The Role of STAT-3 in Androgen-Dependent Prostate Cancer

by

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B.Sc., McGill University, 1999

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
Department of Medicine, Experimental Medicine Program

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

October 2003

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Title of Thesis: The Role of STAT-3 in Androgen-Dependent Prostate Cancer

Degree: M.Sc. Year: 2003
Abstract

Prostate cancer is the third-leading cause of male cancer-related deaths and the prostate gland is the leading site of new male cancer cases in Canada. Prostate tumour growth and survival is initially androgen-dependent and primary treatments include radiation and surgery. Androgen withdrawal therapy is used as a secondary treatment, to decrease the circulating supply of androgens in the body. While primary and secondary treatment can reduce tumour growth, in many cases the epithelial cells can begin to proliferate again and the tumour will acquire an androgen-independent phenotype.

The objective of this thesis is to clarify the biochemical interactions that occur in IL-6 and AR pathway cross-talk. Since EGF also activates STAT-3, we included analysis of EGF cross-talk with the IL-6 and AR pathways. We investigated the potential for pathway interaction in LNCaP cells by using AR and STAT luciferase reporter constructs in the presence of IL-6, R-1881 and EGF stimulation. To verify the necessity of STAT-3 to the observed pathway interactions, we used shRNA to downregulate STAT-3 expression. In addition, we studied the role of STAT-3 in LNCaP xenograft tumour growth and serum PSA production in male nude mice.

Our findings confirm that androgen can augment STAT-mediated gene transcription and growth factors such as IL-6 and EGF can augment AR-mediated gene transcription. Based on the ability of STAT-3 antisense ODN treatment to suppress serum PSA levels and tumour growth in vivo, and our in vitro findings that indicate an inability of STAT-3
shRNA to suppress EGF and IL-6-mediated augmentation of androgen-stimulated AR-mediated gene transcription, we propose that STAT-3 activation may not be directly involved in AR transactivation. Other growth factor pathways that occur in parallel to STAT-3 activation are likely responsible for AR transactivation via AR co-regulators. We conclude that STAT-3 has a role in regulating prostate cancer growth and survival, under androgen-deprived conditions, that indirectly affects AR activity.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>ARE</td>
<td>Androgen response element</td>
</tr>
<tr>
<td>ARR</td>
<td>Androgen response region</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign prostatic hyperplasia</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
</tr>
<tr>
<td>CSS</td>
<td>Charcoal-stripped fetal bovine serum</td>
</tr>
<tr>
<td>CT-1</td>
<td>Cardiotrophin-1</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double-stranded RNA</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>Gp130</td>
<td>Glycoprotein 130</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse-raddish peroxidase</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-6R</td>
<td>Interleukin-6 receptor</td>
</tr>
<tr>
<td>IL-6Ra</td>
<td>Interleukin-6 receptor, 80 kDa transmembrane subunit</td>
</tr>
<tr>
<td>sIL-6Ra</td>
<td>Soluble interleukin-6 receptor, 80 kDa transmembrane subunit</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JH</td>
<td>JAK homology</td>
</tr>
<tr>
<td>kb</td>
<td>kilo base pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukaemia inhibitory factor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MOE</td>
<td>2’-O-(2-methoxy)ethyl</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NE</td>
<td>Neuroendocrine</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxynucleotide</td>
</tr>
<tr>
<td>OSM</td>
<td>Oncostatin M</td>
</tr>
<tr>
<td>P-</td>
<td>Phosphorylated</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PIN</td>
<td>Prostatic intra-epithelial neoplasia</td>
</tr>
<tr>
<td>PLB</td>
<td>Passive lysis buffer</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute media</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SIE</td>
<td>STAT-inducible element</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour Necrosis Factor-α</td>
</tr>
<tr>
<td>TRPM-2</td>
<td>Testosterone-repressed prostate message-2</td>
</tr>
<tr>
<td>TYK</td>
<td>Tyrosine kinase</td>
</tr>
</tbody>
</table>
Acknowledgements

I would like to thank my supervisor, Dr. Michael E. Cox, for his guidance and financial support throughout my Masters degree. Mike, your enthusiasm and energy is inspiring. Thank you for taking me into your lab, for always having an open door and for teaching me so much about all aspects of research.

I cannot begin to express my gratitude for the unconditional support my family has given me throughout my education. To my parents, Jane and Peter Gardiner, thank you for encouraging me to learn, whether in school or in the “real world”. You have always supported my goals and aspirations as well as my adventures. To my sister Rachel, thank you for always listening and always caring.

Thank you to the staff at the Prostate Centre for all their expertise advice. A special thank you to all the members of the Cox Lab: Gina Rossi, Sandra Kruekl, Kevin Figueiredo, Jodie Palmer, Zhihong Wen and Nikita Ivanov. You are a fantastic team to work with and your help has been invaluable.

Finally, thank you to Tim McPhee. You have offered me so much support and encouragement and I can’t thank you enough.
Chapter 1. Introduction

1.1 Introduction

The prostate gland is the leading site of new male cancer cases and is the third-leading cause of male cancer-related death in Canada (Canadian Cancer Society, 2003). The Canadian Cancer Society estimates that 18,800 men in Canada will be diagnosed with prostate cancer in 2003 and there will be 4,200 deaths from the disease. Incidence rates have increased by 30% since 1998 while deaths have decreased by 10%. Current statistics predict that 1 in 8 Canadian men will develop prostate cancer in his lifetime and for 1 in 28 men, it will be the cause of death (Canadian Cancer Society, 2003).

Most prostate cancer cases occur in men over the age of 50 years and the majority of those cases take place in men over 70 years of age. While early symptoms can include difficulty with urination or pain in the back, hips or groin, prostate cancer is slow to develop and may be asymptomatic for years. Pathologies of the prostate gland manifest with various degrees of severity. Symptoms of benign prostatic hyperplasia (BPH) are often similar to those of prostate cancer, however, this condition does not progress to prostate cancer and symptoms can be relieved with treatment or surgery. Prostatic intra-epithelial neoplasia (PIN) may be a precursor to prostate adenocarcinoma, a potentially metastatic form of prostate cancer.
Prostate cancer first develops as an androgen-dependant tumour and, primary treatments include “watchful waiting”, surgery and radiation. Hormone ablation therapy is a secondary treatment, after all forms of primary treatment have failed. Pre-treatment of tumours with androgen ablation therapy prior to surgery, also known as neo-adjuvant hormone therapy, is a new therapeutic strategy that is in clinical trails at present (Hurtado-Coll et al., 2002). Intermittent androgen suppression is a new treatment strategy for administering anti-androgen therapy in order to reduce the side effects of long-term androgen suppression. Patients receive anti-androgens in an on-therapy, off-therapy cycle and reports from the Phase II clinical trials appear promising (Pether et al., 2003).

Initially, growth and survival of the tumour cells is dependant on the presence of androgen, which causes tumour growth by stimulating cell proliferation, while concurrently preventing apoptosis (Feldman et al., 2001). In most cases the tumour returns after a latent period, exhibiting an androgen-independent phenotype that can metastasize and is typically lethal (Feldman et al., 2001; Smith et al., 2001).

There is building evidence to suggest that interleukin-6 (IL-6) is a candidate marker of prostate cancer morbidity. Cases in which serum levels of IL-6 increase with disease progression are well correlated with tumour burden (Hoosein et al., 1995; Smith et al., 2001; Twillie et al., 1995). As increased levels of IL-6 have been associated with metastatic prostate cancer, IL-6 could be considered as a surrogate marker for androgen-independent prostate cancer and the extent of bone metastasis (Adler et al., 1999; Smith et al., 2001; Wise et al., 2000).
IL-6 signals via the IL-6 receptor (IL-6R) and the gp130 receptor, recruiting JAK1, JAK2 and TYK2 to phosphorylate signal transducer and activator of transcription-3 (STAT-3), a nuclear transcription factor (Heinrich et al., 1998). It has been shown that STAT-3 can associate with the androgen receptor (AR) and that cross-talk between the IL-6 pathway and the androgen pathway occurs. IL-6 can augment androgen-stimulated AR transcriptional activity and that androgen can augment IL-6-stimulated STAT-3 transcriptional activity (Chen et al., 2000; Hobisch et al., 1998; Matsuda et al., 2001). Recent investigations have explored a co-operative role for STAT-3 and the AR in development of androgen-independent prostate cancer (Ueda et al., 2002).

The biochemical pathways that influence acquisition of androgen independence have yet to be well described. This thesis aims to define the role of STAT-3 in an androgen-dependant prostate cancer cell line, LNCaP, and its interactions with several key signalling pathways including the AR pathway. The ability of IL-6 to activate androgen-regulated gene transcription in the absence of androgen, or in the presence of very low quantities of androgen, may be an essential step in developing androgen-independent tumour growth. In vivo inhibition of STAT-3 expression in established LNCaP xenograft tumours further explore the effects of STAT-3 interaction with the androgen pathway. Clarification of the role of STAT-3 in principal signal transduction pathways, within the LNCaP cell line and in xenograft tumours, will be important for developing new therapeutic strategies to prevent and treat androgen-independent prostate cancer.
1.2 The Prostate

The prostate is a small, walnut-sized gland that surrounds the urethra and is located below the bladder in the male pelvis. The location of the prostate is illustrated in figure 1.1.

![Male Genitourinary System diagram](http://www.cancer.ca/ccs/internet/standard/0,2939,3172,10175,87671_langId-n,00.html)

**Figure 1.1 Male Genitourinary System**
Figure was reproduced with permission from the Canadian Cancer Society website: [http://www.cancer.ca/ccs/internet/standard/0,2939,3172,10175,87671_langId-n,00.html](http://www.cancer.ca/ccs/internet/standard/0,2939,3172,10175,87671_langId-n,00.html)

The main role of the prostate is to produce fluid for semen. The prostate is composed of both muscular and glandular tissue and, at the beginning of ejaculation, fluid is squeezed from the prostate into the urethra by muscular contractions. The sperm, produced in the testes, also enter the urethra and are propelled from the penis during ejaculation.

1.2.1 Prostate Specific Antigen

Prostate-specific antigen (PSA) is a serine protease produced by prostate ductal and acinar epithelium. It is a significant protein component of the semen with a concentration
of 0.5 to 2.0 mg/ml (Balk et al., 2003). PSA is a member of the tissue kallikrein family and its primary role is in the lumen of the prostate to cleave semenogelin I and II in the seminal coagulum (Balk et al., 2003). PSA can also be found in blood serum and is used as a diagnostic marker of prostate disease.

The AR positively regulates PSA gene transcription by binding to a DNA consensus sequence, GG(A/T)ACA nnn TGTTCT, called an androgen response element (ARE). Strong ARE consensus sequences can be found at -156 to -170 from the PSA gene transcriptional start site while a weaker consensus sequence, called the androgen responsive region (ARR) exists at -365 to -400 with an additional PSA distal enhancer 4.2 kb upstream of the PSA gene (Balk et al., 2003). The structure of the PSA proximal promoter is illustrated in figure 1.2.

![Figure 1.2 PSA promoter](image)

**Figure 1.2 PSA promoter**
The proximal PSA promoter region consists of an ARE and an ARR as well as a distal enhancer which contains an ARE itself. AR binds to the AREs as a homodimer and simultaneous AR binding to multiple AREs in the promoter region tightly regulates transcriptional activity of the PSA gene (Balk et al., 2003).

PSA is initially secreted as prepro-PSA from the basal epithelium of the prostate, with a 17 amino acid leader sequence that is cleaved to an inactive pro-PSA protein, 244 amino acids in length (Balk et al., 2003). Pro-PSA is cleaved after the N-terminal 7 amino acids
to produce the active enzyme, PSA, a 33 kDa protein that is 237 amino acids long (Balk et al., 2003). PSA that enters the blood plasma is immediately inactivated by the protease inhibitor alpha-1-antichemotrypsin (Balk et al., 2003), however antibodies used in prostate disease screens do not detect the PSA-protease inhibitor complex. A small percentage of circulating PSA is called free PSA because it was previously inactivated in the lumen of the prostate, by cleavage, and therefore does not complex with protease inhibitors. An increase in AR activation during prostate cancer can increase PSA production. In addition, proteolytic inactivation of PSA as well as cleavage of pro-PSA to PSA becomes slower with the onset of prostate cancer and results in a higher proportion of active PSA and therefore less free PSA entering the blood stream (Balk et al., 2003). The fraction of free PSA relative to total PSA in the blood serum is the calculation used to diagnose prostate cancer. A lower proportion of free PSA relative to total PSA is indicative of prostate disease. A higher PSA value is used as a marker of disease progression.

1.2.2 Prostate Cancer: Acquisition of Androgen Independence

Initially, prostatic tumour growth and survival is dependent on androgens. Cell proliferation and inhibition of apoptosis are mediated by androgen stimulation and, together, contribute to tumour growth (Feldman et al., 2001). Thus early treatment of prostatic tumours involves decreasing tumour proliferation by radiation or decreasing circulating androgen in the body by surgery. Androgen-withdrawal therapy is used following primary treatment and anti-androgens in the United States and Canada include
flutamide, bicalutamide and nilutamide. While androgen withdrawal therapy can reduce tumour growth, in many cases the epithelial cells will eventually begin to proliferate again, in an androgen-independent manner, due to cancer cell mutations. Androgen-independent tumours tend to metastasize and are typically lethal (Feldman et al., 2001; Smith et al., 2001).

Testosterone is the principal androgen in the circulation, the primary source being the testes, and a secondary, smaller source of circulatory androgen is produced from the conversion of adrenal steroids (Feldman et al., 2001). The enzyme 5α-reductase acts in prostatic epithelial cells to convert testosterone to dihydrotestosterone (DHT), a more active form of the androgen. Ninety percent of testosterone is converted to DHT and its affinity for the androgen receptor is five-fold greater than that of testosterone.

Tumour regression, as a result of androgen withdrawal therapy, is thought be associated with cell cycle arrest rather than apoptosis. Mouse models indicate that an androgen-independent phenotype may be a result of release from cell cycle arrest (Agus et al., 1999; Knudsen et al., 1998). Early events exhibited during androgen withdrawal therapy include decreased androgen receptor expression, increased p53 to initiate a cellular stress response, increased p21 expression to initiate a G1/G0-phase growth arrest, a decrease in the Ki67 proliferative index, with a low rate of apoptosis. Mid- to late events included decreased Rb and cyclin D1 expression and increased p27 and p16 expression, to maintain growth arrest and a low rate of apoptosis. After 80-100 days, serum PSA increased and androgen-independent tumour growth began, characterized by an increase
in cyclin D1 and mdm2 expression, as would be expected with a return to the cell cycle (Agus et al., 1999).

From the evidence presented, there are several possible mechanisms for acquisition of an androgen-independent prostate cancer phenotype:

(1) The hypersensitivity pathway: an increased sensitivity to the lower circulating levels of androgen during androgen ablation therapy would allow an androgen-independent tumour to develop. Amplification of the AR gene leads to an increased expression of the AR and an improved opportunity for DHT binding. In animal models of androgen-independent tumours, 30% have increased AR expression and increased sensitivity of the AR itself (Feldman et al., 2001). Much less DHT was required to stimulate growth in the hypersensitive AR-containing cells. On the other hand, an apparent hypersensitivity of the AR could be the result of increased expression of AR-co-activators, which would increase transactivation of the AR in the presence of low levels of androgen (Feldman et al., 2001). While androgen ablation therapy decreases the total amount of circulating androgen, a compensatory local increase in androgen production at the tumour site could develop and cause an androgen-independent phenotype. Any of the above hypersensitivity mechanisms could also be influenced by an increase in the conversion of adrenal steroids to circulating testosterone (Feldman et al., 2001).

(2) Acquisition of a promiscuous receptor through AR gene mutations would permit receptor activation in the absence of circulating androgen. Non-androgen steroids could
cause deviant receptor activation because of decreased ligand specificity (Feldman et al., 2001).

(3) Unregulated receptor activity could result from receptor activation through a ligand-independent mechanism. Growth factors may have the potential to activate the androgen receptor in the absence of androgens. Over-expression of receptor tyrosine kinases, such as HER2/Neu, can activate transcription of AR-controlled genes in the absence of androgen, although the androgen receptor must be present (Craft et al., 1999; Feldman et al., 2001).

(4) An alternative pathway could maintain tumour growth and inhibition of apoptosis in the absence of androgens, effectively producing the same outcome as the AR pathway but bypassing the AR and the requirement for androgen (Feldman et al., 2001). The alternative pathway could be upregulated to compensate for the downregulation of the AR pathway that would usually inhibit apoptosis and promote cell survival.

(5) Finally, it is possible that a sub-population of androgen-independent tumour cells may exist before androgen ablation therapy begins and that sub-population is selected for survival as circulatory androgen levels decrease (Feldman et al., 2001).

The molecular components that cause the transition of prostate cancer from an androgen-dependant phenotype to androgen-independent phenotype is poorly understood and the mechanisms involved in the conversion are currently under investigation.
1.2.3 LNCaP Prostate Cancer Model

The LNCaP cell line is a model for androgen-responsive prostate cancer. The cell line was established from material withdrawn in a needle aspiration biopsy of a supraclavicular lymph node (Horoszewicz et al., 1980; Horoszewicz et al., 1983). The patient was a 50-year-old Caucasian male, diagnosed with metastatic carcinoma, who survived 18 months from the date of initial diagnosis.

LNCaP cells maintain malignancy, hormone sensitivity and, at low passage numbers (below passage 80) are an excellent *in vitro* model for early-stage prostate cancer (Igawa et al., 2002). After passage 81, LNCaP cells begin to lose androgen dependence although their genetic markers and AR protein expression remain constant. The major change seen in cultured LNCaP cells at higher passage numbers is an increase in PSA secretion, similar to the increase exhibited by hormone-independent prostatic tumours. The close imitation of androgen-dependent tumour characteristics by the LNCaP cell line makes it a practical and useful *in vitro* model for studying early prostatic tumour progression.

LNCaP cells can be injected into mice to create an androgen-dependent, xenograft tumour. PSA secretion is initially androgen-dependent and directly proportional to tumour volume (Miyake et al., 2001). Following castration, tumour growth decreases for a short time and, at the same time, serum PSA decreases by 80% (Miyake et al., 2001). After several weeks, tumour growth rate and PSA levels increase once more - characteristic of hormone-independent prostate cancer. As an *in vivo* model, LNCaP
tumours reflect clinical characteristics seen during the transition from androgen-dependent to androgen-independent tumour progression.

1.3 Interleukin-6

Interleukin-6 (IL-6) is a 21–28 kDa protein consisting of a 184 amino acid glycoprotein and a 28 amino acid cleaved signal peptide (Smith et al., 2001). Homeostatic levels of IL-6 are very low and increased levels can have diverse effects throughout the body. IL-6 is a pleiotrophic cytokine that regulates gene expression in organs and promotes osteoclast activity. IL-6 also functions as a bone marrow megakaryopoetic factor and a colony stimulating factor, and induces growth and differentiation of T-cells and antibody production in B-cells. Further, IL-6 regulates synthesis of liver acute phase reactants and induces thrombopoiesis (Chung et al., 1999; Hirano, 1998; Smith et al., 2001). IL-6 belongs to the IL-6-type cytokine sub-family, which includes LIF, CNTF, OSM, IL-11 and CT-1. All of the IL-6-type cytokines share the receptor subunit gp130 (Hirano, 1998) along with their cognate receptors.

1.3.1 IL-6 Receptor Signalling

IL-6 interacts with the IL-6 receptor (IL-6R) complex which consists of two elements: an 80 kDa transmembrane receptor (IL-6Ra) responsible for cytokine binding and an
associated 130 kDa membrane glycoprotein (gp130) responsible for signal transduction (Chung et al., 1999; Smith et al., 2001).

