Characterisation of the Wnt signalling Components in Prostate Cancer Cells

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By

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Abstract

The Wnt signalling pathway is altered in tumours of many tissues, including the prostate. Activation of the Wnt signalling pathway leads to stabilisation of β-catenin and the formation of β-catenin/Tcf complexes, which can increase expression of genes involved in cell growth, such as cyclin D1. Interest in the role of β-catenin in prostate cancer (PCa) has developed following reports that it is mutated in 5% of primary prostate tumours, that it is aberrantly expressed in the cytoplasm and/or nucleus in up to 38% of advanced tumours, and that it can associate with the androgen receptor (AR), thereby enhancing AR-mediated transcription and reducing its ligand-specificity. The aim of this work was to understand the role of β-catenin and other intracellular components of the Wnt signalling pathway in PCa cells. Initially, a characterisation of a panel of PCa cell lines with respect to their ability to respond to activation of Wnt ligands was carried out and the crosstalk that occurs between the Wnt and AR signalling pathways was studied. Confirming the work of others, β-catenin was found highly expressed in PCa cells and endogenous β-catenin/AR complexes were detected. In support of the hypothesis that β-catenin activates AR, overexpression of Axin, which promotes the degradation of β-catenin, reduced AR activity. In contrast, inhibition of β-catenin by RNAi increased rather than decreased AR activity, suggesting that the role of Axin in regulating AR is independent of β-catenin. Deletion analysis of Axin indicated that this inhibition involves the GSK3β-binding domain of Axin. The GSK-3 inhibitors SB415286 and SB216763 also inhibited AR activity, as did expression of FRAT, a protein that sequesters GSK3β. Importantly, inhibition of GSK3β using chemical inhibitors reduced PCa cell growth. Moreover, overexpression of GSK3β increased AR activity in PCa cells with low endogenous GSK3β activity. Immunoprecipitation studies suggested that GSK3β associates with AR and that the GSK3β-binding domain of Axin prevents formation of this complex. Lastly, mutation analysis suggested that residue serine 94 is a potential GSK3β phosphorylation site of AR and is required for the activation of AR by GSK3β. Taken together, my work has uncovered a novel role for GSK3β in the regulation of AR signalling and a potential new therapeutic application for GSK-3 inhibitors in the treatment of PCa.

Contents

List of Figures

Introduction-

Results-

List of tables

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Abbreviations

AD	Androgen-dependent
AF-1	Activation function-1
AF-2	Activation function-2
AI	Androgen-independent
APC	Adenomatous polyposis coli
AR	Androgen receptor
ARE	Androgen responsive element
ATP	Adenosine triphosphate
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
CBP	CREB-binding protein
CDK	Cyclin dependent kinase
CKI	Casein kinase I
CREB	cAMP-response element binding protein
CSS	Charcoal-Stripped Serum
DBD	DNA-binding domain
DHT	Dihydrotestosterone
Dkk	Dickkopf
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribose nucleic acid
Dvl	Dishevelled
EDTA	Ethylenediamine-Tetraacetic Acid
EGF	Epidermal growth factor
ER	Estrogen receptor
FBS	Foetal Bovine Serum
FGF	Fibroblast growth factor
FRAT	Frequently rearranged in advanced T-cell lymphomas
Fz	Frizzled
GBP	GSK-3-binding protein
GFP	Green fluorescent protein
GR	Glucorticoid receptor
GSK-3	Glycogen synthase kinase-3

HAT	Histone acetyltransferase activity
HCC	Hepatocellular carcinomas
Hsp	Heat shock protein
HMG	High mobility group
IGF	Insulin-like growth factor
IMDM	Iscove's Modified DMEM
IMEM	Richter's Improved MEM
IP	Immunoprecipitation
JNK	c-Jun N-terminal kinase
KDa	Kilodaltons
KGF	Keratinocyte growth factor
LBD	Ligand binding domain
LDL	Low density lipoprotein
Lef	Lymphocyte enhancer binding factor
LH	Leuteinizing hormone
LHR	Leuteinizing hormone receptor
LHRH	Leuteinizing hormone-releasing hormone
LHRHR	Leuteinizing hormone-releasing hormone receptor
LiCl	Lithium chloride
LRP	LDL receptor related protein
MAPK	Mitogen activated protein kinase
MMTV-Luc	Mouse mammary tumour virus promoter-driven luciferase
NLS	Nuclear localisation signal
NP-40	Nonidet P40
OT-Luc	OT promotor-driven luciferase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PCa	Prostate cancer
PI3K	Phosphatidylinositol 3-kinase
$PI(3,4,5)P_3$	Phosphatidylinositol-3,4,5-trisphosphate
PIN	Prostatic intraepithelial neoplasia
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C

PMA	Phrobol 12-myristate 13-acetate
PP2A	Protein phosphatase 2A
PR	Progesterone receptor
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homology
RAR	All trans-retinoic acid receptor
RGS	Regulation of G-protein signalling
RNA	Ribose nucleic acid
RNAi	RNA interference
RSV-β-Gal	Rous sarcoma virus promoter-driven β-galactosidase
SDS	Sodium Dodecyl Sulphate
sFRP	Secreted Frizzled related proteins
SRC-1	Steroid receptor coactivator-1
STAT	Signal transducers and activators of transcription
TBS	Tris Buffered Saline
Tcf	T-cell factor
TGF	Transferring growth factor
TR	Thyroid hormone receptor
βTrCP	β-tarnsducin repeat-containing protein
VDR	Vitamin D_3 receptor
Wnt	Wingless type protein
w.t	Wild-type

1. Introduction

Carcinoma of the prostate is one of the leading causes of male cancer-related deaths in western countries. Worlwide, more than 670,000 men were diagnosed with prostate cancer (PCa) in 2002 (Parkin *et al.*, 2005). Most prostate tumours are initially androgen dependent, and androgen ablation treatment usually leads to tumour regression. However, the cancer often recurs despite the continued treatment, and no current therapy has been shown to increase survival once androgen deprivation is no longer effective. The increase in the number of PCa cases in the past century can be attributed to several reasons- 1. Differentiation of PCa from other types of urinary obstruction since early 1900s. 2. Rapid increase in PCa incidence with age compare to any other cancer type, combined with increase in average life expectancy over the past century. 3. Western lifestyle- the variation in incidence rates across the world indicates that some environmental and dietary factors are likely to be involved. The disease is significantly lower in asian populations and it is increased in men who have emigrated to western nations. The increased incidence has lead to significant changes in the diagnosis and treatment of PCa (reviewed in Denmeade and Isaacs, 2002).

