/receptor inhibitors

Antibodies have been developed that specifically target either IL-6 or IL-6R to block their activity. These therapies have been shown to inhibit tumor growth in patients either alone or in combination with cytotoxic chemotherapies in a range of carcinomas. For example, an IL-6 ligand-blocking antibody (CNTO-328) has been used in a number of phase I/II clinical trials, including myeloma, breast, prostate and ovarian cancer (Trikha et al., 2003, Voorhees et al., 2007, Garcia-Tunon et al., 2005, Voorhees et al., 2009, Tomillero and Moral, 2009, Wallner et al., 2006, Dorff et al., 2010, Puchalski et al., 2010). Similarly, an anti-IL-6 antibody (Siltuximab) was shown to decrease ovarian tumor proliferation in a xenotransplantation model (Coward et al., 2011). An IL-6R blocking antibody (tocilizumab), which binds to the ligand binding site of human IL-6Rα and so inhibits IL-6 binding, has recently been approved for Castelman's disease and rheumatoid arthritis (Nakashima et al., 2010, Garnero et al., 2010), providing an additional option in this regard. Given the results of this Thesis, the development of inhibitory anti-G-CSFR and anti-OBR would also provide an attractive option for ovarian cancer therapy.

6.6.2.2 JAK2/STAT3 Inhibitors

A number of JAK2 and STAT3 inhibitors have now been developed and used in clinical trials for various diseases, such as multiple lymphoma (Younes et al., 2012), prostate cancer (Shodeinde and Barton, 2012), renal cancer (Rossi et al., 2010) and psoriasis (Papp et al., 2012). Inhibition of this pathway has already shown promise in the context of ovarian cancer. For example, inhibition of JAK2/STAT3 with diindolylmethane induced apoptosis in ovarian tumors (Kandala and Srivastava, 2012). In addition, the JAK inhibitor AZD1480 was able to increase the chemosensitivity of ovarian cancer cells lines by inhibiting STAT3 signaling induced by
IL-6 and oncostatin M (Hedvat et al., 2009), while inhibition of STAT3 activation by siRNA led to reversal of paclitaxel resistance in ovarian cancer cell lines (Lee et al., 2011). The JAK-STAT pathway has also been implicated in ‘cancer stem-like cells’ (Kiger et al., 2001), making the use of such inhibitors even more enticing, as they may eliminate the cell population that facilitates the development of recurrent tumors. Therefore, inhibitors of the JAK2/STAT3 pathway holds considerable promise as a future therapeutic agents in ovarian cancer.
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Appendix A ~ Cisplatin resistance generates tumor cells demonstrating epithelial to mesenchymal transition in vivo
A.1 Introduction

EMT potentially plays a role in cisplatin-induced resistance in ovarian carcinomas (Wang et al., 2009). Cisplatin was able to enhance activation of ERK2 and induced EMT and CSC-like phenotypes in ovarian cancer cells \textit{in vitro}. Inhibition of ERK2 activation with a specific MEK inhibitor resulted in the partial suppression of EMT and CSC (Latifi et al., 2011). This Appendix describes studies that sought to confirm these results \textit{in vivo} using a zebrafish xenotransplantation model.

A.2 Results

A.2.1 Cisplatin enhanced the migratory potential of ovarian cancer cells \textit{in vivo}

To confirm the \textit{in vivo} relevance of cisplatin-induced EMT, an established zebrafish xenotransplantation model of metastatic behaviour was employed. Cells were fluorescently labelled prior to injection into the yolk sac of 2-day-old zebrafish embryos and visualized by confocal microscopy. Distinct fluorescent cells were observed from 12 hours post injection (hpi) predominantly in the tail region (Fig. A.1A, A.1B), in a pattern similar to that observed with pancreatic tumor cells (Marques et al., 2009). Cell migration into the tail was analyzed and quantified at both 24 and 48 hpi (Fig. A.1A). In three independent experiments, the average numbers of cisplatin-induced EMT transformed cells migrating towards the tail of zebrafish were significantly more than for the control untreated cells (Fig. A.1.A).

A.2.2 ERK inhibition blocks the effects of cisplatin treatment

Inhibition of ERK2 with the MEK inhibitor U0126 significantly reduced the migration of cisplatin-treated cells to below the basal levels (Fig. A.1.A).
Figure A.1: Xenotransplantation of ovarian cancer cells in zebrafish embryos

The migratory ability of control untreated and cisplatin-treated OVCA 433 cells was evaluated by xenotransplantation in zebrafish. Zebrafish embryos were injected with CM-Dil labeled OVCA433 cells that were untreated, treated with cisplatin or treated with cisplatin and the MEK inhibitor U0126, and the tail region imaged at 0 and 48 hpi, with manual quantification of fluorescent cells in the tail region of zebrafish performed at 24 and 48 hpi (A). Autofluorescence (yellow arrows) was observed in all embryos, primarily in the yolk, pigment cells, and the eye, with xenotransplanted cells evident in the tail region (blue arrows) and at the site of injection in the yolk sac (pink arrows) (B). Significant differences between cisplatin-treated cells compared to control untreated cells are indicated: *p < 0.05.
A.3 Discussion

Using a robust *in vivo* xenotransplantation model, these experiments demonstrated enhanced migratory ability of cisplatin-treated ovarian cancer cells. The enhanced migration of cisplatin-transformed cells in zebrafish likely reflects an increased metastatic ability of these cells. These results are consistent with other animal models where only a small subset (~2%) of metastatic cells has been shown to survive and colonize at distant sites (Weiss, 1990). Since pre-treatment with the MEK inhibitor U0126 was able to inhibit the migration of cisplatin-transformed cells, it appears that ERK plays a critical role in cisplatin-induced EMT *in vivo*, supporting the *in vitro* studies. Moreover, the assessment of the migratory and invasive abilities of tumor cells in this zebrafish model provides a complementary approach to long-term and more expensive mouse models, and is likely to be valuable in evaluating the therapeutic efficacy of novel agents generally.