The IL-6Ra is a member of the type I cytokine receptor superfamily (Heinrich et al., 1998) characterized by a conserved domain of four cysteine residues and a tryptophan-serine-X-tryptophan-serine (WSXWS) motif, also called the WS motif, that is located in the extracellular portion of the receptor adjacent to the transmembrane domain (Hirano, 1998). A soluble form of the IL-6Ra (sIL-6Ra) exists at a concentration of 30-70 ng/ml in human plasma (Heinrich et al., 1998). sIL-6Ra binds to IL-6 with a similar affinity to that of the membrane-bound receptor and can sensitize cells to IL-6, regardless of their IL-6R expression, by binding the ubiquitously expressed gp130. The ability of sIL-6Ra to sensitize cells to IL-6 broadens the biological effects of the cytokine by increasing the number of possible target cells (Shariat et al., 2001).

The shared gp130 receptor is also a type I cytokine receptor and is responsible for signal transduction from the IL-6R complex. The structure of IL-6Ra and gp130 are illustrated in figure 1.3. When IL-6 binds the IL-6R, a hexameric complex is formed consisting of two molecules each of IL-6, IL-6Ra and gp130 (Hirano, 1998; Ward et al., 1994). Initially, IL-6 binds to the IL-6R with low affinity to form the IL-6-IL-6R complex and then induces homodimerization of gp130 molecules. The disulfide-linked homodimerization of gp130 increases the IL-6R binding affinity for IL-6. Dimerization of the gp130 molecules and high affinity binding of IL-6, results in signal transduction through the gp130 molecules and downstream elements in the cell (Smith et al., 2001).
The gp130 molecules lacks inherent tyrosine kinase activity and, following dimerization, recruit cytoplasmic tyrosine kinases JAK1/2 and TYK2 of the Janus kinase family (Heinrich et al., 1998; Imada et al., 2000; Schindler, 1999). JAKs have seven JAK homology (JH) domains. JH-1, at the C-terminal of the JAK, is the tyrosine kinase domain, JH-2 is a kinase-like domain whose function is unclear and JH-3, -4, -5, -6 and -7 in the N-terminal are involved in receptor association (Heinrich et al., 1998).

IL-6R dimerization brings the associated JAK residues into close proximity. Trans-phosphorylation and activation of the JAK molecules results in reciprocal tyrosine phosphorylation on the cytoplasmic portion of the gp130 receptor subunits, mediated by the association of JAKs. The region of gp130 that contains box 1 and box 2 is sufficient for recruitment and activation of JAKs (Hirano, 1998). JAK1/2 and TYK2 phosphorylate downstream proteins. The JAKs phosphorylate a specific binding site for src-homology 2 domains (SH2) at the base of the receptor cytoplasmic domain Y5 or Y6 (Darnell, 1997; Hirano, 1998). These two tyrosine residues bear the motif tyrosine-X-X-glutamine (YXXQ) and are critical for recruitment of the SH2 domain on signal transducer and activator of transcription 3 (STAT-3) (Smith et al., 2001). Y2 phosphorylation is involved in tyrosine phosphorylation of SHP-2, an intra-cellular tyrosine phosphatase.
Figure 1.3 Structure of the Human IL-6 Receptor: IL-6Ra and gp130
Both IL-6Ra and gp130 are members of the type I cytokine receptor superfamily, characterized by three components: an Ig-like domain, a four-cysteine repeat and a WSXWS domain. While the cytoplasmic domain of IL-6Ra is not required for signal transduction, the cytoplasmic domain of gp130 contains several important features for signalling: box 1 (an eight amino acid proline-rich motif), box 2 (a stretch of hydrophobic amino acids followed by positively charged amino acids) and six tyrosine (Y) residues the role of which will be explained below.

1.3.2 The Role of STAT-3 in IL-6 Signalling

Tyrosine phosphorylation of JAKs generates docking sites for STAT-3 molecules (Imada et al., 2000). STAT-3 binds to phosphorylated YXXQ motifs on JAK via a Src homology 2 (SH2) domain (Hirano, 1998; Imada et al., 2000). STAT-3 is an 86 kDa protein that is 770 amino acids in length (Akira, 1999; Zhang et al., 1995). The N-terminal portion of STAT-3 contains a tetramerization domain and a leucine-zipper-like domain, known as a coiled-coil domain. There is a central DNA-binding domain and at the C-terminal there is an SH3-like domain (Linker domain), an SH2 domain and a transactivation domain (TAD) (Heinrich et al., 1998). The structure of STAT-3 is illustrated in figure 1.4.

STAT-3 is phosphorylated by JAK1/2 and TYK2 at tyrosine residue 705 (Y705), which induces homo- and heterodimerization of STAT-3 with other members of the STAT family: STAT-1, STAT-3 and STAT-5 (Darnell, 1997). Un-phosphorylated STAT-3 is primarily drawn to tyrosine residues in gp130. STAT-3 has a higher affinity for other STAT-3 molecules when phosphorylated on Y705 (P-STAT-3 Y705). Dimerization occurs between the SH2 domain on one STAT-3 molecule and the phospho-Y705 on the second STAT-3 molecule (Darnell, 1997). Following dimerization, STAT-3 translocates to the nucleus of the cell. A second STAT-3 phosphorylation takes place on serine
Figure 1.4 Structure of STAT-3
The N-terminal region of STAT-3 contains a coiled coil domain and a DNA binding domain. The C-terminal region of STAT-3 contains a linker sequence, an SH2 domain and a transactivation domain.

Residue 727 (P-STAT-3 S727) before or during nuclear translocation (Decker et al., 2000). While phosphorylation on Y705 is obligatory for STAT-3 activation and DNA binding, phosphorylation on S727 is required for maximal activation of STAT-3 and enhances transcriptional activity (Wen et al., 1997; Wen et al., 1995).

There are currently two proposed mechanisms for serine phosphorylation of STAT-3 at the S727 residue. Several studies have demonstrated that mitogen activated protein kinase (MAPK) phosphorylates the S727 residue of STAT-3 (Decker et al., 2000; Lim et al., 2001; Turkson et al., 2000). The MAPK target phosphorylation sequence is the proline-methionine-serine-proline motif (P(M)SP) (Decker et al., 2000). Recent evidence indicates, however, that in LNCaP cells while MAPK may play a role in regulation of STAT-3 serine phosphorylation at a basal level, it may not function in IL-6-induced phosphorylation (Deeble et al., 2001). When cells were treated with PD098059, a compound that inhibits all MAPK activity, a degree of serine phosphorylation was still detected (Deeble et al., 2001). The level of serine phosphorylation was lower in the PD098059-treated cells than in control cells treated with vehicle control, however, in response to IL-6 stimulation, the degree of phosphorylation increased to the same extent.
in both cases. These results indicate that another mechanism for S727 phosphorylation may exist in prostate cancer cells.

Mammalian target of rapamycin (mTOR) and p70 S6 kinase have recently been proposed as alternate mechanisms for S727 phosphorylation of STAT-3 (Yokogami et al., 2000). In ciliary neurotrophic factor (CNTF)-activated neuroblastoma cells, S727 phosphorylation was not inhibited by PD098059 or by H7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine), a protein kinase C inhibitor (Hidaka et al., 1984; Yokogami et al., 2000). Rapamycin, an mTOR inhibitor, did produce a significant decrease in S727 phosphorylation. This result indicates that either mTOR or a downstream-activated kinase of mTOR, such as p70 S6 kinase, may be directly responsible for IL-6-mediated S727 phosphorylation of STAT-3 in LNCaP cells. This hypothesis is tested in Section 6.2 of Chapter 6.

Studies by Abe et al. have further differentiated between MAPK and mTOR S727 phosphorylation of STAT-3 (Abe et al., 2001). The YXXQ motif of gp130 not only recruits STAT-3 to the receptor for Y705 phosphorylation, but also activates an H7-sensitive pathway that phosphorylates STAT-3 at S727 in the presence of low concentrations of IL-6. The kinase involved in the H7-sensitive pathway is unknown but functions independently of MAPK and requires the presence of a site between amino acids 533 and 711 on STAT-3. JNK, p38, ERK and PKCγ are not involved in the H7-sensitive pathway. Addition of rapamycin did not affect the ability of the H7-sensitive pathway to phosphorylate STAT-3. The tyrosine-serine-threonine-valine (YSTV) motif
on gp130 mediates the MAPK pathway for phosphorylation of STAT-3 at S727. These findings suggest that the H7-sensitive pathway is physiologically more important than the MAPK pathway for phosphorylation of STAT-3 at S727, however, the serine-threonine kinase responsible for the H7-sensitive pathway is still unknown (Abe et al., 2001). The IL-6 pathway and STAT-3 signalling are illustrated in figure 1.5.

S727 phosphorylation may contribute to an improved DNA-binding ability by STAT-3, but more likely S727 phosphorylation enhances transcriptional activity of STAT-3 (Decker et al., 2000; Zhang et al., 1995). STAT-3 controls transcription of several genes including gp130, c-Fos, Jun B and interferon regulatory factor-1 (IRF-1) (Heinrich et al., 1998).

1.3.3 IL-6 Is a Candidate Mediator of Prostate Cancer Morbidity

A candidate marker of morbidity must reflect the hypothetical physiological conditions. The marker must be detectable in the systemic circulation of patients with advanced disease. It must be a secretory gene product of normal and cancerous prostate epithelium. Finally, the marker must have a measurable biological effect on the patient that reflects one of the characteristic signs of morbidity from advanced prostate cancer (Twillie et al., 1995).
Figure 1.5 The IL-6 Signalling Pathway
IL-6 binds to the IL-6Ra, which causes dimerization of the gp130 receptors. A hexameric complex is formed consisting of two molecules each of IL-6, IL-6Ra and gp130 (Ward et al., 1994). Dimerization of gp130 induces cross-phosphorylation of tyrosine residues on the receptors. JAK, a cytoplasmic tyrosine kinase, is recruited to the box 1 and box 2 regions on gp130. JAK is phosphorylated and activated and, in turn, phosphorylates tyrosines on gp130. STAT-3 is recruited to the SH2-binding motif on gp130 and is phosphorylated by JAK at Y705, which induces STAT-3 dimerization. MAPK and/or an H7-sensitive kinase then phosphorylate STAT-3 at S727, which may improve DNA binding ability. The STAT-3 dimer with phosphorylations at Y705 and S727 then enters the nucleus where it acts as a transcription factor and induces gene expression.

Investigation of IL-6 as a candidate mediator of morbidity in prostate cancer progression was logical for several reasons. First, IL-6 has been previously associated with poor prognosis in ovarian cancer, lymphoma, melanoma and renal cell carcinoma (Smith et al., 2001) (Siegall et al., 1990). Secondly, IL-6 production is stimulated by tumour necrosis
factor-α (TNFα), which is not an exocrine product of prostate cancer cells, but is a mediator of cachexia. Thirdly, IL-6 has been associated with wasting syndromes in other diseases (Twillie et al., 1995). It is important to note that IL-6 levels and PSA levels are unrelated in prostate cancer patients. Although both markers show a trend of increasing levels as disease progresses, they are likely influenced by different pathways (Hoosein et al., 1995; Twillie et al., 1995).

Disease progression and tumour burden are correlated with serum IL-6 expression (Giri et al., 2001; Hoosein et al., 1995; Smith et al., 2001; Twillie et al., 1995). IL-6 may be a marker of prostate cancer morbidity, androgen-independent cancer and bone metastasis (Adler et al., 1999; Smith et al., 2001; Wise et al., 2000). A study of cytokine variations in prostate cancer by Wise et al. indicated that in hormone responsive prostate cancer, a stable level of PSA (0.41 ± 0.57 ng/ml) was seen accompanied by a normal level of pro-inflammatory (Th1) cytokines (IL-1, IL-2, interferon-gamma, TNFα and anti-inflammatory (Th2) cytokines (IL-4, IL-6, IL-10) (Wise et al., 2000). Hormone refractory cancer, on the other hand, was characterized by an increase in PSA levels (20.65 ± 40.3 ng/ml) and an increase in anti-inflammatory cytokines while pro-inflammatory cytokines remained at a normal level. Whether increased anti-inflammatory cytokines contribute to prostate cancer progression, or reflect an inability of the immune system to respond to advancing disease, is yet to be determined (Wise et al., 2000).

Under normal conditions, IL-6 levels increase in response to acute stress such as bacterial sepsis or surgery. Chronic exposure to systemic IL-6 causes cachexia, a wasting
syndrome of accelerated fat loss due to hypocholesterolemia. The correlation between chronic IL-6 exposure and induction of cachexia also exists in the absence of prostate cancer (Twillie et al., 1995). Characteristic signs of morbidity associated with prostate cancer include cachexia, anaemia, anorexia, hypoalbuminemia, edema, anergy, diffuse bone pain and elevated acute phase proteins (Smith et al., 2001; Twillie et al., 1995).

Continuous circulatory ejaculate poisoning may cause or hasten death in prostate cancer patients (Twillie et al., 1995). This hypothesis, originally considered by Twillie et al., suggests that there is an increasing presence of ejaculate proteins, for example PSA, in the circulatory system during hormone-refractory prostate cancer and that although the proteins serve a normal role in the genitourinary system, they are pathologic in the circulation.

A study of IL-6 and IL-6R localization in benign, pre-malignant and malignant prostate samples, by Hobisch et al., revealed that IL-6 expression in epithelial and stromal cells increases as prostate cancer progresses (Hobisch et al., 2000). This pattern of IL-6 and IL-6R expression suggests that IL-6 autocrine and paracrine loops may be present in prostatic tumours.

1.3.4 The Role of STAT-3 in Growth and Differentiation of Prostate Cancer Cells

STAT-3 is essential for normal, early-embryonic development. Deletion of the STAT-3 SH2 domain and Y705 residue prevent activation of the STAT-3 protein. Homozygous
mutation of the \textit{STAT-3} gene is embryonic lethal and gastrulation does not occur (Darnell, 1997; Takeda et al., 1997). The role of STAT-3 in adult tissues was assessed \textit{in vivo} using the Cre-loxP recombination system surrounding the Y705 site (Akira, 1999). STAT-3 was found to play a critical role in cell survival and inhibition of apoptosis.

There has been much controversy over whether IL-6 and STAT-3 promote growth or differentiation in LNCaP cells. A conclusive role for IL-6 in prostate cell growth and differentiation has yet to be determined. Dhir et al. demonstrated that STAT-3 activity is significantly higher in cancerous prostatic tissue and the surrounding normal tissue than in prostatic tissue from patients without cancer (Dhir et al., 2002).

Experiments by Chung et al. reflect a role for the IL-6 signalling pathway in cell survival involving inhibition of apoptosis (Chung et al., 2000). \textit{In vitro}, IL-6 initially acts as a paracrine growth inhibitor in androgen-dependent cells but becomes an autocrine growth stimulator after the cells take on an androgen-independent phenotype (Chung et al., 1999). Many of the proliferation assays performed by Chung et al. were performed in cells that artificially over-express IL-6 and, therefore, may not reflect physiological conditions, as the cells under study did not express endogenous levels of IL-6.

Lou et al. also report that IL-6 stimulates prostate cancer cell growth \textit{in vitro}. In LNCaP cells ectopically expressing IL-6, activation of STAT-3 by IL-6 was confirmed and it was concluded that IL-6 acts as an autocrine growth factor (Lou et al., 2000). This is further supported by Ni et al., who found that ectopic expression of a dominant-negative STAT-3
suppressed growth in vitro and tumourigenicity in vivo (Ni et al., 2000). Blocking STAT-3 signalling results in the inhibition of cell growth and initiation of apoptosis (Turkson et al., 2000) and points to a role for STAT-3 as a therapeutic target.

The issue concerning the models put forward by Chung et al. and Lou et al. is that when ectopically overexpressing IL-6 in cells and then selecting for clones that express IL-6, the system must, by definition, produce cells that grow in the presence of high levels of IL-6. Nevertheless, potential mechanisms for IL-6 promoted cell growth have been put forward. Steiner et al. demonstrated that tumour growth is caused by chronic exposure to IL-6 and that uncontrolled growth was caused by preventing Rb-mediated growth control and activating the MAPK signalling (Steiner et al., 2003). Liu et al. have also attributed IL-6 stimulation to prostatic tumour growth. Prostatic intraepithelial neoplasia and prostate cancer cause increased Cyclooxygenase (COX)-2 expression, which in turn causes increased release of prostaglandin E2 (PGE2). Increased PGE2 secretion causes increased IL-6 secretion which, in turn, contributes to tumour growth (Liu et al., 2002).

In contrast, reports from Spiotto et al. indicate that STAT-3 mediates both growth inhibition and neuroendocrine (NE) differentiation in LNCaP cells (Spiotto et al., 2000a, 2000b). Deeble et al. suggest that IL-6 has a growth inhibitory effect on LNCaP cells and promotes NE differentiation (Deeble et al., 2001). Growth of neurite-like processes, rounding of the cell body, inhibition of mitotic activity, expression of NE markers such as neuron-specific enolase, chromogranin A and parathyroid hormone, are all characteristics of neuroendocrine differentiation (Deeble et al., 2001; Mori et al., 1999;
Wu et al., 1996). Elevation of NE differentiation was found to correlate with increased IL-6 expression in metastatic prostate cancer (Hoosein et al., 1995).

While the work presented in this thesis does not specifically address the role of IL-6 and STAT-3 alone in growth and differentiation, our in vivo experiments, described in Chapter 4, indicate that STAT-3 indirectly mediates LNCaP xenograft tumour growth. Results from experiments to ablate STAT-3 expression in vitro, described in Chapter 5, indicate that STAT-3 mediates growth via a downstream molecule or pathway rather than via direct transactivation of the AR.

### 1.4 The Role of Epidermal Growth Factor in Prostate Cancer

Epidermal growth factor (EGF) is a member of the polypeptide growth factor family and is a single chain polypeptide. The 53 amino acid EGF protein contains three intramolecular disulfide bonds to maintain the ternary structure (Boonstra et al., 1995) and is a stable, non-glycosylated protein.

The EGF receptor (EGFR) is a 170 kDa transmembrane glycoprotein and is activated by ligand-induced dimerization (Boonstra et al., 1995). EGFR is a receptor tyrosine kinase and ligand binding causes receptor auto-phosphorylation and tyrosine phosphorylation of other proteins. EGFR activates the MAPK pathways through Grb2 and SOS, which then activate Ras and Raf. The MAPK pathway causes cell proliferation through
phosphorylation of c-Fos, a transcription factor that controls AP-1 and ELK-1. The EGFR is also referred to as the ErbB family of receptor tyrosine kinases. Only ErbB1/EGFR, ErbB2/Neu and ErbB3 are present in normal and diseased prostate tissue (Grasso et al., 1997; Ware, 1998). Overexpression of ErbB2 and ErbB3 has a role in neoplastic transformation of prostate cancer cells and stimulation of LNCaP cells with IL-6 has been shown to induce tyrosine phosphorylation of ErbB2 and ErbB3 but not ErbB1 (Qiu et al., 1998). Following IL-6 stimulation, ErbB2 can bind gp130 of the IL-6R and may play an important role in IL-6 activation of the MAPK pathway (Qiu et al., 1998).

EGFR also recruits JAK1, which phosphorylates and activates STAT-1 and STAT-3, causing cell proliferation. STAT-1 and STAT-3 can form homo- or heterodimers and the dimer then translocates to the nucleus and leads to STAT-1/-3-mediated gene transcription (Zhong et al., 1994). The EGF pathway and the IL-6 pathway both activate STAT-3 and the effects of these two pathways may be co-operative and may mutually enhance gene transcription.

EGF is present in the prostatic fluids of normal human prostates and appears to have a role in growth regulation (Russell et al., 1998). Expression of EGF is regulated by androgen in prostate cells and EGF expression is upregulated in androgen-independent cell lines (Russell et al., 1998). Autocrine expression of EGF and signalling through the EGFR may contribute to autonomous growth of human prostate cancer cells. Epithelial cells in human prostate tissue avoid paracrine dependence on stromal EGF by
demonstrating autocrine expression of EGFR ligands, such as TGFα, EGF and heparin-binding EGF. (Djakiew, 2000). EGF may also promote invasiveness of the prostate cancer cells (Russell et al., 1998). The pleiotrophic effect of EGF on prostate cancer cells is to promote both growth and invasiveness (Djakiew, 2000).