1.1 The prostate

The prostate is a male accessory reproductive gland whose development begins in fetal life and is completed at sexual maturity. Its main function is to produce seminal fluid that expels into the urethra during ejaculation. Normal development of prostatic ducts requires many co-ordinated cellular processes including ductal branching morphogenesis, epithelial proliferation, ductal canalisation, and epithelial and mesenchymal differentiation. The first indication of prostate formation occurs during week 10 of embryonic development when epithelial buds are induced in the prostatic urethra. Then levels of testosterone rise and cells differentiate into the different cell types. Maturation of the gland continues while testosterone levels are high, and as levels fall during the third trimester the gland enters a quiescent state. This state persists until puberty when testosterone levels increase again, the epithelium proliferates and the gland folds to its mature state. The prostate doubles in size during this time, androgen receptors (AR's) are expressed by the epithelial cells and

the full secretory phenotype is established. By the age of 45 to 50, testosterone levels decline again and the prostate enters a period of involution. After this age, atrophy of the gland may continue, though commonly, benign prostatic hyperplasia (BPH) occurs. The initial developmental processes are dependent on androgens, which act through the mesenchymal cells. The epithelium, in turn, stimulates the mesenchymal cells to differentiate into smooth muscle and fibroblasts. Later in development androgens act directly on epithelium to induce secretory protein production (Cunha, 2004).

1.1.1 Prostate anatomy and cells

The prostate is located between the bladder and the levator ani muscle. The prostatic urethra runs through the centre of the gland and bends anteriorly at the verumontanum. The paired ejaculatory ducts enter the base of the gland and join the urethra at the verumontanum (for anatomical relations of the prostate gland, see Figure 1a)(Colville, 2004).

In contrast to the rodent, the human prostate is not subdivided into lobes (dorsal, ventral, lateral), but instead organised into zones- the central zone (CZ), the peripheral zone (PZ) and the transition zone (TZ). The organisation of the prostate was defined by McNeal, based on embryological findings, response to oestrogens and susceptibility to disease (McNeal, 1972). PZ in the normal gland comprises the majority of the prostatic volume (65%), and is characterised by small, simple, acinar spaces lined by tall columnar secretory epithelial cells. The second largest component of the prostate is the CZ, which takes 25% of the volume. The CZ surrounds the ejaculatory ducts and makes up the majority of the prostatic base. It consists of large acini that are lined by low columnar cuboidal epithelial cells and small smooth-muscle stroma cells. The TZ is the smallest zone (5-10%), and is composed of two bilaterally symmetrical lobules found on the two sides of the prostatic urethra. TZ is separated from the other two zones by a narrow band of fibromuscular stroma, which extends from the posterior urethra to the anterior of the gland. The TZ acini are similar to the PZ acini and the stroma cells resemble the stroma cells of the CZ. Histologically, the differences between the zones are subtle in the absence of disease. The clinical significance of zonal anatomy is important in terms of the development of both BPH and PCa. BPH usually originates within the

TZ, and then expands through the PZ. In contrast, even though malignancy can affect all three zones, the majority of cancers originate in the PZ.

The mature prostate is made of epithelial cells and fibromuscular stroma cells. The two cell types maintain an important cross talk that regulates the growth, development and hormone response of the prostate. The epithelium, which gives rise to prostate adenocarcinoma, has three types of cells; basal, luminal secretory and neuroendocrine cells. The luminal cells secrete components of prostatic fluid, and prostatic specific antigen (PSA). They also express AR and are dependent on androgens for survival. The basal cells secrete components of the basement membrane, express low levels of AR and are androgen-independent. The function of the neuroendocrine cells is mainly unknown. It is believed that they induce proliferation of adjacent cells by secretion of neuropeptides (van Leenders and Schalken, 2001). The stroma is composed of fibroblasts, smooth muscle cells, endothelial cells, dendritic cells, nerves and some infiltrating cells and lymphocytes. Androgens regulate the expression of growth factors in prostate stromal cells, which usually have a positive effect on epithelial cell proliferation. KGF (keratinocyte growth factor), for example, can induce growth and is upregulated by androgens. Other growth factors that affect the epithelial cells in a paracrine manner include IGF-II (insulin-like growth factor-II), FGF (fibroblast growth factor), TGFα and TGFβ (transferring growth factor α and β) and the nerve growth factor. TGFβ inhibits epithelial cell growth and is downregulated by androgens (reviewed in Feldman and Feldman, 2001).

1.1.2 PCa stages and development

The ability to recognise premalignant change is important for the cure of PCa (for stages of PCa progression, see Figure 1b). BPH is a differentiated nodular hyperplastic growth that affects the prostate and usually originates from the TZ. BPH occurs in 50% of men between the age of 51-60 years old and increases further with age (Berry *et al.*, 1984). Most men who undergo examination will harbour histological BPH. In the benign prostate, the epithelial cells consist of two cellular layers; basal and luminal cells. This important feature helps to distinguish benign from malignant tissue, as carcinoma of the prostate always devoid of the basal-cell layer.

The earliest stage of malignancy is detected by lesions exhibiting nuclear pleomorphism and nucleolar prominence similar to that seen in PCa. This precursor of malignancy is called prostatic intraepithelial neoplasia (PIN). TZ cancers are not usually related to PIN and on the other hand PIN is very frequently found adjacent to carcinomas in the PZ. Apart from nuclear pleomorphism, PIN also shares other characteristics of malignancy such as loss of basal layer continuity (Le Marchand *et al.*, 1994) and production of acid mucin (Whittemore *et al.*, 1995). In common with other intraepithelial neoplasias, PIN is divided into low and high grade neoplasia based on cytological features. High-grade PIN lesions are diagnosed by increasing proliferation and cytological changes such as nucleolar enlargement (Kirby Roger S, 2001; Colville, 2004).