### 1.5 The Androgen Receptor in Prostate Cancer

The AR is a 110 kDa protein that is a member of the nuclear receptor superfamily (Keller et al., 1996; Lin et al., 2001). Expression of AR is important for male sexual development and, at maturity, for maintenance of accessory sexual organs (Culig, Klocker et al., 2002). The AR contains a C-terminal ligand binding domain, an N-terminal transactivation domain, a nuclear localization domain, and a DNA binding domain. Following binding of androgen, the AR is phosphorylated and dimerizes before translocating to the nucleus where it binds to the androgen response element (ARE), a specific DNA binding sequence, and activates gene transcription (Matsuda et al., 2001; Wong et al., 1993). Transcription is regulated by a variable number of polyglutamine and polyglycine repeats in the N-terminal region and by the ligand binding domain (Culig, Klocker et al., 2002). Two zinc fingers each consisting of four cysteine residues bound to a zinc ion comprise the DNA binding domain, which is also involved in receptor dimerization (Culig, Klocker et al., 2002). Co-activators of the AR include cAMP response element (CREB) binding protein or p300 and SRC-1, both of which have intrinsic histone acetyltransferase activity (Heinlein et al., 2002).
Huggins and Hodges first recognized the hormone dependent nature of prostate cancer in 1941 (Huggins et al., 1941). Androgen ablation therapy is based on the observation that withdrawal of androgens led to regression of prostate cancer (Culig, Klocker et al., 2002). Mutations in the AR are seen in later stages of prostate cancer but not early in the disease. LNCaP cells contain a mutation in the AR in exon H which causes a threonine to alanine substitution at amino acid 877 (Culig, Klocker et al., 2002). The substitution of threonine for alanine changes the binding pocket stereochemistry and broadens the ligand specificity of the AR (McDonald et al., 2000).

1.5.1 Cross-talk Between STAT-3 and the AR in Prostate Cancer

There is abundant literature to suggest a direct interaction between the AR and STAT-3. Androgen-stimulated AR has been found to augment IL-6-induced STAT-3 transcriptional activity (Culig, Bartsch et al., 2002; Matsuda et al., 2001). Conversely, IL-6-stimulated STAT-3 activity has been found to augment androgen-induced AR transcriptional activity (Chen et al., 2000; Culig, Bartsch et al., 2002; Hobisch et al., 1998; Matsuda et al., 2001). Signalling interactions between the IL-6 pathway and the AR pathway are illustrated in figure 1.6.
Figure 1.6 Interaction Between AR and IL-6 Pathways Via STAT-3

IL-6 can activate AR-mediated gene expression in the absence of androgen through a direct interaction between the AR and STAT-3. Conversely, androgens can activate STAT-3 mediated gene transcription in the absence of IL-6 by the same pathway.

Immunoprecipitation of STAT-3 or AR by Matsuda et al. retrieved a STAT-3-AR complex, following dihydrotestosterone stimulation of 293T cells, regardless of which component was targeted in the immunoprecipitation (Matsuda et al., 2001). Ueda et al. demonstrated that the IL-6 activates the N-terminal of the AR in LNCaP cells and that STAT-3 can be co-immunoprecipitated with amino acids 234 to 558 of the AR N-terminal domain in cells stimulated with IL-6 (Ueda et al., 2002).
Lin et al. found that IL-6-influence on the AR is dependent on the MAPK pathway. In the absence of androgens, IL-6 increased AR gene expression and activated the AR in LNCaP cells (Lin et al., 2001). Hobisch et al. suggest that PKA, PKC and MAPK are all necessary for IL-6 to activate AR in the absence of androgen (Hobisch et al., 1998). On the other hand, experiments by Chen et al. show that MAPK activation may not be necessary for IL-6 to influence AR activation but that STAT-3 is required for IL-6-induced upregulation of AR and AR-mediated genes (Chen et al., 2000). Stratton et al. support the idea that IL-6 influence on the AR is not mediated by STAT-3. Activation of the AR does not prevent STAT-3-mediated gene transcription (Stratton et al., 2002). That is to say that AR-STAT-3 binding either does not occur with AR stimulation and the AR does not sequester STAT-3 from the IL-6 signalling pathway, or, there are sufficient STAT-3 molecules in the cell that AR-STAT-3 binding can occur without downregulating the IL-6 signalling cascade.

1.6 Antisense Oligodeoxynucleotides

Antisense oligodeoxynucleotides (ODN) are single strand sequences of DNA, complementary to the target gene mRNA sequence (Miyake et al., 2001). Antisense ODN are rationally designed molecules that inhibit the expression of a protein, by physically blocking the ribosome’s translational activity and reducing the total protein level of the target gene’s product (Agrawal et al., 2000). The activity of antisense ODN in the cell is illustrated in figure 1.7.
Figure 1.7 Antisense Oligodeoxynucleotide Effect on Protein Expression

Antisense ODN bind a short target sequence, approximately 20 base pairs, on the mRNA. Binding the mRNA blocks the ribosomal complex formation and protein translation.

Phosphorothioate ODN have a sulphur substitution for a non-bridging phosphoryl oxygen (Gleave et al., 2002; Miyake et al., 2001) and are stable, water soluble and resistant to nuclease digestion. A second modification is necessary to prolong the in vivo half-life of ODN with a phosphorothioate backbone. An increase in resistance and binding affinity results from a 2'-O-(2-methoxy)ethyl (MOE) ribose modification (Gleave et al., 2002; McKay et al., 1999; Wagner, 1995). In human clinical trials, non-MOE ODN require continuous infusion to ensure efficacy whereas MOE ODN can retain efficacy with weekly administration. The addition of antisense ODN therapy to a chemotherapy regimen has synergistic effects that improve clinical outcome (Miyake et al., 2001).
Several antisense molecules have already been investigated in experimental and clinical trials. The \textit{BCL-2} gene was first characterized as a proto-oncogene in B-cell lymphomas (Miyake et al., 1999). A translocation of \textit{BCL-2} to the immunoglobulin heavy chain locus causes overexpression of BCL-2 protein and results in a resistance to apoptosis and consequent tumour cell survival. BCL-2 has been associated with prostate cancer and was an ideal target for an ODN design. Ideally, reduction of BCL-2 expression decreases cell survival and would be a beneficial augmentation to chemotherapy. Results from Webb et al. in non-Hodgkin lymphoma and Miyake et al. in Shionogi tumours, indicate that BCL-2 ODN can reduce BLC-2 expression in tumour cell, with minimal toxicity, and oligo treatment delayed recurrence of androgen-independent prostatic tumours compared to mismatch ODN treatment (Miyake et al., 1999; Webb et al., 1997).

Testosterone-repressed prostate message-2 (TRPM-2) has been associated with an increased resistance to apoptosis, increased Gleason score and androgen-independent tumour recurrences (Miyake et al., 2000). Similar to BCL-2, TRPM-2 was also a good target for antisense ODN design. Miyake et al. found that antisense ODN treatment reduced TRPM-2 expression in Shionogi tumours and tumour regression occurred more quickly in antisense ODN-treated tumours than in mismatch ODN-treated tumours. Reduction in TRPM-2 expression permitted earlier onset of post-castration apoptosis (Miyake et al., 2000).

An important question to address is whether administration of antisense ODN against a specific molecular target prevalent in several organs, would reduce target gene expression
in all relevant organs. Miyake et al. found that TRPM-2 expression was only reduced in tissues undergoing castration-induced apoptosis. The sensitivity of apoptotic tissues to antisense ODN therapy is perhaps due to increased membrane permeability in those cells (Miyake et al., 2000).

### 1.6.1 Use of Antisense ODN to Block AR Expression

Eder et al. successfully blocked AR protein expression in LNCaP cells in vitro (Eder et al., 2002). A 150-mer phosphorothioate ODN was designed to hybridize to the polyglutamine region of the AR mRNA. Blocking AR protein expression caused significant cell growth inhibition and downregulation of PSA secretion in the LNCaP cells. Tumour growth was inhibited by 52% and tumour weight was reduced to 43% of the original weight. Successful downregulation of AR by antisense treatment clearly demonstrates potential for downregulation of other pathways that contribute to cell growth, in particular, the IL-6 pathway.

### 1.7 RNAi and shRNA

RNA interference (RNAi) is a double-stranded RNA (dsRNA)-induced gene silencing mechanism that was first studied in *Caenorhabditis elegans* and *Drosophila* (Hannon, 2002; Paddison et al., 2002). There are two steps in the RNAi process. First, the dsRNA is recognized by Dicer, an RNaseIII family nuclease. Dicer cleaves the dsRNA into small
interfering RNA (siRNA), which are 20-23 nucleotides in length. Next the siRNA are integrated into the RISC complex, a nuclease complex, which identifies mRNA for destruction based on their homology to the integrated siRNA sequence (Paddison et al., 2002).

Short hairpin RNA (shRNA) can also trigger gene silencing via the RNAi mechanism (Paddison et al., 2002). The shRNA are processed by Dicer to generate siRNA, similar to the effect of Dicer on dsRNA. Gene silencing can be achieved by shRNA, not only in Drosophila cells, but also in mammalian cells including 293T, HeLa, COS-1 and NIH 3T3 murine fibroblast cells (Paddison et al., 2002). Gene silencing by shRNA and siRNA has been effective in selectively silencing mutant but not wild type SOD-1 alleles in amyotrophic lateral sclerosis (ALS) (Ding et al., 2003), and mutant acetylcholine receptor subunits in slow channel congenital myasthenic syndrome (Abdelgany et al., 2003). The primary advantage of using shRNA instead of siRNA is the potential for stable expression of shRNA by a cell line and possibly in vivo.

1.8 Specific Experimental Objectives

The effects of interactions between the IL-6, AR and EGF pathways will be examined in this thesis. The AR and IL-6 pathways connect through direct interaction between STAT-3 and the AR. Since EGF also activates STAT-3 it is likely that EGF-activated STAT-3 may also directly interact with the AR.
Unpublished experiments by Dr. Michael Cox and research presented in this thesis indicate that STAT-3 S727 can be phosphorylated in the absence of prior Y705 phosphorylation in the EGF signal cascade. Further, in the presence of the MAPK inhibitor PD098059, S727 phosphorylation was detected. Not only is serine phosphorylation possible in the absence of tyrosine phosphorylation, a kinase other than MAPK is responsible for the phosphorylation event.

The specific experimental goals of this thesis are to:

1. Clarify downstream effects of interactions between the IL-6, AR and EGF signalling pathways, using luciferase reporter assays to examine activation of gene transcription

2. Investigate the role of STAT-3 in prostate cancer progression in vivo, by treating nude mice with antisense ODN targeted to STAT-3 and examine the efficacy of antisense treatment in preventing increase in serum PSA levels and tumour growth rate

3. Investigate the importance of STAT-3 in facilitating cross-talk between the pathways by preventing its expression in LNCaP cells using antisense ODN and shRNA targeted to STAT-3
Chapter 2. Materials and Methods

2.1 Tissue Culture

2.1.1 Cell Passage

The LNCaP cell line was used as a model of androgen-dependent prostate cancer. LNCaP cells were provided by Dr. L.W. Chung (Emory University, Atlanta, GA). Cells were maintained in T-media (Invitrogen, Burlington, ON) supplemented with 5% fetal bovine serum (FBS) (Invitrogen, Burlington, ON) and 1% penicillin-streptomycin (Invitrogen, Burlington, ON). We were unable to obtain T-media during the growth factor assays for SIE-luc and Gal4Elk-1/5xGal4Erb1-luc and all \textit{in vitro} antisense experiments. Roswell Park Memorial Institute Media (RPMI) supplemented with 5% FBS and 1% penicillin-streptomycin was used for cell culture during these experiments.

Cells were passaged at 70% confluence or approximately every four days. Cells were cultured in 100 mm tissue culture plates (Nalge Nunc International, Rochester, NY) at 37°C and 5% CO$_2$ in humidified air.

To passage cells, the media was aspirated and cells were washed once in 2 ml of phosphate buffered saline (PBS) and 1 ml of 1x Trypsin-EDTA [10% 10x Trypsin-EDTA (Invitrogen, Burlington, ON) and 90% PBS] was added to the plates and incubated for one minute to loosen adherent cells. Cells were triturated from the plates with 5 ml of
media and added to a larger volume of media for re-plating. LNCaP cells have a tendency to form clumps (Horoszewicz et al., 1983), therefore after the cells were added to the final volume of media, the solution was pipetted approximately 10 times in order to break up any cell clumps before re-plating. Cells were passed at a 1:4 ratio in 100 mm plates or one 100 mm plate into one 6-well plate (Nalge Nunc International, Rochester, NY) for transfections.

2.1.2 Thawing Cells

Frozen cells stored at -180°C were thawed, rapidly, in a 37°C water bath and immediately added to 9 ml of T-media with 5% FBS and 1% penicillin-streptomycin in a 100 mm plate. The plate was passaged the following day at a ratio of 1:4.

2.1.3 Plasmid Transfections

The transfection efficiency of Lipofectin (Invitrogen, Burlington, ON) and Lipofectamine 2000 (Invitrogen, Burlington, ON) was compared (data not shown). LNCaP cells were transfected with 1 μg GFP using either 2 μl Lipofectin or 2 μl Lipofectamine 2000. Transfection efficiency was consistently greater when Lipofectamine 2000 was used as the transfection reagent.

Cells were transfected in 6-well tissue culture plates and the following protocol was followed for each well. The following quantity of each plasmid was used: 1 μg of GFP,
luciferase construct or shRNA, 0.25 μg of pRL-TK or pRL-TS. The plasmids were added to 100 μl of Opti-MEM I Reduced Serum Media (Invitrogen, Burlington, ON) and incubated for 5 minutes. At the same time, 2 μl of Lipofectamine 2000 (Invitrogen, Burlington, ON) was incubated 100 μl of Opti-MEM for 5 minutes. Following incubation, the two solutions were combined and incubated for a further 20 minutes. The media was aspirated from the cells but they were not rinsed in PBS, as washing the cells caused dislodging and decreased confluence of the well. 1 ml of Opti-MEM was added to each well and the transfection reaction was added by distributing drops of the reaction over the surface of the well. The transfected cells were incubated for 5 hours and then the transfection reaction was aspirated and 1 ml of phenol red-free RPMI Medium-1640 (Invitrogen, Burlington, ON) containing 5% charcoal-stripped FBS (CSS) was added to each well.

2.1.4 Oligodeoxynucleotide Transfections

Cells were transfected in 6-well tissue culture plates and the following protocol was followed for each well. 500 nM or 100 nM of ODN was added to 61.8 μl of Opti-MEM I Reduced Serum Media (Invitrogen, Burlington, ON) and incubated for 5 minutes. At the same time, 3 μl of Oligofectamine Reagent (Invitrogen, Burlington, ON) was incubated 9.75 μl of Opti-MEM for 5 minutes. Following incubation, the two solutions were combined and incubated for a further 20 minutes. The media was aspirated from the cells but they were not rinsed in PBS, as washing the cells caused dislodging and decreased confluence of the well. 300 μl of Opti-MEM was added to each well and the transfection
reaction was added by distributing drops of the reaction over the surface of the well. The transfected cells were incubated for 4 hours and then the transfection reaction was aspirated and 375 µl of RPMI Medium-1640 (Invitrogen, Burlington, ON) containing 5% CSS was added to each well. A second transfection was performed 24 hours later.

When luciferase constructs were transfected into cells that were co-transfected or previously transfected with ODN, the same protocol was followed and 1 µg of SIE-luc or ARR3-luc and 0.25 µg of pRL-TK were added to the initial incubation with Opti-MEM.

2.2 Cell Stimulation

2.2.1 Reagents

Reagents used in cell stimulation including the stock concentration and the working concentration, are listed in Appendix A.

2.2.2 Stimulation for Luciferase Experiments

Cells were stimulated during re-feeding with CSS. Stimulation took place for 18 hours at 37°C. Lysates for Western blots were prepared from luciferase experiments unless otherwise stated and therefore received 18 hours of stimulation.
2.2.3 Acute Stimulation for Western Blotting

For lysates that were prepared separately from luciferase experiments, cells received acute stimulation for 20 minutes at 37°C.

2.2.4 Inhibitors

Cells were starved for 24 hours in RPMI Media-1640 containing 5% CSS. Inhibitors and growth factors were added to the cells at the same time and stimulation occurred, for 20 minutes at 37°C.

2.3 Luciferase Assays

2.3.1 Luciferase Constructs

The ARR$_3$-luc construct (ARR$_3$-tk-luc) (Snoek et al., 1998) was provided by Dr. Paul Rennie (The Prostate Centre at Vancouver General Hospital, Vancouver, BC). The SIE-luc construct was provided by Corinne Silva (University of Virginia, Charlottesville, VA). The pcDNA3a-GAL4-Elk1-COOH (Roberson et al., 1995) construct and the 5xGAL4-E1b-luc (Sun et al., 1994) construct were provided by Dr. Michael Weber (University of Virginia, Charlottesville, VA). Plasmid maps for ARR$_3$-tk-luc, pcDNA3a-
GAL4-Elk1-COOH and 5xGAL4-E1b-luc are illustrated in figure 2.1. pRL-tk-luc and pRL-ts-luc were both obtained from Promega (Madison, WI) The SIE-luc construct consists of a triple SIE element.

2.3.2 Luciferase Assay

The media was aspirated from the cells and 200μl of 1x Passive Lysis Buffer (PLB) (5x PLB diluted 1:5 in dH2O) (Promega Corporation, Madison, WI) was added to each well and incubated on ice for 5 minutes. Cells were scraped down, added to eppendorf tubes and incubated for 5 minutes on ice. 20 μl of each lysate was added to a 96-well luminometry plate. The Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI) was used for the luciferase assay.

The luminometer was set to inject 100 μl of Luciferase Assay Reagent after a delay of 1.6 seconds and measured over an interval of 30 seconds. After the first measurement, 100 μl of Stop & Glo Reagent was injected after a delay of 5 seconds and measured over an interval of 10 seconds.

As pRL-TK and pRL-TS exhibited an androgen-responsive nature, all luciferase values are expressed as the luciferase reading alone corrected for the baseline reading from 1x PLB, (luciferase value = luciferase reading for sample – luciferase reading for 1x PLB). In experiments performed in duplicate, the luciferase values are expressed as an average of the two replicates and the error bars represent the standard deviation between replicates. In experiments performed in a greater number of replicates, the luciferase
values are expressed as an average of all replicates and the error bars represent the standard error between replicates.
Figure 2.1 Plasmid Maps for Luciferase Constructs
(a) ARR3-tk-luc, (b) pcDNA3a-GAL4-Elk1-COOH, (c) 5xGAL4-Erb1-luc
2.4 Western Blots

2.4.1 Preparation of Whole Cell Lysates

The media was aspirated from the cells and 200 μl of RIPA lysis buffer (20 mM of 1 M Tris-HCl/SDS, pH 7.4, 1% Triton-X-100, 0.1% of 1% deoxycholic acid, 1 mM of 0.5 M EDTA, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 0.5 mM sodium vanadate, 2 μM microcystin) was added to each well and incubated on ice for 5 minutes. Cells were scraped down, added to eppendorf tubes and incubated for 5 minutes on ice. Lysates were cleared by centrifugation at 10,000 g for 10 minutes at 4°C and the supernatant was assayed for protein content using a BCA assay (Pierce, Rockford, IL). 50μg of each sample was prepared for loading. 1/6 volume of 6x SDS sample buffer (35% 1 M Tris-HCl/SDS pH 6.8, 30% glycerol, 10% SDS, 9.3% DTT, 0.012% bromophenol blue) was added to each sample and boiled for 2 minutes at 100°C. Whole cell lysates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (Schleicher and Schuell, Keene, NH). Samples were analyzed by immunoblotting.