Another lesion type which has been suggested to represent a premalignant change is atypical adenomatous hyperplasia (AAH). This entity is focally associated with disruption of the basal-cell layer.

PIN (and possibly also BPH and AAH) can progress to a localised prostate tumour (Colville, 2004). As all other classical adenocarcinomas, prostate adenocarcinoma is made up of epithelial cells with different degrees of grandular architecture. There is a departure from two layers of cells, and the basal-cell layer is absent. There is also an infiltrative growth pattern that makes it difficult to determine the edge and origin of the tumour. Another common feature of prostate carcinomas is enlarged pleomorphic nuclei and prominence of nucleoli (Kirby Roger S, 2001). Invasion and metastasis of PCa cells involves processes like matrix remodelling and cell-cell detachment and attachment like in other invasive malignancies. In addition to these processes, PCa cells need to be able to grow and survive in the absence of the secreted stromal factors. Large prostate-confined tumours and lymph node metastases do not have a lot of stromal cells and are less dependent on stromal growth factors. PCa typically metastasises to the bone. This could be due to the fact that the bone stroma produces androgen resistance factors that induce growth and survival of the cancer. This possibility might explain why tumours in patients with metastatic disease continue to grow and migrate even with androgen ablation therapy (reviewed in Jenster, 1999).

The grade of malignancy has been an important prognostic marker in PCa diagnosis and treatment. A number of grading systems have been established, the most widely used is the Gleason, which is based on microscopy findings that define

the pattern of the tumour. A 5-step grading system is used, and the two most prominent grades are added together as the 'Gleason score', which ranges from 2-10. The Gleason system offers significant prognostic information and was found to be reproducible between pathologists (Miller, 1990; Gleason, 1992).

Figure 1a- Prostate anatomy

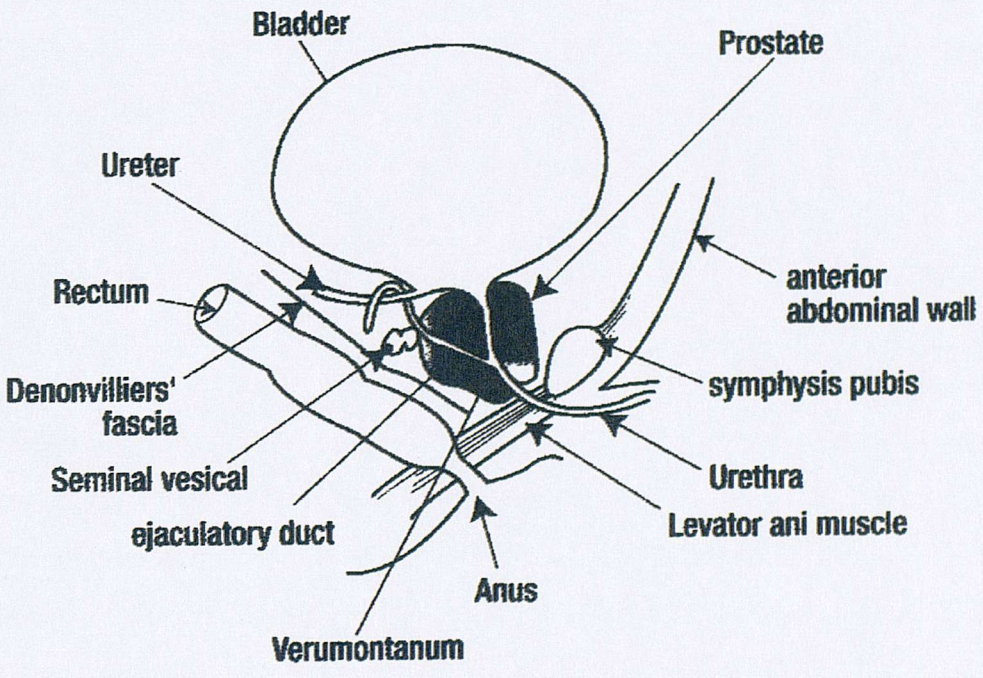


Fig 1a. Anatomical relations of the prostate gland (viewed from the side).
Taken from Prostate Cancer, Clinical and Scientific Aspects -Bridging the Gap. Edited by Abel PD and Lalani El-Nasir (2004) (Colville, 2004)

Figure 1b-PCa progression from pre-malignancy to malignancy

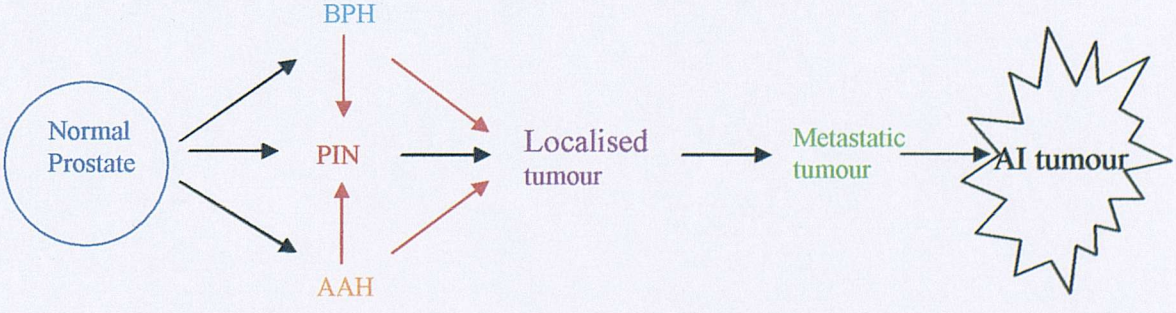


Fig 1b. Possible sequential changes occurring during PCa progression. Abnormal growth in the normal prostate can lead to progression of different levels of malignancy. PIN (prostatic intraepithelial neoplasia), BPH (benign prostatic hyperplasia) and AAH (atypical adenomatous hyperplasia) are the earliest stages of malignancy. These can develop to a localised tumour, metastatic tumour and then to androgen-independent (AI) tumour. Red arrows indicate equivocal links.

1.2 Androgen production and action

Androgens bind to AR in the hypothalamus and stimulate production of luteinizing hormone (LH)- releasing hormone (LHRH) (for androgen production cascade, see Figure 2). LHRH travels to the pituitary where it interacts with LHRH receptors and stimulates the release of LH. Binding of LH to the LH receptors expressed on the leydig cells of the testis induces the production of the main circulating androgen, testosterone, by stimulating steriodogenesis via a cAMP-mediated pathway. In this pathway the parental compound cholesterol is hydrolysed to various androgenic steroids in several enzymatic stages, which end in the conversion of androstenedione to testosterone by the enzyme 17β-Hydroxylase (Robinson S, 2002). Increased testosterone levels can also decrease LHRH and LH production through negative-feedback loops and in this way maintaining the circulating testosterone at physiological levels (reviewed in Denmeade and Isaacs, 2002). Testosterone circulates in the blood, where it is bound to albumin and sex-hormone-binding globulin (SHBG) and only a small fraction is dissolved freely in the serum. When free, testosterone enters the prostate cells and 90% of it is converted to dihydrotestosterone (DHT) by the 5α-reductase enzymes. Among the two enzyme isoforms, 5α-reductase type II is expressed in the male urogenital tract, is active in the prostate and crucial for prostate development and function. DHT is the more active form of testosterone with a fivefold higher affinity for AR. Although testosterone is primarily secreted by the testis, it is also produced by peripheral conversion of adrenal steroids. The adrenals secrete large amounts of the inactive steroid dehydroepiandrosterone (DHEA), its sulfate DHEAS, and the weak androgen androstenedione. These precursors can be converted to testosterone in most peripheral tissues including the prostate (reviewed in Jenster, 1999).

Figure 2-Androgen production

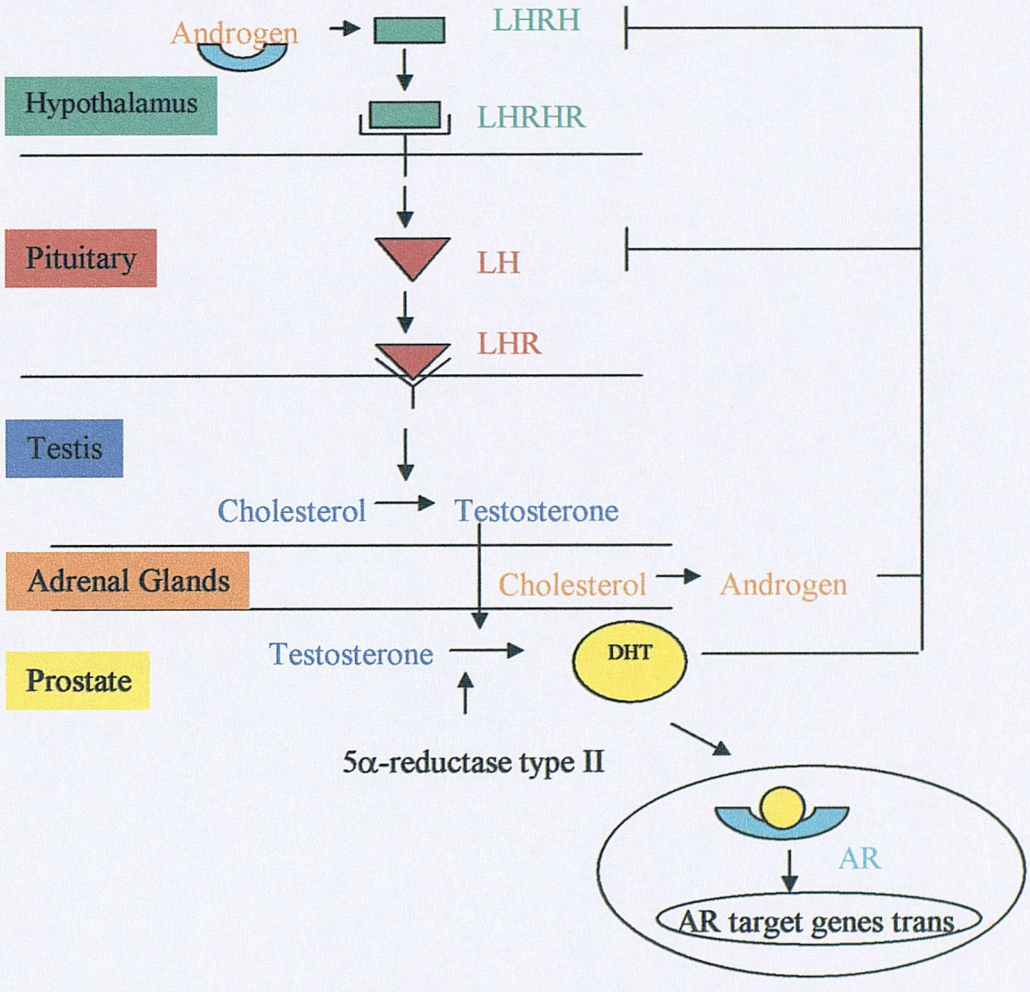


Fig 2. Androgen production cascade. Stimulation of luteinizing hormone (LH)-releasing hormone (LHRH) in the hypothalamus starts a cascade of events through the pituitary, the testis and ends up in prostate cells where dihydrotestosterone (DHT) binds to AR and initiates transcription of AR target genes. The adrenal glands are also able to secrete androgens. Androgens can also inhibit LHRH and LH production through negative-feedback loops. LHRHR= luteinizing hormone-releasing hormone receptor, LHR= luteinizing hormone receptor.