2.4.2 Immunoblotting

Membranes were incubated in blocking buffer [100 ml of 10x TBST pH 7.6 (2.42% Tris base, 8% sodium chloride, 0.5% Tween-20) in 1000 ml dH2O containing 5% Bovine Serum Albumin Fraction V (BSA) (Roche Diagnostics Corporation, Indianapolis,
Indiana) for a minimum of 2 hours at room temperature or overnight at 4°C. Primary antibody (see table below) was added to blocking buffer, at the appropriate working concentration, and incubated for 1 hour at room temperature or overnight at 4°C. Membranes were washed three times for 10 minutes in 1x TBST. Primary antibodies were detected with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (DAKO, Glostrup, Denmark). Secondary antibody was added at a 1:2000 dilution in 1x TBST for 30 minutes at room temperature. Membranes were washed again three times for 10 minutes in 1x TBST and visualized by chemiluminescence (ECL, Amersham Pharmacia, Buckinghamshire, England). Primary antibodies used in Western blotting, including working concentrations, are listed in Appendix B.

2.4.3 Preparation of Tumour Lysates

Tumours were thawed on ice and placed in a dounce homogenizer. 300 μl of RIP A buffer (containing 1% SDS and protease inhibitors) was added to the dounce and the tumour was homogenized. The dounce walls were rinsed with 100 μl of RIP A buffer and the lysate was removed and cleared by centrifugation, three times, at 10,000 g at 4°C. The lysates were diluted 1:10 in RIP A buffer and the diluted lysates were assayed for protein content using a BCA assay. For the second in vivo experiment, IgG was pre-cleared from the lysates. 500 μl of whole cell lysate was incubated with 40 μl of protein A agarose beads (Invitrogen, Burlington, ON) for 30 minutes at 4°C while rotating. 50 μg of diluted lysate was loaded onto the gel for the first in vivo experiment and 100 μg of diluted lysate was loaded for the second in vivo experiment.
2.5 *In Vivo* Antisense Experiments

2.5.1 Oligodeoxynucleotides

ODN were provided by ISIS Pharmaceuticals (Carlsbad, CA). A human STAT-3 antisense ODN (5'-GCT CCA GCA TCT GCT GCT TC-3'-) (ISIS 113176) and a 5 base mismatch control ODN (5'-GCT CCA ATA CCC GTT GCT TC-3') (ISIS 129987) were used in the *in vivo* experiments (Mora et al., 2002).

2.5.2 Injection Protocol

Thirty male, nude mice were injected with LNCaP cells (human) to establish a xenograft tumour. There were two sub-cutaneous injection sites in the hind flank and approximately 6x10^6 cells were mixed with Matrigel (BD Biosciences, San Jose, CA) and injected at each site. Following injection, serum PSA was monitored and castration took place once serum PSA exceeded 50 ng/ml. Group A consisted of mismatch ODN-injected animals, Group B consisted of antisense ODN-injected animals and Group C consisted of saline-injected animals. Injections for Group A and Group B began the day after castration. Animals were injected five times in the first week and three times per week in the following weeks. Group C began saline injections once serum PSA had returned to pre-castrate levels. Intra-peritoneal injections were administered at a dose of 10 mg/kg of body weight or 0.3 mg/30 g mouse.
Serum PSA was measured weekly by an enzymatic immunoassay kit (Abbott IMX, Montreal, QC). Blood samples for analysis were obtained from the tail vein of each mouse. Tumour volume was measured weekly, with callipers, and calculated by the formula volume = length x width x depth x 0.5236. Values for serum PSA and tumour volume are expressed as an average of all animals measured each week, with error bars representing average deviation. The end-point of the experiment was determined by a tumour volume greater than 15% of body weight or signs of toxic side effects.

2.6 shRNA Preparation

2.6.1 Selection of STAT-3 shRNA Sequences

Design of the shRNA construct began with selection of appropriate target sequences within the STAT-3 mRNA. Sequences were selected by an shRNA design program located at the website http://python.penguindreams.net/Xeragon_Order_Entry/SearchBySequence.do. Each sequence was run through a BLAST search to ensure that it was unique to STAT-3, both as a 20-mer and as a 10-mer. The six highest-ranked unique sequences were used to design oligonucleotides. The oligo sequences are inserted into the pSHAG vector in order to produce a plasmid for transfection into cells.

To select a sequence for oligo design, several considerations must be taken into account. The target sequence from the mRNA coding region should have a GC ratio of 50%.
sequence should not be within 50 to 100 nucleotides of the AUG start codon or the termination sequence. There should be no more than three G or C in a row within the sequence to prevent hyperstacking.

To design oligo inserts, a series of modifications must be applied to the selected sequence, as determined Dr. Greg Hannon and colleagues (Cold Spring Harbor Laboratory, Cold Spring Harbour, NY). (1) The selected sequence is a 20-mer but RNA pol III will transcribe a final 31-mer hairpin RNA. To produce a 31-mer, the core 20bp sequence is extended by 11bp in such a way that the oligo ends in a C followed by two base pairs (5'-N1 NN...NN C N30 N31-3'). Pol III initiates at a G residue in the U6 promotor on the antisense strand. (2) The reverse complement of the sequence for the antisense strand is obtained (5'-N31' N30' G N'N'...N'N1'-3'). (3) Residues N30' and N31' are removed from the reverse complement sequence. (4) The sequence 5'-GAAGCTTG-3' is added to the 3' end of the reverse complement (5'G N'N'...N'N1'GAAGCTTG-3'). (5) The sense sequence is added to the 3' end of the modified reverse complement sequence to obtain a long hairpin sequence (5'-G N'N'...N'N1'GAAGCTTG1N...NN C N30 N31-3'). (6) A pol III terminator sequence TTTTTT is added to the 3' end of the long hairpin sequence. (7) The G residue is dropped from the 5' end of the long hairpin and the resulting sequence is designated “oligo A” of the pair that will be ligated into the vector (5'-N'N'...N'N1'GAAGCTTG1N...NN C N30 N31 TTTTTT) (8) The reverse complement of oligo A is generated and modified, such that GATC is added to the 5' end and CG is added to the 3' end, resulting in “oligo B” (5'-GATC AAAAAA (reverse
complement of N31' N31 C NN...NN1)CAAGCTTG(reverse complement of N1'N'...N'N' CG-3'). Oligo A and oligo B are now hybridized and the result is a hybridized pair with overhangs that are appropriate for cloning into BseRI and BamHI restriction sites in the pSHAG plasmid.

Our six selected sequences, termed shRNA#1 through shRNA#6, were modified according to the above procedure and the oligo pairs were generated by the Nucleic Acids Protein Services Unit (NAPS) at the University of British Columbia. The selected shRNA sequences are listed in Appendix A.

2.6.2 Ligation of Oligos and pSHAG Vector

The pSHAG vector was provided by Dr. Alice Mui (University of British Columbia, Vancouver, BC). The vector was prepared by cutting with the restriction enzymes BseRI and BamHI. The oligos were dissolved at 100 mM and were combined in pairs at 50 μM each in the final volume. The oligo pairs were boiled at 95°C for 5 min and cooled by removing the heat block and leaving it at room temperature overnight for slow re-annealing.

The ligation reaction is performed with a 10:1 ratio of insert to vector and the volume of insert required for the ligation reaction was calculated as shown below, given that the size of the vector is 4 kb, the size of the oligo insert is 80 bp and the concentration of vector was 50 ng/μl.
Mass of insert needed

\[
\text{Mass of insert needed} = \text{ng vector} \times \text{kb size of insert} \times \text{insert: vector ratio}
\]

\[
\text{kb size of vector}
\]

(Use 50 ng of vector)

\[
= 50 \text{ ng vector} \times 0.08 \text{ kb insert} \times 10/1
\]

\[
= 4 \text{ kb vector}
\]

\[
= 10 \text{ ng insert needed for ligation}
\]

Concentration of insert

\[
\text{Concentration of insert} = 50 \mu\text{M} = 50 \mu\text{mol/L} \times 1 \text{ mol/1x}10^{-6} \mu\text{mol} = 50 \times 10^{-5} \text{ M}
\]

\[
= 50 \times 10^{-6} \text{ moles/L}
\]

\[
dada = 660 \text{ g/mole/base}
\]

\[
\text{insert} = 660 \text{ g/mole/base} \times 80 \text{ bp insert} = 52,800 \text{ g/mole}
\]

so \[
52,800 \text{ g/mole} \times 50 \times 10^{-6} \text{ moles/L} = 2.64 \text{ g/L}
\]

concentration of insert = 2.64 \mu\text{g/ul}

Volume of insert

\[
\text{Volume of insert} = 10 \text{ ng of 2.64 \mu\text{g/ul insert so 1:1000 dil} = 2.64 \mu\text{g/ul}}
\]

\[
10 \text{ ng} = 3.79 \text{ ul (volume of insert needed for reaction)}
\]

\[
2.64 \mu\text{g/ul}
\]

The following oligo combinations were used: 31, 32, 33, 34, 35, 36, control (vector only, no insert) representing shRNA #1 to shRNA #6. The ligation reaction was 20 \mu\text{l in volume. Each reaction consisted of 1 \mu\text{l of vector (50 ng), 3.79 \mu\text{l of oligo insert (10 ng), 4 \mu\text{l of 5x T4 ligation buffer (Invitrogen, Burlington, ON), 1 \mu\text{l T4 DNA ligase (Invitrogen, Burlington, ON), 10.21 \mu\text{l TE. Ligation was performed at room temperature for 3 hours. Following ligation, each reaction was transformed into DH5}\alpha\ E. coli cells. Ligations that contained the insert were determined by cutting the ligated plasmid with the restriction enzyme HindIII. HindIII cuts within the inserted oligo sequence and produces a linearized plasmid or a single band on a 1% agarose gel.}
2.7 Molecular Techniques

2.7.1 Transformation

Plasmids were transformed into DH5α E. coli cells. 1 µl and 25 µl of DH5α cells were incubated on ice for 30 minutes. The solution was heat shocked at 42°C for 45 seconds and 1 ml of LB broth was added to the transformation. The solution was incubated at 37°C for 1 hour, shaking. The transformation was plated on LB plates inoculated with ampicillin or kanamycin. 10 µl or 100 µl of the transformation solution was spread on the plate and incubated overnight at 37°C for colony formation.

2.7.2 Plasmid Purification

Plasmids were purified using the Qiagen Plasmid Mini or Maxi Kit (Qiagen, Mississauga, ON).
Chapter 3. Investigating the Role of STAT-3 in Cell Signalling Cross-Talk in LNCaP Cells Using Luciferase Constructs

3.1 Introduction

Interaction between STAT-3 and the AR has been demonstrated in several publications. Culig et al. and Hobisch et al. have examined androgen receptor activity in IL-6-stimulated DU145 cells, an androgen-independent prostate cancer cell line, and have demonstrated that IL-6 is a ligand-independent activator of the AR and of AR-mediated gene transcription (Culig, Bartsch et al., 2002; Hobisch et al., 1998). Matsuda et al. have examined pathway cross-talk in LNCaP cells using a STAT-3 luciferase construct and -285PB-luc, a probascin reporter. Their results indicate that androgen amplifies IL-6-stimulation of STAT-3 luc and that IL-6 amplifies androgen-stimulation of -285PB-luc. Chen et al. have investigated the ability of IL-6 to activate ARE-luc in LNCaP cells and demonstrated that STAT-3 associates with the AR in an androgen-independent, but IL-6-dependent, manner (Chen et al., 2000).

Several authors have demonstrated that STAT-3 and the AR have a direct interaction. Matsuda et al. demonstrated that in LNCaP cells co-stimulated with IL-6 and androgen, the AR and STAT-3 can be co-immunoprecipitated (Matsuda et al., 2001). Ueda et al. demonstrated that IL-6 activates the N-terminal of the AR in LNCaP cells and that STAT-3 can be co-immunoprecipitated with amino acids 234 to 558 of the AR N-terminal domain in cells stimulated with IL-6 (Ueda et al., 2002).
Results described above as well as other findings in the literature have been used to propose a model of STAT-3 and AR association and co-potentiation of both ARE and SIE-mediated transcription. At the time of the above publications, we were performing the same experiments with the additional goal of distinguishing between IL-6 and EGF-mediated transactivation of the AR. Our experiments build on the above findings, by using both the ARR3-luc reporter and the SIE-luc reporter in LNCaP cells, to investigate their response to stimulation by IL-6, R-1881 and EGF.

We include EGF as a stimulant, in addition to IL-6 and R-1881, because EGF is a crucial activator of STAT-3 in a number of cell systems. Other growth factors, such as platelet-derived growth factor (PDGF) (Yellaturu et al., 2003), basic fibroblast growth factor (bFGF) (Deo et al., 2002), and IGF-1 (Culig et al., 1994) can also activate STAT-3, however, we chose EGF to include in our model because it can transactivate the AR (Culig et al., 1994). In LNCaP cells, as well as in other cell systems, EGF and IL-6 both cause Y705 phosphorylation of STAT-3 and may have complementary effects on the AR. Our study of AR and STAT-3 interaction in LNCaP cells focuses, not only on IL-6 activation of STAT-3, but also EGF activation of STAT-3 and the interaction of STAT-3 and the AR.

Throughout this thesis, luciferase data is expressed as relative luciferase units not as fold-change in induction of luciferase activity. Our results were consistently reproducible and expressing the data as relative luciferase units presents a more rigorous analysis of our results.
3.2 Optimizing Experimental Conditions for ARR₃-luc and SIE-luc Activation

Two luciferase constructs were used to examine activation of gene expression: ARR₃-luc and SIE-luc. To establish stimulation conditions for the luciferase constructs, a dose-response analysis was performed for each construct with varying concentrations of the appropriate stimulant. Since the AR response to the steroids present in fetal bovine serum (FBS) was a concern in LNCaP cells, the effect of stimulation in media containing 5% FBS or 5% CSS was also compared. We had to ensure that depletion of steroids in the assay, by using CSS, did not kill the cells.

R-1881 induced a measurable response by ARR₃-luc at a concentration of 1x10⁻¹¹ M as represented in figure 3.1. Stimulation of ARR₃-luc with 1x10⁻⁸ M produces a similar degree of activation to stimulation with 1x10⁻⁹ M (personal communication from Dr. Michael Cox and Zhihong Wen). We considered 1x10⁻⁹ M to be the maximal dose of R-1881 for cell treatment and therefore 1x10⁻⁹ M R-1881 was considered to be the saturating dose of androgen for the luciferase construct. Half-maximal activation of ARR₃-luc occurred at an R-1881 concentration between 1x10⁻¹⁰ M and 1x10⁻⁹ M. Sub-maximal activation of the luciferase construct was chosen for experiments that include other stimulants, to allow for augmentation or suppression of inducible signal. An R-1881 concentration of 1x10⁻¹⁰ M was selected as the sub-saturating concentration for use in future luciferase experiments.
Figure 3.1 Dose Response Curve for ARR3-luc Stimulated with Increasing Concentrations of R-1881 and SIE-luc Stimulated with Increasing Concentrations of IL-6

LNCaP cells were transfected with (a) 1 μg of ARR3-luc or (b) 1 μg of SIE-luc and 0.25 μg of pRL-TK. Cells were incubated in RPMI-1640 with 5% FBS or 5% CSS. Cells were stimulated with increasing concentrations of (a) R-1881 or (b) IL-6 for 18 hours and results were determined by luciferase assay. Results shown are a representative replicate of two replicates.
IL-6 induced a measurable response from SIE-luc at a concentration of 0.04 nM. The saturating dose of IL-6 for maximal SIE-luc activation was 0.2 nM. Experiments in the literature that stimulated SIE-luc with IL-6 used a concentration of 100 ng/ml (Matsuda et al., 2001). Stimulation of ARE-luc with IL-6 was performed with concentrations of 10 ng/ml (Hobisch et al., 1998), 50 ng/ml (Hobisch et al., 1998; Ueda et al., 2002) or 100 ng/ml (Chen et al., 2000). Since the luciferase construct response did not increase between 0.2 nM and 2 nM, we selected 2 nM or 40 ng/ml as the concentration of IL-6 for use in luciferase assays. Our concentration approximates the median concentration of IL-6 used in the literature. Subsequent experiments demonstrated that signal from SIE-luc, stimulated with 2 nM IL-6, can be successfully augmented by other stimulants and we continued to use a 2 nM concentration of IL-6 for the rest of our experiments.

In both IL-6-stimulated and R-1881-stimulated cells, the use of CSS rather than FBS during cell stimulation reduced the background signal caused by the presence of low levels of steroids in FBS. Without background signal, increases in luciferase construct activation and Western blot signals were more pronounced.

### 3.3 pRL-TK and pRL-TS Renilla Constructs Are Androgen-Responsive

Luciferase assays are designed to compare the response of an experimental luciferase construct to the response of a control plasmid that expresses *Renilla* luciferase protein.
The control plasmid should express *Renilla* protein at a constant level, independent of the experimental conditions.

*Renilla* plasmids pRL-TK (Promega, Madison, WI) and pRL-TS, provided by O. Fronlich (Emory University School, Atlanta, GA) were used in our luciferase assays. pRL-TK contains the HSV thymidine kinase promoter and pRL-TS contains a thromboxane promoter. We observed that *Renilla* levels increased in pRL-TK-transfected samples stimulated with R-1881 and in pRL-TS-transfected samples stimulated with IL-6. The androgen-responsive nature of pRL-TK and pRL-TS has been described in the literature (Ibrahim et al., 2000).

Experiments with LNCaP cells transfected with pRL-TK and stimulated with increasing doses of R-1881, represented in figure 3.2a, confirmed the findings of Ibrahim et al. (Ibrahim et al., 2000). R-1881 does not increase pRL-TK activity at concentrations of 1x10^{-13} M to 1x10^{-11} M. At a concentration of 1x10^{-10} M, pRL-TK activity increases by 1.6-fold and at a concentration of 1x10^{-9} M, pRL-TK activity increases further by 28-fold. IL-6 and EGF alone or in combination do not alter pRL-TK activity substantially, as illustrated in figure 3.2b, when compared to the effect of R-1881 on pRL-TK activity. Although the effect of R-1881 on pRL-TK activity was modest when a concentration of 1x10^{-10} M, our working concentration, was compared to 1x10^{-9} M, there was still a significant fluctuation in pRL-TK activity when 1x10^{-10} M R-1881 stimulation was compared to EGF or IL-6 stimulation.
Figure 3.2 Expression of pRL-TK Is Influenced by R-1881 Stimulation
LNCaP cells were transfected with 1 μg ARR3-luc and 0.25 μg pRL-TK. (a) Cells were stimulated with increasing concentrations of R-1881 for 18 hours. (b) Cells were stimulated with 2 nM IL-6, 1x10^{-10} M R-1881 and 100 ng/μl EGF for 18 hours. Results were determined by luciferase assay and are expressed as an average of two replicates. Error bars represent standard deviation.
Fluctuations in pRL-TK and pRL-TS expression can be attributed to the fact that LNCaP cells are healthier in the presence of androgen (Mulholland, in press). IL-6 can activate AR-mediated gene expression and, therefore, LNCaP cells will also benefit from IL-6-stimulated AR gene expression. Since our objective is to investigate pathway cross-talk between the AR and IL-6 pathways, neither Renilla construct can be used as a statistical control at our working concentration of $1 \times 10^{-10}$ M R-1881. However, pRL-TK and pRL-TS were used to monitor transfection efficiency, taking into account the androgen-responsive nature of the constructs.