1.3 PCa therapy

1.3.1 Androgen–ablation therapy

As in normal prostate development, primary prostatic cancers are largely dependent on androgens for growth. Therefore, androgen ablation and antiandrogen therapy have been standard treatments for patients with metastatic PCa. In the 1940s Charles Huggins showed that orchiectomy (removal of the testis) induced the regression of

PCa. Since then, androgen-ablation has been the main therapeutic intervention for the treatment of PCa. However, although orchiectomy is effective in depleting androgens, it causes significant cardiovascular and thromboembolic toxicity. In addition, androgens can still be produced by the adrenal gland and maintain a low level of androgens in the serum. Therefore, during the 1960-1980s new approaches that either block adrenal androgen production or inhibit androgen interaction in the target tissue were developed. These include: chronic administration of LHRH agonists (and LHRH antagonists), AR antagonists, suppression of adrenal steroidogenesis, treatment with 5α-Reductase inhibitors and total androgen-ablation by a combination treatment of anti-androgens and LHRH agonists. 1. LHRH agonists produce inhibitory effects and downregulate the pituitary receptors for LHRH (for example; leuprolide, nafarelin, buserelin). This leads to supression of LH (and the follicle stimulating hormone (FSH)) release and inhibition of testosterone secretion from the testes. However, treatment with LHRH agonists causes side effects such as hot flushes, loss of libido and impotence. LHRH antagonists that immediately antagonise LH release, avoiding the initial stimulation of testosterone (known as 'testosterone flare') were also developed (for example; cetrorelix, abarelix, orgalutran). 2. AR antagonists (anti-androgens) competitively inhibit the binding of DHT or testosterone to AR. However, anti-androgens also bind to receptors that are expressed in the hypothalamus and pituitary and therefore block the negative feedback route of androgens, leading to increase in serum testosterone levels. One steroidal anti-androgen, cyproterone, overcame this problem by adding an acetate group. This creates a binding site for the progesterone receptor in the pituitary and thereby indirectly decreases testosterone levels by inhibiting the release of LH and also directly acts as an anti-androgen in the prostate. The limitations of this drug are loss of libido and sexual potency. Non-steroidal anti-androgens that do not affect libido and potency were later developed (for example; flutamide, bicalutamide, nilutamide), and these are the main antiandrogens in use today. 3. Compounds that suppress adrenal steroidogenesis and subsequent androgen production are used as second-line hormone therapy (for example, ketoconazole). 4. 5α-Reductase inhibitors block the conversion of testosterone to DHT (for example, finasteride). 5. Total androgen-ablation combines anti-androgens with LHRH agonists. The idea of this therapy is to reduce both the amount of testosterone released from the testis

using LHRH agonists and neutralise androgens produced by the adrenal with anti-androgens. Although the combined treatment is more effective than any of the monotherapy treatments with advanced cancer, it has not yet been shown to prolong survival.

These treatments benefit 60-80% of patients; however, the duration of response is only 1-2 years. Androgen-ablation therapy provides significantly effective therapy for most patients with androgen-dependent (AD) cancer. However, these cancers eventually become androgen-independent (AI), progress and metastasise. One of the main reasons for that is the fact that prostate tumours are composed of a heterogeneous population of cells that are either dependent or independent of androgens. The AI cells will keep progressing and find ways to grow in the presence of low levels of androgens therefore, treatment can never eliminate this type of cells and cure the cancer completely. The strategy taken today is to minimise the duration of androgen-ablation by delaying therapy until patients have clear evidence of metastases or by treating on an intermittent basis to delay the transition to an AI carcinoma. In addition to that, since the realisation that androgen-ablation has failed to be totally curative, two alternative approaches have been taken. First, to find better treatment for systemic disease. Second, to develop a method to aggressively screen for PCa in early stages where the cancer is still localised in the prostate and potentially easier to treat (reviewed in Feldman and Feldman, 2001; Denmeade and Isaacs, 2002).

1.3.2 Screening for PSA and other PCa markers

PSA was first described in 1971. It is a serine protease that is encoded by an androgen-responsive gene located on chromosome 19q13.3-13.4 (Riegman *et al.*, 1989). It is secreted from the prostate epithelial cells and its main function is to liquefy human semen through its proteolitic action. After the discovery that PSA levels are higher in patients with PCa, a limit of 4 ng/ml serum concentration for men was established. PSA serum levels higher than 4 ng/ml are considered to be an indicator for the presence of PCa and require further clinical evaluation and treatment. Measuring PSA levels combined with digital rectal examination has become a useful diagnostic tool for detecting the presence of PCa. However, the PSA test was revealed to have sensitivity problems. One of them is that serum PSA levels can be elevated as a result of conditions other than PCa, such as BPH and the

prostatic inflammatory condition, prostatitis. In addition, 20-30% of men with PCa have serum PSA levels in the normal range, resulting in undiagnosed disease. An interesting study also revealed that preoperative PSA levels do not correlate with cancer volume and Gleason grade. Despite the disadvantages of the PSA test, it is currently the best clinical marker available for PCa and is approved for monitoring disease recurrence and treatment. Many studies are underway in search of a better marker for PCa. These markers are required to show some level of correlation with one or more of the following factors: presence of PCa, disease progression, cancer recurrence, prediction of response to therapy or disease-free survival. The ideal candidate for detection of disease would be one that is easily accessible in biological fluids such as human serum, urine or prostate fluid and is able to distinguish between normal, BPH, PIN and cancerous prostate tissues. Examples for potential markers to date include: chromogranin A (GRN-A), glutathione S-transferase π 1 (GSTP1), prostate stem cell antigen (PSCA), prostate-specific membrane antigen (PSMA) and telomerase reverse transcriptase (TERT) (reviewed in Tricoli *et al.*, 2004).

1.3.3 Surgical and radiological treatments

During the past several years the surgical techniques used in localised PCa treatment have significantly improved. Various surgical procedures to remove the prostate (radical perineal prostatectomy) have been reported to be in use since the early 1900s. However, they were not commonly used as they left most of the patients impotent. In 1983, a new technique was developed on the basis of anatomical approach – radical retropubic prostatectomy. This approach avoids injury of nerves around the penis and therefore does not affect sexual potency. This procedure was developed at the same time as the development of the PSA screening test as well as the use of biopsies obtained from patients. Together, early detection methods have lead to an increase in number of patients who were treated with prostatectomy and this has prevented death especially in men under the age of 75 (reviewed in Denmeade and Isaacs, 2002).

1.3.4 Radiation therapy

Brachytherapy is a radiation therapy where the patient's prostate is implanted with radioactive seeds that deliver radiation in order to destroy the tumour cells. The

radiation is delivered over a short distance, thereby minimising the amount of radiation delivered to the normal tissue. Since 1983 this technique was also used under the guidance of transrectal ultrasonography and today is still used as a common approach for treating localised PCa.

Another type of radiation therapy is external beam radiotherapy. This therapy is applied from outside to a defined area of the body. The use of this technique increased in the 1950s when higher-energy cobalt machines that could penetrate to deeper levels became available. Today improved radiographic and data processing capabilities that help planning treatment are available. This allows the prostate to be treated with a high dose of radiation while sparing the surrounding normal tissue. The standard care today is to incorporate androgen-ablation therapy before, during and after radiation in order to improve survival and delay relapse time (reviewed in Denmeade and Isaacs, 2002).

1.3.5 Cytotoxic chemotherapy

The interest in cytotoxic chemotherapy started after the realisation that many patients fail androgen-ablation therapy and the need for treatment of hormone-refractory PCa has increased. Although studies on chemotherapies are still underway there are agents that are already in use and have been proved to be effective in treating hormone-refractory PCa. The main indicator of response in recent studies has been the fall in PSA levels in treated patients. Using this criterion on a number of chemotherapy combinations resulted in a more than 50% decline in serum PSA levels in a significant proportion of patients. The approved chemotherapeutic combination for metastatic disease today is mitoxantrone and corticosteroid. More combinations that have shown a significant survival advantage and fall in serum PSA are still being studied (reviewed in Denmeade and Isaacs, 2002).

1.4 The androgen-receptor

AR is an 110 kDa AD transcription factor and is a member of the nuclear receptor superfamily. This family includes receptors for steroids (androgen, estrogen, progesterone, glucocorticoid and mineralocorticoid), retinoids, thyroid hormone, vitamin D, fatty acids and other small hydrophobic molecules. The nuclear receptors function as ligand-inducible transcription factors that regulate the expression of

target genes in response to ligands specific to each receptor. The receptors can be subdivided to three different types; Type 1- the classical steroid receptors such as AR, estrogen receptor (ER), progesterone receptor (PR), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). They form ligand-induced homodimers and bind to inverted repeat DNA response elements of target genes. Type 2- these include the receptors for vitamin D_3 (VDR), thyroid hormone (TR), all trans- retinoic acid (RAR), and the peroxisome proliferator-activated receptors (PPAR). The DNA response elements of this group are characterised as direct repeats. Type 3- the orphan receptors such as testicular orphan receptor 2 (TR2), TR4, and chicken ovalbumin upstream promotor transcription factor (COUP-TF). The ligands for these receptors remain unclear (Giguere, 1999). Post-translational modifications such as phosphorylation, acetylation and ubiquitilation have been shown to modify the ligand-induced activity of steroid receptors. However, some nuclear receptors can become active without being modified (Fu *et al.*, 2003b; Nawaz and O'Malley, 2004). Prostate epithelial cells express multiple members of the nuclear receptor superfamily that regulate transcription of genes involved in proliferation and differentiation. Interestingly, the VDR ligand 1α, 25-dihydroxyvitamin D_3, has antiproliferative effects in prostate epithelial cells. These effects were retained in some PCa cell lines due to cellular resistance and altered levels of the corepressor SMRT (silencing mediator for retinoic and thyroid hormone receptor), suggesting a role for 1α, 25-dihydroxyvitamin D_3 in prostate tumour growth (Peehl *et al.*, 1994; Campbell *et al.*, 1997; Khanim *et al.*, 2004). Although AR normally functions as a homodimer, it can sometimes be found in a heterodimer with TR4 (Lee *et al.*, 1999), or ERα (Panet-Raymond *et al.*, 2000). In these cases, however, AR transcriptional activity was found to be reduced (reviewed in Heinlein and Chang, 2002).

1.4.1 *AR structure*

The family of nuclear receptors, including AR, share a unique modular structure that can be divided into four functional domains (for AR structure, see Figure 3): the N-terminal domain which harbours transcription activation functions, the centrally located DNA-binding domain (DBD), hinge region, and the C-terminal ligand-binding domain (LBD). Two transcriptional activation function domains were found on the nuclear receptors; the N-terminal activation function-1 (AF-1) is a ligand-

independent domain, and can create a constitutively-active receptor when separated from the LBD. The ligand-dependent AF-2 domain is located on the LBD and mutations in this region dramatically reduce transcriptional activity in response to ligand. Both the DBD and LBD share homology among the different receptors. The N-terminal domain and the hinge region are more receptor-specific due to variations in size and differences in the primary sequence. In the case of AR, there are two discrete regions within the N-terminal domain that contribute to transactivation. The first is a region located between amino acids 141 and 338, which contains a polymorphic polyglutamine repeat that ranges from 8 to 31 in normal individuals. Longer polyglutamine tract length results in decreased AR activity *in vitro* and causes infertility and impaired spermatogenesis. If the tract expands to more than 40 repeats it can lead to some rare neuromuscular disorders. In addition, the polyglutamine tract forms part of the interaction surface for the AR coactivator ARA24 (AR-associated protein 24 KDa). In contrast, shorter AR is more active and associated with growth advantage. The second important region within the AR N-terminal domain is the AF-1 domain, which is located between amino acids 360-494. A specific motif within the AF-1, 433(WXXLF)473, has been shown to interact with the AR LBD. The AR N/C-terminal interaction is important in stabilising the bound ligand and known to be facilitated by several coactivators. Possibly, the AF-1 domain interacts with these coactivators and provides the interaction surface for the AR C-terminal domain. AF-1 was also found active in AR fragments that lack the LBD domain, therefore it is thought to be able to function on its own by recruiting coactivators or transcriptional factors (Jenster *et al.*, 1995).

The hinge region links the DBD and LBD. AR, as all other steroid receptors, has a ligand-dependent nuclear localisation signal (NLS) located between the C-terminus of the DBD and the hinge region (aa 617-633).

The DBD of all nuclear receptors consists of two zinc fingers that recognise specific DNA consensus sequences. AR binds as a dimer to the consensus inverted repeat androgen response elements, GGTACAnnnTGTTCT, as well as to other elements. Some coregulators affect AR activity by modulating its ability to bind to this sequence. The coactivator receptor accessory factor (RAF), for example, binds to the AR N-terminal domain and in this way enhances AR DNA binding. Corepressors can inhibit AR transactivation by binding to the AR DBD and preventing its binding to DNA (for example, calreticulin).