### 3.4 IL-6 and EGF Amplify the Activity of ARR$_3$-luc in Response to Androgen Stimulation

In order to clarify the roles of EGF and IL-6 in transactivating the AR, we examined the influence of IL-6 and EGF on R-1881-induced ARR$_3$-luc signalling. LNCaP cells were transfected with ARR$_3$-luc and stimulated with combinations of 2 nM IL-6, $1 \times 10^{-10}$ M R-1881 and 100 ng/µl EGF. IL-6 and EGF were used to activate STAT-3 in order to look at the ability of STAT-3 to augment R-1881-induced ARR$_3$-luc signalling. The eight combinations of stimulants used in the assay were: no stimulants, EGF alone, R-1881 alone, EGF and R-1881, IL-6 alone, IL-6 and R-1881, IL-6 and EGF, and IL-6, EGF and R-1881 together.
R-1881 stimulation of ARR\(_3\)-luc produced a 650-fold increase in signal over no stimulation. Although IL-6 and EGF alone did not activate ARR\(_3\)-luc signalling to a significant degree, both stimulants can augment R-1881-mediated ARR\(_3\)-luc signalling as represented in figure 3.3. IL-6 augmented R-1881-induced ARR\(_3\)-luc signalling by 1.7-fold whereas EGF augmented signalling by 1.3-fold. IL-6 and EGF together augmented R-1881-induced ARR\(_3\)-luc signalling by 2.8-fold.

Significant differences between treatments were calculated by Student’s \(t\)-test, (p<0.05). Only stimulation with R-1881 alone, not EGF alone or IL-6 alone, significantly activated ARR\(_3\)-luc above the signal from unstimulated cells.

Since EGF and IL-6 activate similar intracellular signalling pathways in LNCaP cells, we assessed STAT-3 activation by these agents. We observed that although EGF phosphorylates STAT-3 at S727 in LNCaP cells, there is no detectable Y705 phosphorylation. We stimulated LNCaP cells with EGF or IL-6 to examine S727 and Y705 phosphorylation differences between the two treatments, as represented in figure 3.4. Neither Y705 nor S727 phosphorylation was visible in the absence of treatment. When cells were stimulated with IL-6, S727 phosphorylation increased 2-fold and Y705 phosphorylation also increased 2-fold. Cells that were stimulated with EGF showed a similar 2-fold increase in S727 phosphorylation but only a baseline amount of phosphorylation on Y705. It is not possible to discern whether baseline Y705 signalling was due to active phosphorylation or simply background signal, as it was of a similar strength to the signal in the unstimulated lane. Evidence in the literature argues against
Figure 3.3 IL-6 and EGF Augment ARR₃-luc Response to R-1881
LNCaP cells were transfected with 1 μg ARR₃-luc and 0.25 μg pRL-TK. Cells were stimulated with 2 nM IL-6, 1x10⁻¹⁰ M R-1881 and 100 ng/μl EGF for 18 hours and results were determined by luciferase assay. Results are expressed as an average of eight replicates from four separate experiments and error bars represent standard error. Student’s t-test was used to establish significant differences between treatments, specific comparisons are detailed in the text, (p<0.05).
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**Figure 3.4 EGF Stimulation Causes S727 But Not Y705 Phosphorylation of STAT-3 in LNCaP Cells**

Whole cell lysates were extracted from LNCaP cells, stimulated with 2 nM IL-6 or 100 ng/μl EGF for 20 minutes. 50 μg of each lysate was separated by 8% SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were immunoblotted for P-STAT-3 S727 (top panel), P-STAT-3 Y705 (middle panel) and total STAT-3 (bottom panel).
the possibility of EGF causing phosphorylation on S727 of STAT-3 without prior Y705 phosphorylation on the same molecule. While phosphorylation of S727 substantially increases the transcriptional ability of STAT-3, phosphorylation of Y705 is essential for dimerization and nuclear translocation, therefore, it is unlikely that EGF stimulation results in a STAT-3 molecule with only a S727 phosphorylation (Darnell, 1997; Wen et al., 1997; Wen et al., 1995). There is a basal level of existing P-STAT-3 Y705 in LNCaP cells and it is likely that EGF phosphorylates S727 on STAT-3 that has received prior Y705 phosphorylation. The mechanism for IL-6-induced and EGF-induced S727 phosphorylation of STAT-3 is described further in Chapter 6.

Our luciferase results indicate that there may also be a functional difference between EGF-stimulated STAT-3 and IL-6-stimulated STAT-3. The ability of EGF to augment R-1881 activation of ARR3-luc demonstrates that the ability of EGF and IL-6 to augment AR activation is correlated with increased STAT-3 S727 phosphorylation, not Y705 phosphorylation (Figure 3.3). However, IL-6 augmented R-1881 activation of ARR3-luc to a greater degree than EGF, which may indicate a deficiency in the ability of EGF-phosphorylated STAT-3 to interact with the AR and to activate ARR3-mediated gene transcription.

IL-6 and EGF together, augmented R-1881-mediated ARR3-luc activation to a greater extent than either growth factor alone. An important factor to consider is that both IL-6 and EGF activate the MAPK pathway. If the MAPK activation level from IL-6 and EGF
co-stimulation is lower than that caused by IL-6, EGF and R-1881 stimulation, MAPK activation may play an important role in ARR3-luc activation.

3.5 R-1881 and EGF Amplify the Activity of SIE-luc in Response to IL-6 Stimulation

As ARR3-luc signalling was influenced by IL-6 and EGF even though EGF did not influence STAT-3 Y705 phosphorylation, we investigated the effect of R-1881 and EGF on IL-6-induced SIE-luc signalling. Reports in the literature, that STAT-3 and the AR have a direct interaction in the cell, led us to believe that signalling cross-talk would occur in both directions. The same experimental conditions were followed for the SIE-luc experiments as described above for ARR3-luc.

IL-6 stimulation of SIE-luc produced a 32-fold increase in signal over no stimulation (Figure 3.5). EGF stimulation of SIE-luc increased signal by 9-fold over baseline while R-1881 had minimal effects on SIE-luc and increased signal by only 1.8-fold. IL-6 stimulation of SIE-luc produced a significantly greater signal than EGF stimulation. Both R-1881 and EGF augmented IL-6-induced SIE-luc activation. EGF stimulation augmented IL-6-induced SIE-luc signalling by 2-fold. R-1881 stimulation augmented IL-6-induced SIE-luc signalling by 1.2-fold. EGF and R-1881 stimulation together augmented IL-6-induced SIE-luc signalling by 2.7-fold. Significant differences between treatments were calculated by Student’s t-test, (p<0.05). Only stimulation with IL-6
Figure 3.5 R-1881 and EGF Augment SIE-luc Response to IL-6

LNCaP cells were transfected with 1 μg SIE-luc and 0.25 μg pRL-TK. Cells were stimulated with 2 nM IL-6, 1x10^{-10} M R-1881 and 100 ng/μl EGF for 18 hours and results were determined by luciferase assay. Results are expressed as an average of seven replicates from four separate experiments and error bars represent standard error. Student’s t-test was used to establish significant differences between treatments, specific comparisons are detailed in the text, (p<0.05).
alone, not EGF alone or R-1881 alone, significantly activated SIE-luc above the signal from unstimulated cells. EGF-mediated activation of SIE-luc was significantly increased by co-stimulation with IL-6 or with IL-6 and R-1881 together. IL-6-mediated activation of SIE-luc was significantly increased by co-stimulation with EGF or EGF and R-1881 together.

The effect of EGF on SIE-luc, alone and in combination with IL-6 and R-1881, demonstrated that EGF-activated STAT-3 could activate SIE-mediated gene transcription, although to a lesser extent than IL-6-activated STAT-3. The functional activity of EGF-activated STAT-3 was confirmed by the luciferase assay, however, the mechanism of transcriptional activation is not known. It is most likely that EGF phosphorylates STAT-3 at S727 on a molecule that has received prior Y705 phosphorylation. If EGF-activated STAT-3 contains only the S727 phosphorylation and no Y705 phosphorylation, perhaps that STAT-3 can work co-operatively with a S727- and Y705-phosphorylated molecule in order to bind DNA and effect transcription.

3.6 MAPK Is A Potential Effector of EGF- and IL-6-Augmented R-1881-Induced ARR3-luc Signalling

EGF and IL-6 both activate MAPK through the Ras pathway. MAPK activation may cause significant augmentation in signalling when EGF, IL-6 and R-1881 are used to co-stimulate LNCaP cells. We used a Gal4ELK1 construct with a 5xGal4Erb1-luc
luciferase reporter construct to identify MAPK-mediated gene activation. R-1881 did not activate MAPK, as expected (Figure 3.6). IL-6 activated MAPK by 12-fold over baseline while EGF activated MAPK by 29-fold over baseline. EGF-activation of MAPK was augmented 1.5-fold by co-stimulation with R-1881 and augmented, further, 1.4-fold by co-stimulation with R-1881 and IL-6. IL-6-activation of MAPK was not augmented by co-stimulation with R-1881 but was augmented 5-fold by co-stimulation with EGF. Activation of MAPK by co-stimulation with IL-6 and EGF was not increased further by the addition of R-1881.

Significant differences between treatments were calculated by Student's \(t\)-test, \((p<0.05)\). Only stimulation with EGF alone or IL-6 alone but not R-1881 alone, significantly activated Gal4ELK1/5xGal4Erb1-luc above the signal from unstimulated cells. EGF-mediated Gal4ELK1/5xGal4Erb1-luc activation was not significantly increased by co-stimulation with IL-6 or R-1881. IL-6-mediated activation of Gal4ELK1/5xGal4Erb1-luc was significantly increased by co-stimulation with EGF or with EGF and R-1881 together.

Western blots, shown in figure 3.7, demonstrate a similar pattern, with regard to magnitude of MAPK activation, and confirm that MAPK is activated by EGF but not further activated by the addition of R-1881. Phosphorylation of MAPK was not detected in cells in the absence of EGF phosphorylation. Y705 phosphorylation of STAT-3 was only present in cells that were stimulated with IL-6 and was absent in cells stimulated with EGF alone. IL-6 stimulation caused S727 and Y705 phosphorylation of STAT-3 and
Figure 3.6 Gal4ELK1/5xGal4Erb1-luc Response to Stimulation by IL-6, EGF and R-1881
LNCaP cells were transfected with 1 μg Gal4ELK1, 1 μg 5xGal4Erb1-luc and 0.25 μg pRL-TS. Cells were stimulated with 2 nM IL-6, 1x10^{-10} M R-1881 and 100 ng/μl EGF for 18 hours and results were determined by luciferase assay. Results are expressed as an average of eleven replicates from six separate experiments and error bars represent standard error. Student’s t-test was used to establish significant differences between treatments, specific comparisons are detailed in the text, (p<0.05).
Figure 3.7 Western Blots Indicate that MAPK is Activated by EGF

Whole cell lysates were extracted from LNCaP cells, stimulated for 20 minutes with 2 nM IL-6, 1x10^{-10} M R-1881 and 100 ng/µl EGF. 50 µg of each lysate was separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was immunoblotted for P-STAT-3 Y705 (top panel), STAT-3 (second panel), STAT-1 (third panel), P-MAPK (fourth panel), and for MAPK (bottom panel).
induced SIE-luc signalling. Co-stimulation with IL-6 and R-1881 did not cause an increase in S727 or Y705 phosphorylation of STAT-3. R-1881 is augmenting IL-6-mediated SIE-luc activation without causing an increase in STAT-3 activation and the increase in signalling may, therefore, be mediated by the AR. EGF and IL-6 co-stimulation of MAPK causes a much greater signal than either stimulant alone, therefore, MAPK signalling may play a role in IL-6 and EGF stimulation of ARR3-luc, but not in the increase in signal seen between IL-6- and EGF-stimulated ARR3-luc and IL-6-, EGF- and R-1881-stimulated ARR3-luc.

MAPK likely does not play a role in R-1881-mediated augmentation of EGF- or IL-6-induced SIE-luc signalling but rather AR association with STAT-3 mediates R-1881 influence over STAT-induced gene transcription. EGF-mediated MAPK activation may play an important role in augmentation of ARR3-luc signalling when cells are co-stimulated with EGF and R-1881. Augmentation of R-1881-induced ARR3-luc signalling by co-stimulation with EGF and IL-6 cannot be attributed to an increase in MAPK activation. The level of MAPK activation induced by co-stimulation of cells with IL-6 and EGF is not augmented by the addition of R-1881. Augmentation of IL-6-stimulated SIE-luc by EGF and R-1881 appears to be mediated by an interaction between STAT-3 and the AR rather than by MAPK. Augmentation of R-1881-stimulated ARR3-luc by EGF and IL-6 may be mediated by either MAPK or an interaction between STAT-3 and the AR.
3.7 Summary

Signalling cross-talk occurs between the IL-6 and AR pathways through direct interaction of STAT-3 and the AR. Signalling by IL-6-stimulated-SIE-luc can be augmented by co-stimulation with R-1881 and EGF. Conversely, signalling by R-1881-stimulated-ARR3-luc can be augmented by co-stimulation with IL-6 and EGF. Our hypothesis that signalling cross-talk occurs between the AR and IL-6 pathways was confirmed. In addition, the AR pathway can influence the IL-6 pathway and vice versa. Activation of the MAPK presented an extra consideration in addition to our primary hypothesis. MAPK is activated by both IL-6 and EGF but not by R-1881. Consequently, augmentation of SIE-luc signalling by R-1881 appears to be primarily mediated by STAT-3 and AR interaction, whereas augmentation of ARR3-luc signalling by IL-6 and EGF may be mediated by STAT-3 and AR interaction or by MAPK activation.

Our results have complemented previous findings in the literature that the AR can activate STAT-mediated gene transcription and IL-6 can activate AR-mediated gene transcription. The results from the MAPK luciferase constructs have added an important dimension to our investigation of the ability of EGF and IL-6 to transactivate AR-mediated gene transcription.

Signalling cross-talk between the IL-6 and AR pathways in the literature have been attributed to an interaction between STAT-3 and the AR by establishing an absence of signalling augmentation in the presence of a STAT-3 mutant. In order to confirm that
STAT-3 is a key mediator of signalling cross-talk between the AR and IL-6 pathway, STAT-3 must be removed from the signalling network. In an effort to ablate STAT-3 protein expression in LNCaP cells, we used both antisense ODN and shRNA to downregulate STAT-3 expression. We examined the ability of STAT-3 and the AR to cause activation of the ARR3-luc and SIE-luc reporter constructs as a reflection of the presence or absence of cross-talk between the AR and IL-6 pathways.
Chapter 4. Downregulation of STAT-3 Expression by Antisense Oligodeoxynucleotides

4.1 Introduction

Our results suggest a model in which STAT-3 can act as a co-regulator for the AR and the AR can act as a co-regulator for STAT-3. The ability of STAT-3 and the AR to associate been demonstrated previously (Culig, Klocker et al., 2002; Matsuda et al., 2001) and a STAT-3-AR complex may play an important role in our model of IL-6 and AR pathway cross-talk.

To determine the necessity of STAT-3 to IL-6 and AR pathway cross-talk we attempted to remove STAT-3 from the cell signalling system. Previous studies of the role of STAT-3 in signalling cross-talk employed a dominant negative STAT-3 construct to prevent STAT-3 activation. A dominant negative construct will still bind to the IL-6 receptor and may prevent activation of other molecules that may otherwise occur in the absence of STAT-3 binding. Rather than over-expressing a dominant negative construct, we decided to prevent STAT-3 expression completely by using an antisense ODN. Human STAT-3 antisense ODN (5'-GCT CCA GCA TCT GCT GCT TC-3') (ISIS 113176) and 5 base mismatch control ODN (5'-GCT CCA ATA CCC GTT GCT TC-3') (ISIS 129987) were provided by ISIS Pharmaceuticals (Carlsbad, California). These ODN were used recently, to effectively inhibit STAT-3 signalling in DU-145 cells by Mora et al. (Mora et al., 2002).
4.2 Antisense and Mismatch ODN Function Was Tested *In Vitro*

The antisense and mismatch ODN were transfected into LNCaP cells, at concentrations of 0 nM, 100 nM, 250 nM and 500 nM, in order to determine the concentration at which STAT-3 protein is downregulated *in vitro*. Previous *in vitro* studies by Miyake et al. used an established antisense ODN transfection procedure that we also followed (Miyake et al., 2001; Miyake et al., 2000; Miyake et al., 1999). Antisense ODN were transfected on two subsequent days for a four hour period on each day. STAT-3 expression could be suppressed by as little as 100 nM and expression was suppressed further by 250 nM and 500 nM concentrations of ODN. 500 nM was the optimal concentration at which to achieve maximal downregulation of STAT-3 by the antisense ODN while maintaining STAT-3 expression by the mismatch ODN, as represented in figure 4.1. Previous antisense ODN dose experiments by Miyake et al., with Testosterone-repressed Prostate message-2 (TRPM-2) antisense ODN and Bcl-2 antisense ODN, indicated that 77% downregulation of TRPM-2 and 81% downregulation of Bcl-2 expression can be achieved by transfecting cells with a 500 nM concentration of ODN (Miyake et al., 2000; Miyake et al., 1999). The STAT-3 ODN effectively and specifically downregulated STAT-3 expression *in vitro* and justified further study in an *in vivo* model.
Figure 4.1 Antisense ODN Most Effectively Downregulate STAT-3 Expression At A Concentration of 500 nM
Whole cell lysates were extracted from LNCaP cells transfected with 0 nM, 100 nM, 250 nM or 500 nM of antisense or mismatch ODN. 50 μg of each lysate was separated by 8% SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were immunoblotted for STAT-3 (top panel), STAT-1 (second panel), AR (third panel) and MAPK (bottom panel).
4.3 Downregulation of STAT-3 Expression In Vivo

4.3.1 Antisense Treatment Downregulated PSA Expression and Tumour Volume in LNCaP Xenograft Tumours In Vivo

To identify the role of STAT-3 in in vivo tumour development, we treated LNCaP xenograft tumours with antisense ODN to downregulate STAT-3 expression and identify the effect on androgen-mediated gene expression and tumour volume. Thirty, six-week-old male, nude mice were injected, sub-cutaneously in the hind flank, with LNCaP cells. Of the thirty mice injected, twenty-seven developed tumours. There were two injection sites and approximately six million cells were mixed with Matrigel and injected at each site. Animals were randomly designated into three groups: Group A was injected with mismatch ODN, Group B was injected with antisense ODN and Group C was injected with a control, saline solution.

The antisense, mismatch and saline treatments were administered by intra-peritoneal injection. The dosage given to each animal was 10 mg/kg of body weight, which was equivalent to 0.3 mg of ODN per 30 g mouse. The concentration of the original stock solutions for each ODN was 100 mM, dissolved in phosphate-buffered saline, and was diluted 1:10 for injection.

Castration took place once serum PSA levels had exceeded 50 ng/ml. Group A and B began treatment the day following castration. In the first week of treatment, ODN were
administered for five days. For the second, third and fourth weeks, the animals were treated three days per week. Treatment of Group C did not begin until the post-castration PSA level had returned to pre-castration levels.

Serum PSA level and tumour volume were measured once a week for the duration of the experiment. Serum PSA was determined by an enzymatic immunoassay kit from blood samples obtained from the tail vein of each mouse. Tumour volume was determined by calliper measurement and calculated as described in Materials and Methods. The endpoint of the experiment was indicated by a tumour that was greater than fifteen percent of the total body weight or toxic effects of the treatment regimen.

Animals were treated with antisense or mismatch ODN or with saline for the first four weeks. After four weeks, ODN treatment ceased and animals were monitored for changes in serum PSA or tumour volume. After six weeks, antisense ODN were administered to saline-treated animals in order to look at the effect of antisense treatment on established, androgen-independent tumours. At ten weeks, the mice were sacrificed and the tumours were removed and flash frozen at -180°C. The tumours were used for in vivo confirmation of STAT-3 downregulation by Western blotting.