The LBD of AR, in addition to forming the ligand-binding pocket, mediates the interaction between AR and heat shock proteins (Hsp's) and interacts with the N-terminal domain. X-ray crystallographic studies indicate that the LBD among the nuclear receptors has a similar structure, with the ligand-binding pocket formed by 11-13 α-helices. When ligand binds to the LBD domain a conformational change is induced in which helix 12 and the AF-2 domain fold back across the ligand-binding pocket. In addition, the N/C-terminal domain interaction stabilises helix 12 across the ligand-binding pocket, resulting in a reduced dissociation rate of bound androgen. Although the crystal structure of AR suggests that ligand-binding induces a similar LBD conformational change as occurs in ER, it does not generate the same coregulator interaction surface and there are distinct differences between the coregulator interaction domains of AR and ER. This is possibly due to the interaction that occurs between the AR N/C-terminal domains, which create a specific coregulator interaction surface (reviewed in Heinlein and Chang, 2002).

Figure 3-AR structure and phosphorylation sites

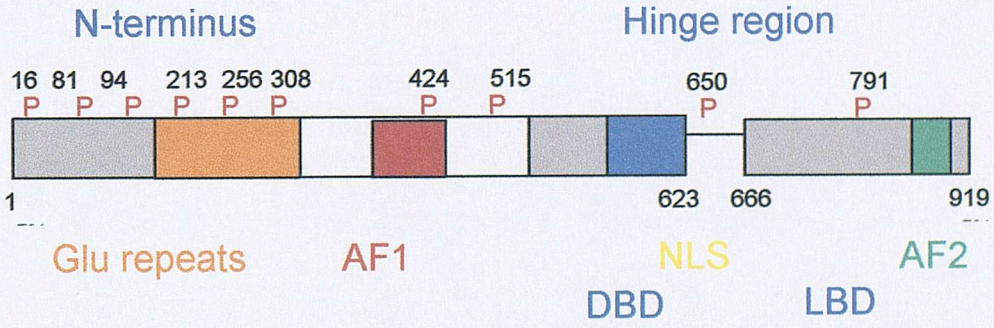


Fig 3. AR structure and phosphorylation sites. AR domains represented include the N-terminus with the glutamine repeat regions (Glu repeats), activation function domains-1 (AF1), the DNA binding domain (DBD) with the nuclear localisation signal (NLS, residues 613-633), the ligand binding domain (LBD, residues 666-919) including activation function domains-2 (AF2) and the hinge region (residues 623-666). Phosphorylation sites are represented as P, and with the residue number above.

1.4.2 AR coregulators

In its basal state, AR, like other nuclear receptors, is bound to a large heterocomplex that contains Hsp's, immunophilins and other proteins (such as the FK51 and FK52 binding proteins, CyP-40 and p23) (reviewed in Pratt and Toft, 1997). Some of these

proteins have chaperone functions (e.g Hsp90 and Hsp70), and the major function of the complex is to facilitate the folding of the receptor LBD into the high-affinity ligand binding conformation. Hsp90 interacts directly with the receptor LBD and holds the receptor in a conformation that prevents DNA binding. Binding to androgens induces a conformational change in AR that leads to dissociation from these proteins and to an interaction between the N/C-terminal domains of AR. The conformational change also induces the formation of AR homodimer complexes that translocate to the nucleus and bind to androgen responsive elements (AREs) in the promotors of target genes. The activated DNA-bound AR complex recruits coregulators that can either enhance or repress transcription of target genes. The receptor also binds directly to several transcription factors. Examples of AR binding to these proteins include a number of transcriptional factors such as activator protein-1 (AP-1), and the nuclear factor κB (NFκB) as well as proteins from the transcription machinery like transcription factor IIF (TFIIF) and cyclin dependent kinase 7 (CDK7). However, the ability of AR to bind to these proteins is not always sufficient to initiate transcription. Using different mechanisms, the coregulators are able to stimulate or inhibit gene transcription. Coregulators can be classified into two groups, depending on their mechanism of action. Type I coregulators function with the receptor at the target gene promotor level and they can either induce DNA binding, chromatin remodelling and/or recruit other transcription factors to associate with the RNA polymerase holocomplex. An example of this type of coregulator is the steroid receptor coactivator-1 (SRC-1), which has histone acetyltransferase activity (HAT); this is associated with transcriptional activity as it allows chromatin opening at the promotor of target gene (Ogryzko *et al.*, 1996; Spencer *et al.*, 1997). In addition, the SRC coactivators are known to recruit additional nuclear receptor coregulators including CBP (CREB-binding protein) and p/CAF (p300/CBP-associated factor) (Chen *et al.*, 1997). Type II coregulators' function enables the AR to be in the right conformation, to be able to bind its ligand and facilitate N/C-terminal interaction. These actions may contribute to AR protein stability and its subcellular distribution. For example, ARA70 (AR-associated protein 70 KDa) and filamin both facilitate the translocation of ligand-bound AR to the nucleus (Yeh and Chang, 1996; Ozanne *et al.*, 2000). ARA70 is also important for AR stability when AR is bound to androgens. Interestingly, ARA70 can enhance AR activity in

response to weak prostate-specific agonists like E2 (Yeh *et al.*, 1998) and also enables the AR antagonists hydroxyflutamide and casodex to behave as AR agonists (Yeh *et al.*, 1997).

Corepressors were originally identified as proteins that associate with unliganded type 2 nuclear receptors and that mediate transcriptional repression through either formation of non-productive interactions with transcription factors, or recruitment of histone deacetylase (HDAC) complexes that maintain a locally closed chromatin structure and thereby suppress target genes transcription (Muscat *et al.*, 1998). Type 2 nuclear receptors can bind to DNA in the absence of ligand, resulting in transcription silencing facilitated by the corepressors. Although type 1 nuclear receptors cannot bind to the DNA in the absence of ligand, there are examples of corepressor recruitment to the androgen-bound AR. 1. Cyclin D1, which reduces AR activity in the presence of the synthetic androgen R1881. Interestingly, Cyclin D1 repression of AR is independent of its role in cell cycle regulation (Knudsen *et al.*, 1999). 2. Calreticulin, which also inhibits AR-dependent transcription in response to R1881 and prevents AR binding to its response elements (Dedhar, 1994). 3. HBO1 (Histone acetyltransferase binding to ORC1), a member of the SAS (something about silencing) protein family, which are known to carry a HAT domain (Reifsnyder *et al.*, 1996; reviewed in Heinlein and Chang, 2002).