As previously described for this xenograft model, the serum PSA level of all animals decreased during the first week following castration (Figure 4.2). For animals treated with mismatch ODN and those treated with saline, serum PSA levels returned to pre-castration levels by the third week following castration. Serum PSA levels continued to
Figure 4.2 Downregulation of STAT-3 by Antisense ODN Suppresses PSA Production in LNCaP Xenograft Tumours in Male Nude Mice

Antisense (ISIS 113176), mismatch (ISIS 129987) and saline treatment were administered by intraperitoneal injection at a dose of 10 mg/kg body weight. Treatment was administered for 4 weeks post-castration. At 6 weeks post-castration, antisense treatment was administered to previously saline-treated animals. Results are expressed as an average of all animals measured and error bars represent standard error. Serum PSA levels for antisense-treated animals were significantly different from either mismatch-treated animals or saline-treated animals from week 3 onward. Significant differences were determined by Student's t-test, (p<0.05). Refer to Table 4.1 for associated data.
### Table 4.1 Associated Data Table and Student’s t-Test for Suppression of PSA Production in LNCaP Xenograft Tumours in Male Nude Mice (Part 1 of 3)

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Table 4.1 Associated Data Table and Student’s $t$-Test for Suppression of PSA Production in LNCaP Xenograft Tumours in Male Nude Mice (Part 3 of 3)

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p≤0.05 in bold lettering

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a: The procedure for this *in vivo* experiment and for serum PSA measurement are described in Figure 4.2 and in the text. Serum PSA is reported as ng/ml. A Student’s $t$-test was used to test for significant differences, where p≤0.05.
b: Tumours were treated with 10 mg/kg antisense ODN
c: Tumours were treated with 10 mg/kg mismatch ODN
d: Tumours were treated with saline
rise in mismatch ODN- and saline-treated animals until they were double the pre-castrate levels at six weeks. Animals treated with antisense ODN maintained suppression of serum PSA levels throughout the experiment. At six weeks post-castration, the serum PSA levels of antisense ODN-treated animals had not yet returned to pre-castration levels.

Antisense ODN treatment effectively slowed the rate of tumour growth whereas mismatch-treated and saline-treated animals did not exhibit a deflection in tumour growth rate (Figure 4.3). In the first week of treatment, tumour growth rate was indistinguishable between treatment groups. After the second week of treatment, the antisense-treated animals exhibited a suppressed growth rate and did not achieve tumour doubling within the 4 week treatment period. Mismatch-treated and saline-treated animals maintained a more rapid tumour growth rate such that tumour volume doubled every 2.5 weeks. Treatment of LNCaP xenograft tumours with antisense ODN suppressed both serum PSA levels and tumour volume. The suppression effects lasted for several weeks after treatment was terminated. The steady increase in serum PSA levels and tumour volume in mismatch ODN and saline-treated animals represents a progression to androgen independence. Early treatment of tumours with antisense ODN, downregulated STAT-3 and, thereby, effectively blocked progression to an androgen-independent phenotype.

To investigate the effect of antisense ODN treatment on androgen-independent tumours, at six weeks post-castration, antisense ODN treatment was administered to animals previously treated with saline. Serum PSA levels were suppressed in these animals but
Figure 4.3 Downregulation of STAT-3 by Antisense ODN Suppresses Tumour Growth Rate in LNCaP Xenograft Tumours in Male Nude Mice

Antisense (ISIS 113176), mismatch (ISIS 129987) and saline treatment were administered by intraperitoneal injection at a dose of 10 mg/kg body weight. Treatment was administered for 4 weeks post-castration. At 6 weeks post-castration, antisense treatment was administered to previously saline-treated animals. Results are expressed as an average of all animals measured and error bars represent standard error. Tumour volume for antisense-treated animals was significantly different from either mismatch-treated animals or saline-treated animals from week 3 onward. Significant differences were determined by Student’s t-test, (p<0.05). Refer to Table 4.2 for associated data.
Table 4.2 Associated Data Table and Student’s *t*-Test for Suppression of Tumour Growth Rate in LNCaP Xenograft Tumours in Male Nude Mice\(^a\) (Part 1 of 3)

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Table 4.2 Associated Data Table and Student’s t-Test for Suppression of Tumour Growth Rate in LNCaP Xenograft Tumours in Male Nude Mice (Part 3 of 3)

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T-Test B:A: 0.2746 0.2037 0.0327 0.0380 0.0222 0.0128 0.0167 0.0482 0.1024 0.0536 0.0353
T-Test B:C: 0.2152 0.5444 0.1911 0.0926 0.0865 0.0456 0.0179 0.0287 0.0735 0.0124 0.0115
T-Test A:C: 0.7111 0.6520 0.7529 0.7406 0.9828 0.7622 0.8309 0.9695 0.6985 0.5208 0.6769
p≤0.05 in bold lettering

a: The procedure for this in vivo experiment, and for tumour volume measurement and calculation, are described in Figure 4.3 and in the text. Tumour volume was measured as mm³. A Student’s t-test was used to test for significant differences, where p≤0.05.
b: Tumours were treated with 10 mg/kg antisense ODN
c: Tumours were treated with 10 mg/kg mismatch ODN
d: Tumours were treated with saline
tumour growth rate remained unchanged. Although PSA, an androgen-mediated gene product, remains sensitive to STAT-3 activation, after the tumour has begun to grow in an androgen-independent manner, the tumour growth rate is no longer sensitive to STAT-3 downregulation. While there were no significant differences detected with the deflected serum PSA and tumour volume curves, our observations do represent an interesting trend in the ability of antisense ODN to decrease serum PSA levels in androgen-independent tumours. Our results demonstrate the importance of early antisense ODN treatment of tumours.

Significant differences between treatments were calculated by Student’s t-test, (p<0.05). Serum PSA levels in antisense ODN-treated animals were significantly different from levels in mismatch ODN-treated animals and in saline-treated animals by week 2 of treatment and remained significantly different throughout the 10 weeks. Tumour volume in antisense ODN-treated animals was significantly different from tumour volume in mismatch ODN-treated animals by week 2 of treatment but lost statistical significance at week 8 before regaining significance at week 10. Tumour volume in antisense ODN-treated animals was significantly different from tumour volume in saline-treated animals between weeks 5 and 10 with the exception of week 8 in which statistical significance was lost. The effects of mismatch ODN treatment and saline treatment were not significantly different for serum PSA levels or tumour volume.
4.3.2 Downregulation of STAT-3 Protein Expression by Antisense ODN Was Confirmed

In the light of encouraging results from the serum PSA levels and tumour volume of STAT-3 antisense ODN-treated animals, we assessed STAT-3 and STAT-1 expression and STAT-3 phosphorylation in the resulting tumours. After ten weeks, tumours were collected from each animal and three tumours from each group were selected for Western blotting. Tumours selected, exhibited the characteristic trend of each group. Results of the immunoblotting analysis demonstrated that STAT-3 was downregulated in the antisense-treated tumours even six weeks after treatment had been terminated, as represented in figure 4.4. It was unexpected that the antisense effect would last for so long after termination of treatment. STAT-1 signal also decreases in antisense ODN-treated tumours. Although downregulation of STAT-1 may be a result of potential cross-reactivity with STAT-3, since a valid loading control was not obtained during Western blotting of these tumours, apparent downregulation of STAT-1 may also have resulted from a decreased protein quantity in these lanes. In vivo findings indicate that STAT-3 antisense treatment can suppress serum PSA levels and tumour volume and biochemical analysis confirms that the outcomes of the in vivo experiment can be attributed to downregulation of STAT-3 protein expression.
Figure 4.4 Analysis of Collected Tumours Confirms That STAT-3 Was Downregulated by Antisense Treatment

Tumours were collected at ten weeks and whole cell lysates were extracted from each sample. 50 μg of each lysate was separated by 8% SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were immunoblotted for P-STAT-3-S727 (top panel), P-STAT-3 Y705 (second panel), STAT-3 (third panel) and STAT-1 (bottom panel).
4.3.3 The Biological Effect of STAT-3 Downregulation by Antisense ODN *In Vivo* Was Studied at Three Time-Points

Tumours from three time-points during ODN treatment of the mice needed to be assessed in order to effectively determine the nature of STAT-3 downregulation by antisense ODN *in vitro*. The second *in vivo* experiment was conducted over a three week period and was designed specifically to generate antisense ODN-treated tumours to assess STAT-3 expression during exposure to the ODN and saline treatments. The same experimental protocol was followed, as above, to establish LNCaP xenograft tumours in twenty-seven male, nude mice. At seven, fourteen and twenty-one days, three animals from each group were sacrificed for tumour harvest. Similar trends of serum PSA suppression (*Figure 4.5*) and tumour volume (*Figure 4.6*) suppression were exhibited by the tumours in the second *in vivo* experiment.

Antisense- and mismatch-treated animals exhibited similar trends for both serum PSA levels and tumour volume until week two. Following week two, a divergence occurred between the two treatments. Consistent with the first *in vivo* experiment, by week three, antisense-treated animals exhibited suppressed serum PSA and tumour volume, whereas mismatch-treated animals exhibited an increase in serum PSA and tumour volume, indicating that these tumours were progressing to an androgen-independent phenotype.

Serum PSA levels in antisense-treated animals decreased to approximately 50% of pre-castrate levels by week two and decreased further, to 25% of pre-castrate levels, by week
Figure 4.5 Downregulation of STAT-3 by Antisense ODN, at Three Time-Points, Suppresses PSA Production in LNCaP Xenograft Tumours in Male Nude Mice

Antisense (ISIS 113176), mismatch (ISIS 129987) and saline treatment were administered by intraperitoneal injection at a dose of 10 mg/kg body weight. Treatment was administered for 3 weeks post-castration. Results are expressed as an average of all animals measured and error bars represent standard error. Significant differences were determined by Student's t-test, (p<0.05).
Figure 4.6 Downregulation of STAT-3 by Antisense ODN, at Three Time-Points, Suppresses Tumour Growth Rate in LNCaP Xenograft Tumours in Male Nude Mice
Antisense (ISIS 113176), mismatch (ISIS 129987) and saline treatments were administered by intraperitoneal injection at a dose of 10 mg/kg body weight. Treatment was administered for 3 weeks post-castration. Results are expressed as an average of all animals measured and error bars represent standard error. Significant differences were determined by Student’s t-test, (p<0.05).
three. The same serum PSA decrease occurred in mismatch-treated animals over the first two weeks of treatment but by week three, serum PSA levels had increased to approximately 60% of pre-castrate levels. Saline-treated animals returned to pre-castrate levels of serum PSA by week two and continued to rise at week three.

Tumour volume increased in all animals during the first week of treatment. Antisense- and mismatch-treated animals exhibited a slower rate of tumour growth than saline-treated animals that became evident after the first week of treatment. At week two, antisense- and mismatch-treated animals exhibited an increase in tumour volume of approximately 140%, whereas saline-treated animals exhibited an increase in tumour volume of approximately 180%. At week three, antisense-treated animals exhibited a decrease in tumour volume that closely approximated pre-castrate levels. Mismatch- and saline-treated animals exhibited an increase in tumour volume of 160% and 220% respectively.

Significant differences between treatments were calculated by Student’s t-test, (p<0.05). Statistical significance was not achieved in this experiment in serum PSA analysis or tumour volume analysis, however, the primary goal of the experiment was to produce tumours for Western blot analysis.
4.3.4 Downregulation of STAT-3 Protein Expression by Antisense ODN at Three Time-Points Was Confirmed

Tumours harvested at one, two and three weeks following castration and treatment, were lysed and prepared for Western blotting. Results of the immunoblotting analysis appeared to confirm that STAT-3 was downregulated in the antisense ODN-treated tumours (Figure 4.7). Due to the background in all STAT-3 blots, analysis by densitometry proved to be extremely difficult. The overall trend in the Western blots appears to be that antisense ODN-treatment downregulates STAT-3 expression in LNCaP xenograft tumours. Again, *in vivo* results indicating that antisense treatment suppresses serum PSA levels and tumour volume can be attributed to downregulation of STAT-3 protein expression in the tumours.

4.3.5 Summary

Antisense ODN were used, *in vivo*, to effectively downregulate STAT-3 expression in LNCaP tumours. As a result of reduced STAT-3 expression, serum PSA levels and tumour volume were suppressed and the suppression was sustained for a short period of time after termination of antisense treatment. Western blotting suggests that the antisense effect may have continued for up to six weeks after termination of antisense treatment.
Figure 4.7 Analysis of Collected Tumours Confirms That STAT-3 Was Downregulated by Antisense Treatment

Tumours were collected at ten weeks and whole cell lysates were extracted from each sample. Tumour lysates were pre-cleared of mouse IgG with protein A agarose beads (Invitrogen). 100 µg of each lysate was separated by 8% SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were immunoblotted for STAT-3 (top panel) and STAT-1 (bottom panel).
Downregulation of STAT-3 by antisense ODN may be a potential therapeutic strategy in prostate cancer treatment.

4.4 Downregulation of STAT-3 Expression *In Vitro*

In an effort to elucidate the role of STAT-3 in IL-6 and AR cross-talk in LNCaP cells, we attempted to co-transfect luciferase reporter constructs and antisense ODN. Antisense ODN has been successfully transfected into cells to downregulate STAT-3 expression by Miyake et al. (Miyake et al., 2000; Miyake et al., 1999). Our hypothesis was that downregulation of STAT-3 by antisense ODN treatment would prevent STAT-3 transactivation of ARR3-luc and AR transactivation of SIE-luc. Downregulation of STAT-3 expression would also clarify the importance of STAT-3 to IL-6 and EGF-mediated potentiation of R-1881-induced ARR3-luc signalling. If STAT-3 was not necessary, perhaps the MAPK pathway plays a key role in IL-6 and EGF-mediated augmentation of AR-mediated gene transcription.

4.4.1 Experimental Conditions Were Optimized for Transfection of LNCaP Cells with SIE-luc Using Oligofectamine Reagent

The Oligofectamine transfection reagent (Invitrogen, Burlington, ON) was recommended for optimal transfection of ODN (personal communication from Miyake et al.) rather than Lipofectamine 2000 or Lipofectin transfection reagents, although all three regents can be
used to transfect ODN. Our previous experiments with luciferase constructs used Lipofectamine 2000 for transfection procedures. To ensure that luciferase constructs could be effectively transfected, the SIE-luc construct was introduced into LNCaP cells using Oligofectamine and was stimulated with IL-6. SIE-luc luciferase activity was activated successfully after transfection with Oligofectamine, as represented in figure 4.8.

In addition to verifying intact function of the luciferase construct after transfection with Oligofectamine, it was important to investigate how different volumes of Oligofectamine in the transfection reaction affected luciferase function. The transfection was performed using 1.5 µl and 3.0 µl of Oligofectamine. Transfecting SIE-luc with 1.5 µl or 3.0 µl of Oligofectamine, in the absence of IL-6 stimulation, did not increase SIE-luc signalling above baseline levels (Figure 4.9). Addition of IL-6 stimulation to cells transfected with SIE-luc using 1.5 µl of Oligofectamine increased SIE-luc signalling by 13-fold. Addition of IL-6 stimulation to cells transfected with SIE-luc using 3.0 µl of Oligofectamine increased SIE-luc signalling further by 2.9-fold. A volume of 3.0 µl of Oligofectamine achieved maximal luciferase signalling by SIE-luc.
Figure 4.8 Luciferase Activity Can Still Be Activated Following Transfection with Oligofectamine Reagent

LNCaP cells were transfected with 1 μg SIE-luc and 0.25 μg pRL-TK, using 1.5 μl Oligofectamine. Cells were stimulated with 2 nM IL-6 for 18 hours and results were determined by luciferase assay. Results are expressed as an average of two replicates and error bars represent standard deviation.
Figure 4.9 Luciferase Function Is Optimal When 3.0µl of Oligofectamine Is Used for the Transfection

LNCaP cells were transfected with 1 µg SIE-luc and 0.25 µg pRL-TK using 1.5 µl or 3.0 µl of Oligofectamine. Cells were stimulated with 2 nM IL-6 for 18 hours and results were determined by luciferase assay. Results are expressed as an average of two replicates and error bars represent standard deviation.
4.4.2 Length of Antisense ODN-Mediated Downregulation of STAT-3 Expression

The length of the antisense effect on STAT-3 expression would determine how the luciferase component of the experiments would be included in the protocol. We studied the ability of antisense ODN to downregulate STAT-3 protein after one, two, three and four days. In the same experiment, we also looked at whether one or two antisense transfections most effectively maintained STAT-3 downregulation. Antisense ODN transfection downregulated STAT-3 expression, slightly, after one transfection and one day of incubation, as represented in figure 4.10. The degree to which STAT-3 was downregulated, after one transfection with antisense ODN, increased with each additional day of incubation before lysis. Two antisense ODN transfections substantially increased the degree to which STAT-3 expression was downregulated. The degree to which STAT-3 expression was downregulated after two transfections with antisense ODN also increased with each additional day of incubation before lysis. STAT-1 levels remained relatively consistent in both antisense and mismatch ODN-transfected cells and variations in STAT-1 levels can be attributed to differences in protein content of the lanes. Cells appeared healthiest when transfected on two subsequent days and lysed on day five. Cells lifted from the plates when incubated for longer time periods and the antisense effect was not as apparent when cells were incubated for shorter time periods. Mismatch ODN transfection of cells downregulated STAT-3 expression after four subsequent days of incubation in cells transfected once, or twice with ODN. Downregulation of STAT-3 by mismatch ODN is likely due to toxicity of ODN transfection. The MAPK control blot for
Figure 4.10 Number of Antisense Transfections and Length of Antisense Effect

LNCaP cells were transfected with 500 nM antisense ODN or 500 nM mismatch ODN either once or on two subsequent days. Whole cell lysates were extracted after one, two, three and four days. 50 μg of each lysate was separated by 8% SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were immunoblotted for STAT-3 (top panel), STAT-1 (second panel), MAPK (bottom panel).
these lanes contained consistent quantities of protein, again reflecting a possible toxic effect of ODN transfection after four subsequent days of incubation.

Once a transfection timeframe had been established for the antisense and mismatch ODN, the time at which the luciferase construct would be transfected into the cells was determined. The luciferase construct could either be added to the cells with the second ODN transfection or in a separate transfection the next day. When the luciferase construct was added to the cells with the second transfection reaction, no luciferase activity could be elicited from the cells, regardless of antisense or mismatch ODN treatment (Figure 11a). When the luciferase plasmid was transfected separately, on the following day, luciferase activity was present and the contrast between antisense and mismatch ODN effects was most evident at the 500 nM concentration of ODN rather than the 100 nM concentration. Western blots of whole cell lysates from the luciferase assay shown in figure 11a, however, indicated that STAT-3 downregulation was achieved most effectively by the 100 nM concentration of ODN (figure 11b). The inconsistency between the luciferase assay data and the Western blot data is an example of the problems that occurred when we attempted to co-transfect luciferase constructs and ODN.
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**Transfection**:
- next day
- same day
- none

**Dose of ODN**:
- 500nM
- 100nM

**Mismatch**

**Antisense**
Figure 4.11 The Luciferase Reporter Plasmid Is Most Effective When Transfected Separately On the Third Day
LNCaP cells were transfected with 500 nM antisense ODN or 500 nM mismatch ODN on day 1 and again, on day 2. The 1 μg SIE-luc and 0.25 μg pRL-TK was transfected either on day 2 with the antisense ODN or separately on day 3.

(a) Cells were stimulated with 2 nM for 18 hours and lysed on day 4. Results were determined by luciferase assay.

(b) Whole cell lysates were extracted on day 4. 50 μg of each lysate was separated by 8% SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were immunoblotted for STAT-3 (top panel) and STAT-1 (bottom panel).
4.2.3 Antisense and Mismatch ODN Cannot Be Reliably Transfected with Luciferase Reporter Plasmids to Produce Consistent Results

Although initial experiments to combine ODN and luciferase reporter constructs were inconsistent, we attempted to co-transfect 500 nM ODN with SIE-luc to downregulate STAT-3 activity in the presence IL-6, R-1881 and EGF stimulation. We transfected cells with antisense or mismatch ODN and SIE-luc, then stimulated cells for 18 hours with 2 nM IL-6, 1x10^{-10} M R-1881 and 100 ng/μl EGF for luciferase assays and Western blots. No attempts at this experiment produced consistent STAT-3 downregulation results that could be seen in both the luciferase assay and the Western blots. An example replicate is shown in which antisense ODN-mediated downregulation of luciferase activity was detected (Figure 4.12a), but downregulation of STAT-3 was not seen in the Western blots (Figure 4.12b). Antisense ODN treatment appeared eliminated cross-talk between the AR pathway and the IL-6 pathway in the luciferase assay. Western blots indicated, however, that no significant downregulation of STAT-3 had occurred in antisense ODN-treated cells. Meetings with ISIS Pharmaceuticals confirmed that co-transfection of antisense and mismatch ODN with luciferase reporter plasmids does not produce reliable results.

4.2.4 Summary

Initial in vitro experiments demonstrated that antisense ODN could be used to reduce activation of SIE-luc in LNCaP cells by downregulating STAT-3 expression.
Figure 4.12 Luciferase Results Demonstrate Downregulated SIE-luc Activation But Western Blots Do Not Exhibit STAT-3 Downregulation by Antisense ODN

LNCaP cells were transfected with 500 nM antisense ODN or 500 nM mismatch ODN on day 1 and day 2 and with 1 μg SIE-luc and 0.25 μg pRL-TK on day 3. Cells were stimulated with 2 nM IL-6, 1×10⁻⁸ M R-1881 and 100 ng/μl EGF for 18 hours.

(a) Cells were lysed on day 4 and results were determined by luciferase assay.

(b) Whole cell lysates were extracted on day 4 and 50 μg of each lysate was separated by 8% SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were immunoblotted for P-STAT-3 S727 (top panel), P-STAT-3 Y705 (second panel), STAT-3 (third panel), STAT-1 (bottom panel).
Although one replicate did confirm our initial findings, Western blotting indicated that antisense treatment had not affected STAT-3 expression. ISIS Pharmaceuticals confirmed that co-transfection of antisense and mismatch ODN with luciferase constructs did not produce reliable results. To address the issue of downregulation of STAT-3 in LNCaP cells, we used shRNA to reduce STAT-3 expression. Results of our experiments using shRNA to downregulate STAT-3 in vitro will be discussed in Chapter 5.
Chapter 5. Downregulation of STAT-3 Expression by shRNA

5.1 Introduction

Our attempts to perform transcriptional reporter assays in the presence of antisense ODN were inconsistent. As an alternative, shRNA targeted to STAT-3 was used in vitro to downregulate STAT-3 expression, in order to examine the importance of STAT-3 to IL-6 and AR pathway cross-talk.

Ueda et al. demonstrated that co-stimulation of ARR-tk-luc with IL-6 and R-1881 amplifies the signal generated by stimulation with R-1881 alone (Ueda et al., 2002). However, the luciferase construct used, in the same paper, to demonstrate that STAT-3 activates the N-terminal of the AR does not reflect physiological conditions. The N-terminal AR construct, AR-(1-558)-Gal4DBD R-1881, containing the N-terminal of the AR receptor and a non-androgen-specific Gal4 DNA binding domain, was used in conjunction with a 5xGal4UAS-TATA-luc construct for signalling. R-1881 stimulation did not elicit luciferase activity from the N-terminal AR construct. IL-6 successfully activated the N-terminal AR construct but IL-6 and R-1881 stimulation did not augment the signal generated by IL-6 stimulation alone. The same construct was used to demonstrate that STAT-3 downregulation prevented activation of the AR N-terminal domain by IL-6. In the downregulation experiment by Ueda et al., dominant negative mutants of STAT-3 were over-expressed in the cells. Dominant negative constructs do not have functional activity but do bind the IL-6 receptor and prevent other potential
factors from binding. Our experiments prevent expression of STAT-3 leaving the receptor free to bind other molecules. In addition, our use of the ARR3-luc reporter in conjunction with shRNA transfection address specific binding of the AR to the ARR and represents directly induced AR-mediated gene transcription activity.

5.2 Optimizing Experimental Conditions for shRNA-Mediated Downregulation of STAT-3 Expression in LNCaP Cells

5.2.1 Preparation of shRNA plasmid

The sequences within the STAT-3 mRNA that were selected for ligation into the pSHAG vector are listed in Appendix A.

The pSHAG vector was provided by Dr. Alice Mui (University of British Columbia, Vancouver, BC). It contains a U6 promoter region, followed by BseRI and BamHI restriction sites for oligo insertion and a kanamycin resistance region (Figure 5.1). The 4 kb plasmid was prepared for ligation by cutting with BseRI and BamHI. The ligation procedure is described in Chapter 2. The shRNA inserts were designated 31, 32, 33, 34, 35 and 36 representing shRNA#1 through shRNA#6. Each ligation reaction was transformed into DH5α cells and the colonies were grown in LB broth with Kanamycin for selection. Plasmids were prepared by QIAprep Miniprep Kit (Qiagen, Mississauga, ON). Minipreps were cut with HindIII in order to ascertain whether the insert was
Figure 5.1 pSHAG Vector
The pSHAG vector is a 4 kb vector and is prepared for ligation by cutting with BseRI and BamHI. shRNA inserts were ligated into the pSHAG vector to create a plasmid for transfection.
contained in the plasmid. HindIII cuts at a restriction with within the insert and will linearize the plasmid. Two colonies from ligation 33 (33a and 33c) and one colony from ligation 36 contained the shRNA insert (Figure 5.2).

5.2.2 shRNA to Downregulates STAT-3 and SIE-luc Activity in LNCaP Cells

The three plasmids from our diagnostic gel that contained the shRNA insert, 33a, 33c and 36, were co-transfected with GFP into LNCaP cells. After 48 hours and 72 hours, the cells were photographed on the microscope under phase contrast and fluorescence to ensure that transfection had occurred (approximately 20% efficiency). Western blotting of whole cell lysates suggests that cells transfected with plasmids 33c and 36 exhibited a greater degree of STAT-3 downregulation than cells transfected with 33a (Figure 5.3). The ability of the shRNA plasmids to downregulate SIE-luc activation will also contribute to plasmid choice. The effects of shRNA transfection were more pronounced at 72 hours than at 48 hours.

Although all three shRNA clones downregulated STAT-3 expression in LNCaP cells, it was also necessary to test the ability of each clone to downregulate SIE-luc activation. SIE-luc, shRNA and GFP were co-transfected into LNCaP cells and at 48 hours post-transfection, cells were stimulated with 2 nM IL-6 for 18 hours. Following stimulation, cells were photographed on the microscope under phase contrast and fluorescence to ensure transfection had occurred (approximately 30% efficiency in unstimulated cells and
Figure 5.2 HindIII Digest of All Colonies Grown From shRNA Insert and pSHAG Vector Ligations

Six ligations were performed, each consisting of the pSHAG vector and one of six selected shRNA oligo pairs. Each ligation was grown on an LB plate containing Kanamycin. From ligation 31, 2 colonies grew, from ligation 32, 2 colonies grew, from ligation 33, 3 colonies grew, from ligation 34, 1 colony grew, from ligation 35, 3 colonies grew and from ligation 36, 1 colony grew. LB broth cultures containing Kanamycin were inoculated with the colonies and the plasmids were extracted and purified. Each ligation was digested with HindIII as a diagnostic cut to ensure the plasmid contained the shRNA insert. Digests were separated on a 1% agarose gel. A HindIII cut of a successful ligation will result in a linearized plasmid, while unsuccessful ligations will exhibit multiple bands when cut. Colonies 33a, 33c and 36 produced plasmids that contained the shRNA insert.
Figure 5.3 shRNA Downregulates STAT-3 Expression

Whole cell lysates were extracted from LNCaP cells transfected with 1 μg shRNA and 1 μg GFP for 48 hours or 72 hours. 50 μg of each lysate was separated by 8% SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were immunoblotted for STAT-3 (top panel) and MAPK (bottom panel).
approximately 40% efficiency in IL-6-stimulated cells) before cells were lysed and prepared for luciferase assay and for Western blotting.

In the absence of shRNA transfection, the addition of IL-6 to SIE-luc increased signalling 2500-fold above signalling in the absence of IL-6 stimulation (Figure 5.4). Ligations 33a, 33c and 36 all downregulated SIE-luc activity when compared to IL-6 stimulation of SIE-luc in the absence of shRNA. Ligation 36 exhibited the least downregulation of SIE-luc activity. Ligation 33a and 33c both exhibited more effective downregulation of SIE-luc activity in the presence and absence of IL-6 signalling than 36. Although transfection with the 33a ligation downregulated SIE-luc activity in the presence and absence of IL-6 to the greatest degree, transfection efficiency of 33a was 2.3-fold lower than 33c. Ligation 33c exhibited less downregulation of SIE-luc in the presence of IL-6 stimulation but the 2-fold increase in signalling caused by IL-6 only achieved a magnitude of 361 relative luciferase units. IL-6 stimulation of SIE-luc in the absence of shRNA activates signalling to a magnitude of 2500 relative luciferase units and, therefore, the relatively small increase in SIE-luc signalling permitted by ligation 33c in the presence of IL-6, will have minimal effects on our comparison of IL-6, R-1881 and EGF-stimulated signalling in the presence and absence of shRNA. 33c was selected for use in luciferase assays with both the SIE-luc and ARR3-luc constructs.
Figure 5.4 shRNA 33c Downregulates SIE-luc Activity More Effectively than 33a or 36

LNCaP cells were transfected with 1 µg shRNA, 1 µg SIE-luc and 0.25 µg pRL-TK. Cells were stimulated with 2 nM IL-6 for 18 hours and results were determined by luciferase assay.
5.3 Downregulation of STAT-3 by shRNA Suppresses IL-6-Induced SIE-luc Activity

In order to assess the importance of STAT-3 in EGF and R-1881 augmentation of IL-6-induced SIE-luc signalling, LNCaP cells were co-transfected with shRNA to downregulated STAT-3 expression and SIE-luc to monitor STAT-3-mediated gene activation. Following transfection with shRNA, SIE-luc and pRL-TK, LNCaP cells were stimulated with IL-6, R-1881 and EGF and incubated for 18 hours. GFP was co-transfected with shRNA in the first replicate of the experiment to identify transfection efficiency by microscopy. Transfection efficiency increased from 30% in wells stimulated with no reagent, EGF, R-1881 or EGF/R-1881 to 50% in wells that were stimulated with IL-6 alone or in combination with R-1881 or EGF. IL-6 may increase general transcriptional regulation of GFP via the CMV promoter. In addition, the anti-apoptotic effects of IL-6 may be contributing to overall cell health and therefore, improved transcription.

The addition of shRNA substantially downregulated SIE-luc signalling in LNCaP cells and reduced augmentation of IL-6-mediated SIE-luc activation by R-1881 or EGF (Figure 5.5a). SIE-luc activity was expected to reduce with the addition of shRNA since STAT-3 is the key mediator of SIE-luc activity. R-1881 and EGF together still augmented the shRNA-downregulated IL-6-induced SIE-luc signal by 3-fold. The inability of R-1881 to augment signalling in the presence of shRNA indicates that STAT-
Figure 5.5 shRNA Suppresses IL-6-Induced SIE-luc Signalling

LNCaP cells were transfected with 1 μg shRNA, 1 μg SIE-luc and 0.25 μg pRL-TK. Cells were stimulated with 2 nM IL-6, 1x10^-10 M R-1881 and 100 ng/μl EGF for 18 hours.

(a) Results are expressed as an average of six replicates from three separate experiments and error bars represent standard error. Student’s t-test was used to establish significant differences between treatments, specific comparisons are detailed in the text, (p<0.05).

(b) Whole cell lysates were extracted and 50 μg of each lysate was separated by 8% SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were immunoblotted for P-STAT-3 S727 (top panel), P-STAT-3 Y705 (second panel), STAT-3 (third panel) and MAPK (bottom panel).
3 is necessary for any cross-talk between the IL-6 and AR pathways and supports the proposed model that STAT-3 and the AR do have a direct interaction in the cell.

Significant differences between treatments were calculated by Student's t-test, (p<0.05). The addition of shRNA to the cells only significantly decreased SIE-luc signalling in cells stimulated with EGF alone or with IL-6 alone. Significant differences within samples that contained shRNA or within samples that did not contain shRNA occurred in a different pattern from those calculated for figure 3.5, however, the trend of relative activation of SIE-luc by each treatment is similar to the pattern of activation established in figure 3.5.

Within the samples that did not contain the shRNA, IL-6 but not EGF or R-1881 alone significantly activated SIE-luc above the signal from unstimulated cells. EGF-mediated activation of SIE-luc was significantly increased when cells were co-stimulated with R-1881 or with IL-6 and R-1881. R-1881-mediated activation of SIE-luc was significantly increased when cells were co-stimulated with EGF or IL-6. IL-6-mediated SIE-luc activation was not significantly increased by co-stimulation with EGF or R-1881 in the absence of shRNA transfection.

Within the samples that did contain shRNA, again only IL-6 alone but not EGF or R-1881 alone significantly activated SIE-luc above the signal from unstimulated cells. EGF-mediated stimulation of SIE-luc was significantly increased by co-stimulation with R-1881, IL-6 or both R-1881 and IL-6 together. R-1881-mediated activation of SIE-luc
was significantly increased by co-stimulation with IL-6 or IL-6 and EGF together. IL-6-mediated activation of SIE-luc was significantly increased by co-stimulation with EGF and R-1881 together. In addition, IL-6 and EGF-mediated SIE-luc activation was increased by the addition of R-1881.

Western blots confirmed that shRNA downregulated STAT-3 expression (Figure 5.5b). Cells transfected with shRNA expressed significantly lower levels of STAT-3 than cells without shRNA. Although the total quantity of STAT-3 protein in the cell decreased in the presence of shRNA, co-stimulation with EGF and IL-6 appears to stabilize the IL-6-mediated Y705 and S727 phosphorylation of STAT-3 and prevents a decrease in phosphorylated STAT-3 in the presence of shRNA. This observation is consistent with both sets of Western blot lysates in Figure 5.5b and Figure 5.6b. Each figure represents a separate set of whole cell lysates. The potential stabilization of phosphorylated STAT-3 would account for the elevated ARR3-luc and SIE-luc signalling in the presence of IL-6 and EGF co-stimulation and shRNA transfection.

5.4 Downregulation of STAT-3 by shRNA Did Not Affect IL-6 and EGF-Mediated Augmentation of R-1881-Induced ARR3-luc Activity

Downregulation of STAT-3 by shRNA reduced SIE-luc signalling and prevented augmentation of IL-6 induced SIE-luc signalling by R-1881 or EGF. In order to test if loss of STAT-3 expression influenced the ability of IL-6 or EGF to augment R-1881-
mediated ARR3-luc activation, we transfected LNCaP cells with ARR3-luc and shRNA to look at the effect of reduced STAT-3 expression on ARR3-luc signalling.

Contrary to our hypothesis, downregulation of STAT-3 did not affect the ability of EGF and IL-6 to augment R-1881-mediated ARR3-luc signalling (Figure 5.5a). Stimulation of ARR3-luc with R-1881 increased signalling to 22700-fold above the signal from unstimulated cells. Cells transfected with shRNA and stimulated with R-1881 exhibited a further increase in ARR3-luc signal of 4-fold but the increase was not statistically significant. Co-stimulation of cells with EGF and R-1881 in the presence and absence of shRNA transfection had similar effects. Stimulation with EGF and R-1881 augmented R-1881-induced ARR3-luc signalling by 3.5-fold. In the presence of shRNA transfection, co-stimulation with EGF and R-1881 further increased R-1881-induced ARR3-luc signalling by 2-fold, but again the increase was not statistically significant. As shown in Chapter 3, co-stimulation of cells with IL-6 and R-1881 augments R-1881-induced ARR3-luc signalling, in this case by 7-fold. Co-stimulation with IL-6, R-1881 and EGF also augments R-1881-induced ARR3-luc signalling, in this case by 20-fold. Surprisingly, transfection of shRNA did not significantly decrease the ability of IL-6 alone and IL-6 and EGF together to augment R-1881-induced ARR3-luc signalling. There was no significant difference, between augmentation of ARR3-luc signalling by growth factors, in the presence or absence of shRNA. Downregulation of shRNA did not affect the ability of R-1881 to activate ARR3-luc.
Figure 5.6 shRNA Does Not Affect IL-6 and EGF Augmentation of R-1881-Induced ARR₃-luc Activity
LNCaP cells were transfected with 1 μg shRNA, 1 μg ARR₃-luc and 0.25 μg pRL-TK. Cells were stimulated with 2 nM IL-6, 1x10⁻¹⁰ M R-1881 and 100 ng/μl EGF for 18 hours.

(a) Results are expressed as an average of six replicates from three separate experiments and error bars represent standard error. Student’s $t$-test was used to establish significant differences between treatments, specific comparisons are detailed in the text, (p<0.05).

(b) Whole cell lysates were extracted and 50 μg of each lysate was separated by 8% SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were immunoblotted for P-STAT-3 S727 (top panel), STAT-3 (second panel) and MAPK (bottom panel).
Significant differences between treatments were calculated by Student’s $t$-test, ($p<0.05$). The addition of shRNA to the cells did not significantly decreased ARR$_3$-luc signalling. Significant differences within samples that contained shRNA or within samples that did not contain shRNA occurred in a different pattern from those calculated for figure 3.3, however, the trend of relative activation of ARR$_3$-luc by each treatment is similar to the pattern of activation established in figure 3.3.

Within the samples that did not contain the shRNA, R-1881 alone but not IL-6 or EGF alone significantly activated ARR$_3$-luc above the signal from unstimulated cells. R-1881-mediated activation of ARR$_3$-luc was significantly increased when cells were co-stimulated with IL-6.

Within the samples that did contain shRNA, again only R-1881 alone but not IL-6 or EGF alone significantly activated ARR$_3$-luc above the signal from unstimulated cells. R-1881-mediated activation of ARR$_3$-luc was not significantly increased by co-stimulation with IL-6 or EGF.

Since IL-6 and EGF were able to augment ARR$_3$-luc signalling in the absence of STAT-3, it is possible that other activated signalling pathways are responsible for transactivating the AR. Although STAT-3 and the AR may have a direct interaction, STAT-3 may not be responsible for augmenting AR-mediated gene transcription, despite its proximity to the AR.
5.5 Summary

Transfection of shRNA into LNCaP cells effectively downregulated STAT-3 expression. Downregulation of STAT-3 reduced SIE-luc signalling in response to IL-6 and EGF. Consequently, downregulation of STAT-3 reduced the ability of EGF and R-1881 to augment IL-6-induced SIE-luc signalling. Downregulation of STAT-3 had no significant effect on the ability of growth factors to amplify the R-1881-induced ARR3-luc signal. Although STAT-3 clearly mediates cross-talk between the IL-6, EGF and the AR pathways with regard to SIE-luc signalling, another protein, such as MAPK, may mediate pathway cross-talk with regard ARR3-luc signalling.
Chapter 6. Phosphorylation of Serine Residue 727 on STAT-3

6.1 Introduction

STAT-3 requires phosphorylation on Y705 in order to achieve dimerization, nuclear translocation, DNA binding and transcriptional activation. Further phosphorylation on S727 augments the transcriptional activity of STAT-3. JAK1/2 and TYK2 phosphorylate STAT-3 at Y705. The mechanism(s) by which S727 phosphorylation takes place has yet to be fully elucidated.

There is evidence in the literature to suggest that MAPK phosphorylates STAT-3 at S727 (Decker et al., 2000; Lim et al., 2001; Turkson et al., 2000). Deeble et al. suggest, however, that while MAPK may play a role in basal S727 phosphorylation of STAT-3, it does not function in IL-6-induced phosphorylation (Deeble et al., 2001). Mammalian target of rapamycin (mTOR), or downstream elements of mTOR, may play a role in S727 phosphorylation of STAT-3 (Yokogami et al., 2000). A kinase that is sensitive to the inhibitor H7 has also been investigated as the molecule that functions in IL-6-induced S727 phosphorylation of STAT-3 (Abe et al., 2001). H7 inhibits the activity of protein kinase C (Hidaka et al., 1984).

We were interested in the mechanism by which STAT-3 is phosphorylated on S727, because of our observations regarding the ability of EGF to phosphorylate S727, without apparent prior Y705 phosphorylation (figure 3.4).
6.2 S727 Phosphorylation of STAT-3 Is Mediated by A Combination of Kinases

In order to clarify the roles of MAPK and mTOR in S727 phosphorylation of STAT-3, LNCaP cells were starved for 24 hours in CSS. Cells were treated with 50 μM PD098059, a MAPK inhibitor, or with 5 ng/ml rapamycin, an mTOR inhibitor, for 20 minutes. At the same time, cells were stimulated with 2 nM IL-6 and 100 ng/μl EGF for 20 minutes. Cells were lysed for Western blotting.

A basal level of S727 phosphorylation was visible in the absence of IL-6 or EGF treatment (Figure 6.1). The level of basal phosphorylation was not significantly decreased by treatment with PD098059, rapamycin, or both inhibitors together. IL-6-induced S727 phosphorylation was decreased by the addition of PD098059 and by rapamycin. Rapamycin reduced S727 phosphorylation slightly more than PD098059. The addition of both inhibitors together did not prevent S727 phosphorylation of STAT-3, instead, a level of S727 phosphorylation remained that was above baseline levels. It appears that another kinase is also involved in IL-6-induced S727 phosphorylation, in addition to MAPK and mTOR. EGF-induced S727 phosphorylation was not decreased by the addition of rapamycin. PD98059 downregulated EGF-induced S727 phosphorylation and had a greater effect on EGF-induced S727 phosphorylation than on IL-6-induced S727 phosphorylation.
Figure 6.1 Phosphorylation of STAT-3 on S727 Is Caused by A Combination of Kinases

Whole cell lysates were extracted from LNCaP cells, stimulated with 50 μM PD098059 or 50 ng/ml and 2 nM IL-6 or 100 ng/μl EGF for 20 minutes. 50 μg of each lysate was separated by 8% SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were immunoblotted for P-STAT-3 S727 (top panel), P-STAT-3 Y705 (second panel), total STAT-3 (third panel), phospho-MAPK (fourth panel), total MAPK (fifth panel), phospho-p70 S6K (sixth panel) and total p70 S6K (bottom panel). The figure is representative of two replicates.
To assess mTOR downregulation by rapamycin, we measured p70 S6 kinase activation. Activation of p70 S6 kinase occurs downstream of mTOR. There was no apparent increase or decrease in phospho-p70 S6 kinase levels in the presence or absence of rapamycin. Experiments by Gina Rossi indicate that the phospho-p70 S6 kinase antibody does not detect a phospho-protein but rather the total protein.

Downregulation of IL-6-induced and EGF-induced phosphorylation of MAPK occurred in the presence of PD098059. The addition of rapamycin to IL-6-stimulated cells caused MAPK to decrease. This surprising result is likely due to rapamycin-mediated suppression of an upstream element in the MAPK activation pathway of IL-6-stimulated LNCaP cells, rather than a direct downregulation of MAPK activation by rapamycin.

Western blot results indicate that MAPK may have a role in IL-6-induced S727 phosphorylation of STAT-3. In addition to MAPK, mTOR also has a role in S727 phosphorylation and perhaps is more important for S727 phosphorylation of STAT-3. MAPK phosphorylation plays a role in EGF-induced S727 phosphorylation of STAT-3. Y705 phosphorylation of STAT-3 in EGF-stimulated cells is visible at a level that is barely above baseline. It is not possible to discern from the blots, in either figure 3.4 or in figure 6.1, whether the Y705 phosphorylation seen in EGF-stimulated cells is a low level of basal phosphorylation or a background artefact of the blots themselves.
6.3 Summary

Although MAPK and mTOR both have a role in S727 phosphorylation of STAT-3, mTOR may have a greater role in IL-6-induced S727 phosphorylation than MAPK. With regard to EGF-induced S727 phosphorylation, MAPK appears to have a greater role than mTOR. Inhibition of both kinases did not, however, completely inhibit S727 phosphorylation in cells stimulated with either IL-6 or EGF, indicating that other kinases may have a role in S727 phosphorylation. An H7-sensitive kinase, for example protein kinase C, may also have a role in S727 phosphorylation of STAT-3.
Chapter 7. Conclusions and Future Directions

STAT-3 and AR have a direct interaction in LNCaP cells stimulated with IL-6 and androgen (Matsuda et al., 2001; Ueda et al., 2002). Evidence in the literature suggests that this direct interaction mediates androgen-stimulated AR transactivation of IL-6-stimulated, STAT-3-mediated gene transcription (Chen et al., 2000; Culig, Bartsch et al., 2002; Hobisch et al., 1998; Matsuda et al., 2001). There is also evidence to suggest that IL-6-stimulated STAT-3 can transactivate androgen-stimulated, AR-mediated gene transcription (Culig, Bartsch et al., 2002; Matsuda et al., 2001; Ueda et al., 2002). We investigated the ability of STAT-3 and the AR to interact in LNCaP cells, a model of androgen-dependent prostate cancer.

In order to establish that signalling cross-talk occurs between the IL-6 and AR pathways, LNCaP cells were transfected with luciferase reporter construct, ARR3-luc and SIE-luc, to assess transcriptional activation. Cells were stimulated with IL-6, R-1881 and EGF alone and in combination, to investigate the ability of IL-6 to cause AR activation, and the ability of R-1881 to cause STAT-3 activation. Our results indicate that IL-6-stimulated SIE-luc signalling can be augmented by co-stimulation with EGF and R-1881. Conversely, R-1881-stimulated ARR3-luc signalling can be augmented by co-stimulation with IL-6 and EGF.

MAPK activation by IL-6, R-1881 and EGF was investigated as a potential mediator of apparent signalling cross-talk between the IL-6 and AR pathways. MAPK is activated by
IL-6 and EGF but not R-1881. As a result, we have inferred that augmentation of SIE-luc signalling by R-1881 appears to be mediated by STAT-3 and AR interaction. Augmentation of ARR3-luc signalling by IL-6 and EGF is more likely mediated primarily by MAPK activation of downstream elements. Our results concur with those of Ueda et al., who indicate that MAPK has a role in IL-6 activation of the AR and that the addition of a MAPK inhibitor decreases AR activation in cells stimulated with IL-6.

The role of STAT-3 as a potential key mediator of IL-6 and AR pathway cross-talk was further investigated by downregulating STAT-3 expression. Previous studies of the role of STAT-3 in AR signalling have used a dominant negative construct to prevent STAT-3 activation (Matsuda et al., 2001; Ueda et al., 2002). While the dominant negative constructs in question cannot be activated and, therefore, block STAT-3 signalling, they are still able to bind to the IL-6R. The ability of the dominant negative STAT-3 construct to bind to the IL-6R prevents potential activation of other factors that may be activated in the absence of bound STAT-3. Our strategy was to prevent expression of STAT-3 using targeted antisense ODN and shRNA, leaving the IL-6R available to activate other factors.

The ability of STAT-3 to affect AR-mediated gene transcription and tumour growth was investigated in vivo using antisense ODN to downregulate STAT-3. Male nude mice, with established LNCaP xenograft tumours, were injected with antisense ODN, mismatch ODN or saline treatment and the effect of the treatments on serum PSA levels and tumour volume was monitored weekly. Antisense treatment not only decreased serum PSA levels and tumour growth rate, but serum PSA and tumour growth remained suppressed for up
to six weeks after treatment had ceased. Tumours removed after 10 weeks exhibited
downregulated STAT-3 levels in Western blots.

Our results indicate that STAT-3 has an important role in AR-mediated gene expression
and in tumour growth. Suppression of STAT-3 expression resulted in a suppression of
serum PSA levels and tumour volume. In order to investigate a specific therapeutic role
for STAT-3 antisense ODN, we would like to combine antisense ODN treatment with
concurrent docetaxel treatment. A co-treatment strategy using BCL-2 antisense ODN and
paclitaxel has already been successfully carried out by Gleave et al. in castrated mice
with established Shionogi or LNCaP tumours (Gleave et al., 2002). Our hypothesis is that
co-treatment of tumours with antisense ODN and docetaxel will decrease serum PSA
levels and tumour growth rate to almost a baseline level. We have also recently become
aware of a potential control antibody for the tumour Western blots in order to make
analysis by densitometry more feasible. Previous attempts to find a control antibody have
been unsuccessful, however, we expect that anti-Grb2 may prove to be effective.

Our efforts to downregulate STAT-3 expression in vitro using antisense ODN were
inconsistent. STAT-3 downregulation by ISIS antisense ODN was studied in DU145 cells
by Mora et al (Mora et al., 2002). DU145 cells exhibit constitutive STAT-3 signalling
and are an androgen-independent cell line. Downregulating STAT-3 expression in these
cells induces growth inhibition and apoptosis. As the cell line is androgen-independent
and AR-negative, a greater emphasis is placed on the role of growth factor pathways to
carry out AR-mediated events. Downregulating STAT-3 eliminates one of the crucial
pathways for cells survival in androgen-independent cells. We chose, instead, to use an shRNA plasmid to downregulate STAT-3 and to investigate the importance of STAT-3 to IL-6 and AR pathway cross-talk in LNCaP cells. Our results indicated that STAT-3 expression is essential for androgen to affect the IL-6 pathway and our hypothesis was that STAT-3 expression is also essential for IL-6 and EGF to affect the AR pathway based on previous findings by Ueda et al. (Ueda et al., 2002).

Our results indicate, however, that downregulation of STAT-3 in LNCaP cells does not affect the ability of IL-6 and EGF to augment R-1881-induced ARR3-luc signalling. This observation offers a novel perspective on the influence of IL-6 and STAT-3 on AR-mediated gene transcription and contradicts previous findings by Matsuda et al. and Ueda et al. (Matsuda et al., 2001; Ueda et al., 2002). STAT-3 downregulation did decrease R-1881 and EGF augmentation of IL-6-stimulated SIE-luc signalling.

Although STAT-3 and the AR may have a direct interaction in the cell and be able to co-immunoprecipitated from cells stimulated with either IL-6 or R-1881, transactivation appears to occur only in one direction. The AR appears to bind STAT-3 and transactivate STAT-inducible gene transcription in IL-6 stimulated cells. While STAT-3 may bind the AR under R-1881-stimulated conditions, transactivation does not appear occur directly through STAT-3. It is likely that a co-activator of the AR is activated by STAT-3 or by other downstream elements of IL-6 and that this co-activator mediates IL-6 and EGF augmentation of R-1881-induced AR-mediated gene transcription. Candidate
co-activators include p300 and SRC-1. We propose a new model for IL-6 and AR pathway cross-talk in LNCaP cells in figure 7.1.

**Figure 7.1 Proposed Model for IL-6 and AR Pathway Cross-talk**
Although it has been demonstrated that STAT-3 and the AR can be co-immunoprecipitated from androgen-stimulated cells, IL-6-mediated transactivation of the AR does not appear to occur directly through STAT-3 activation.

Our *in vivo* results confirm that loss of STAT-3 represses AR-mediated gene transcription, however, it is likely that this repression is indirect, suggesting that transactivation occurs through a downstream molecule rather than STAT-3 itself. MAPK is a likely mediator of and IL-6 augmentation of R-1881-induced AR-mediated gene transcription. Both EGF and IL-6-mediated augmentation is MAPK dependent (Ueda et
al., 2002) and although MAPK cannot be activated by R-1881, IL-6 and EGF both activate MAPK significantly. We have also demonstrated MAPK activation by EGF and IL-6 in Figure 3.5. The necessity of MAPK activation, in AR signalling, to potential augmentation of the signal by growth factors needs to be established. An ideal experiment to determine the role of MAPK in growth factor augmentation of R-1881-stimulated ARR3-luc, would be to treat cells with PD098059 or U-0126 to inhibit MAPK while stimulating cells with IL-6, EGF and R-1881. In addition to the possible role of MAPK, the potential for other mediators of AR-transactivation must be investigated. We would also like to treat ARR3-luc-transfected LNCaP cells with protein kinase C inhibitors and PI-3 kinase inhibitors while stimulating with IL-6, R-1881 and EGF to investigate the ability of other factors to mediate AR-transactivation by IL-6 and EGF.

While we have demonstrated that STAT-3 is required for R-1881-mediated augmentation of IL-6-induced SIE-luc signalling, we also need to confirm the role of AR in the same system. An AR-targeted shRNA is available in the Prostate Centre and we would like to use it, in combination with SIE-luc and stimulation with IL-6, R-1881 and EGF, to investigate the role of AR in R-1881-mediated augmentation of IL-6-induced SIE-luc signalling.

As part of our ongoing studies of IL-6 and AR pathway cross-talk in LNCaP cells, we became aware of a unique STAT-3 phosphorylation pattern caused by EGF stimulation. It appears that in LNCaP cells, EGF phosphorylates STAT-3 on S727 in the absence of prior Y705 phosphorylation. It is not possible to discern from the Western blots (Figure
3.4 and Figure 6.1) whether any visible Y705 phosphorylation in EGF-stimulated cells can be attributed to an existing basal level of Y705 phosphorylation in the cells that is always present or whether bands simply represent a background artefact of the blot. In order to clarify whether EGF is phosphorylating a STAT-3 molecule with prior, baseline Y705 phosphorylation or whether a naïve STAT-3 molecule is being S727 phosphorylated, we would like overexpress a mutant tyrosine to phenylalanine STAT-3 to prevent Y705 phosphorylation and to see whether EGF phosphorylates the mutant protein on S727. We would also like to perform an EMSA assay using STAT-3 protein that has only been phosphorylated on S727 to assess the ability of that protein to bind DNA. We have shown, in the results of this thesis, that EGF-stimulated STAT-3 can activate STAT-mediated gene transcription in figure 3.5 and figure 5.5.

As a result of our observations regarding EGF-mediated S727 phosphorylation of STAT-3, we also investigated the mechanism by which IL-6-induced S727 phosphorylation and EGF-induced S727 phosphorylation of STAT-3 occurs. Our results indicate that, in IL-6-induced S727 phosphorylation of STAT-3, mTOR has a more significant role in S727 phosphorylation than MAPK. However, our results also indicate that a baseline level of S727 phosphorylation remains even in the presence of mTOR and MAPK inhibitors. There appear to be other kinases that have a role in S727 phosphorylation of STAT-3. Contrary to IL-6-induced S727 phosphorylation, EGF-induced S727 appears to be primarily mediated by MAPK rather than by mTOR. However, baseline levels of S727 phosphorylation also exist in EGF-stimulated cells in the presence of MAPK and mTOR inhibitors, indicating that other kinases may also be responsible for EGF-induced S727
phosphorylation. An H7 sensitive kinase, potentially protein kinase C, has been identified as a mediator of IL-6-induced S727 phosphorylation of STAT-3 (Abe et al., 2001). In order to make our analysis of STAT-3 S727 phosphorylation in LNCaP cells, induced by IL-6 or EGF, more complete, we would like repeat the experiment using PD098059, rapamycin and H7. We would also like to immunoblot for protein kinase C activation to investigate the potential role of protein kinase C in S727 phosphorylation of STAT-3.

The results in this thesis have added an important, novel perspective on IL-6 and AR pathway cross-talk. Downregulation of STAT-3 does not decrease IL-6 and EGF augmentation of R-1881-induced AR-mediated gene transcription. Although in vivo downregulation of STAT-3 by antisense ODN reduced AR-mediated gene transcription and tumour growth rate, our results from shRNA-mediated downregulation of STAT-3 indicate that STAT-3 does not directly transactivate the AR.
Bibliography


Appendix A Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Source</th>
<th>Stock Conc.</th>
<th>Working Conc.</th>
</tr>
</thead>
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<tr>
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<td>2 nM</td>
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<td>100 ng/µl</td>
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<td>R-1881</td>
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<td>1x10^{-10} M</td>
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<td>PD098059</td>
<td>Biomol Research Laboratories, Plymouth</td>
<td>50 mM</td>
<td>50 µM</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>Sigma, Saint Louis, Missouri</td>
<td>5 µg/ml</td>
<td>5 ng/ml</td>
</tr>
</tbody>
</table>
## Appendix B Antibodies

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Source</th>
<th>Working Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-STAT-3, Phospho-Specific (Ser(^{727}))</td>
<td>Calbiochem, San Diego, California</td>
<td>1:1000</td>
</tr>
<tr>
<td>Human, rabbit polyclonal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-phospho-STAT3 (Y705), rabbit polyclonal</td>
<td>Upstate Biotechnology, Lake Placid, New York</td>
<td>1:1000</td>
</tr>
<tr>
<td>Stat-3 (H-190), rabbit polyclonal</td>
<td>Santa Cruz Biotechnology, Inc., Santa Cruz, California</td>
<td>1:1000</td>
</tr>
<tr>
<td>Stat1 p84/p91 (E-23), rabbit polyclonal</td>
<td>Santa Cruz, Biotechnology Inc., Santa Cruz, California</td>
<td>1:1000</td>
</tr>
<tr>
<td>Phospho-p44/42 MAP Kinase (Thr202/Tyr204), rabbit polyclonal</td>
<td>Cell Signalling Technology, Beverly, Massachusetts</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-MAP Kinase2/Erk2, clone 1b3b9, mouse monoclonal</td>
<td>Upstate Biotechnology, Lake Placid, New York</td>
<td>1:5000</td>
</tr>
<tr>
<td>Phospho-p70 S6 kinase (Ser411) (A-6)</td>
<td>Santa Cruz, Biotechnology Inc., Santa Cruz, California</td>
<td>1:1000</td>
</tr>
<tr>
<td>p70 S6 kinase (H-9)</td>
<td>Santa Cruz, Biotechnology Inc., Santa Cruz, California</td>
<td>1:1000</td>
</tr>
</tbody>
</table>
Appendix C shRNA Sequences

shRNA#1
oligo A

```
aaactgccgcagctccattgggaagctggaagcttgcagcttcccaatggagctgcggcagtttctgtttttt
```
oligo B

```
gatcaaaaaacagaaactgcggcagctccattgggaagctgcaagcttccagcttcccaatggagctgcggctgtttcg
```

shRNA#2 (uniqueness rated #1)
oligo A

```
ccccatatgcccaatcttgactctcaagaagcttgttgagagtcaagattgggcatatgcggccagtttttt
```
oligo B

```
gatcaaaaaacagaaactgcggcagctccattgggaagctgcaagcttccagcttcccaatggagctgcggctgtttcg
```

shRNA#3 (uniqueness rated #2)
oligo A

```
tcactgtagagctgatggagctgctccagaagcttgttgagcagctccatcagctctacagtgacagtttttt
```
oligo B

```
gatcaaaaaacagaaactgcggcagctccattgggaagctgcaagcttccagcttcccaatggagctgcggctgtttcg
```

150
shRNA #4
oligo A
tgtcaagctgtgtagctgattccattggaagttgcaagtgaatctacagctacagcttgacacactttttt
oligo B
gatcaaaaaagtgtcaagctgtgtagctgattccattgcaagttcaatggaatcagctacagcttgacacg

shRNA #5 (uniqueness rated #4)
oligo A
ggcaatctccattgtcagctgaagcttggtgtagttttaatgaaagccaaatggagatatgcggtttttt
oligo B
gatcaaaaaaccgggcaatctccattgtgtaagtagcagttttagaaatgcaacagattgcacg

shRNA #6 (uniqueness rated #3)
oligo A
aacattcgactcttgaggagcagggcctggcaggtctgcagttcatttcaggaagtttgagcttctctctctttttt
oligo B
gatcaaaaaagagaacattcgactcttgaggagcagggcctggcaggtctgcagttcatttcaggaagtttgagcttctctctctctg