1.4.3 AR phosphorylation

Steroid receptor phosphorylation is well recognised from studies on PR, GR and ER, with phosphorylation occurring at serine or threonine residues. Nuclear receptor phosphorylation has been suggested to have a role in receptor cycling between the nucleus and the cytoplasm (Mendel *et al.*, 1990; Hu *et al.*, 1994) and in receptor transcriptional activity (Ali *et al.*, 1993; Gioeli *et al.*, 2002). PR is phosphorylated on multiple residues (S81, S102, and S162) in its basal state with a rapid induction in the phosphorylation of these sites in the presence of steroid (Zhang *et al.*, 1994). Another phosphorylation site identified in PR was found in the hinge region, S676 (Knotts *et al.*, 2001). In GR, at least seven phosphorylation sites have been identified, and the relative level of phosphorylation on these sites appears to be cell-cycle regulated (Bodwell *et al.*, 1991). Additionally, the phosphorylation status of GR seems to have a role in receptor stability (Webster *et al.*, 1997). Phosphorylation on S118 of ER is believed to be a ligand-dependent modification but can also result

from activation of the mitogen-activated protein kinase (MAPK) cascade in a ligand-independent manner (Kato *et al.*, 1995). Another phosphorylation site on ER is S167, which is important for the regulation of the transcriptional activity of the AF-1 domain (Joel *et al.*, 1998). Phosphorylation sites have generally not been reported in the DBD or LBD of steroid receptors, however one exception is the vitamin D receptor, which was found to be phosphorylated by (protein kinase C) PKC in the DBD (Hsieh *et al.*, 1991).

Exposure of cells to androgens leads to an increase in AR basal phosphorylation (van Laar *et al.*, 1990; van Laar *et al.*, 1991; Gioeli *et al.*, 2002). Moreover, AR phosphorylation correlates with three AR isoforms on SDS-PAGE gels (Jenster *et al.*, 1994; Wang *et al.*, 1999). Phosphorylation of the 110 KDa isoform of AR occurs rapidly, resulting in the formation of a 110-112 KDa doublet, and a third 114 KDa isoform appears upon addition of androgens (Kuiper *et al.*, 1991; Jenster *et al.*, 1994). Activation of the protein kinase A (PKA) pathway by forskolin leads to a rapid dephosphorylation of the AR, resulting in a decrease in the amount of the 112 KDa isoform (Blok *et al.*, 1998). Several studies have identified candidate phosphorylation sites on AR using *in vitro* phosphorylation assays and/or by identifying kinase consensus sites and then mutating them. The sites identified are S81, S94, S213, S515, S650 and S791 (Jenster *et al.*, 1994; Zhou *et al.*, 1995; Yeh *et al.*, 1999; Wen *et al.*, 2000; Lin *et al.*, 2001). Three of these sites (S81, S94 and S650) were identified as *in vivo* sites by another group (Gioeli *et al.*, 2002), and are homologous to the major phosphorylation sites found in PR (Denner *et al.*, 1990; Poletti and Weigel, 1993). Gioeli *et al.* (2002) did not observe phosphorylation of S515 in LNCaP cells. Interestingly, one report suggested that the S81 phosphorylation site in AR is involved in nuclear export (Black *et al.*, 2004). Mutant forms of AR that are not efficiently exported, which were found in patients with androgen insensitivity syndrome (AIS), are found to accumulate in subnuclear foci and have reduced AD phosphorylation on S81, but not on S650. In addition, one study identified S308 as a novel *in vivo* phosphorylation site (Zhu *et al.*, 2001). In a comprehensive study that was conducted in COS-1 cells expressing AR and in LNCaP cells (Gioeli *et al.*, 2002), three additional *in vivo* phosphorylation sites were identified (S16, S256 and S424). In addition, this work also showed that S650 phosphorylation is increased by forskolin, epidermal growth factor (EGF) and phrobol 12-myristate 13-acetate (PMA). This suggests that PKA, EGF and PKC

signalling have a role in the regulation of AR phosphorylation on S650. Phosphorylation at all of these sites, apart from S94, is regulated by androgen treatment of the cells. However, Gioeli *et al.* (2002) were unable to detect direct phosphorylation at any of these sites by either MAPK or PKB, although a possible role for PKC in the phosphorylation of S81 and S650 was suggested. The kinetics of the labelling of these phosphorylation sites was relatively slow, peaking at 2 hours or later, suggesting that the phosphorylation might be involved in a later event in AR signalling such as receptor recycling. Importantly, support for the role of S650 phosphorylation affecting AR transcriptional activity comes from a report showing that substitution of this site with an alanine results in a 30% reduction in AR activity in CV-1 cells (Zhou *et al.*, 1995). In contrast, substituting S81 and S94 with alanine had little effect on AR activity in another study. The positive effect of hormone on overall AR phosphorylation has been reported, although this is in parallel with an increase in the amount of AR, due to ligand-dependent stabilisation (van Laar *et al.*, 1991; Kemppainen *et al.*, 1992; Gregory *et al.*, 2001a). (for AR phosphorylation sites, see Figure 3 and table 1).

Table 1- A summary of reports on AR phosphorylation sites

All AR phosphorylation sites reported to date (indicated by ✔), *in vitro* or *in vivo* study, the cell type or method they were found by (COS-1=COS, 293=293T, LN=LNCaP, Mas. spec= mass spectrometry) and the relevant reference.

In vivo/ In vitro	S16	S80/ S81	S93/ S94	S210/ S213	S256	S308	S424	S515	S650	S790	Reference
In vitro (COS)		✔	✔								(Jenster *et al.*, 1994)
In vivo (COS, CV-1)		✔	✔						✔		(Zhou *et al.*, 1995)
In vitro								✔			(Yeh *et al.*, 1999)
In vitro (293)				✔					✔		(Wen *et al.*, 2000)
In vitro+ vivo (COS)				✔						✔	(Lin *et al.*, 2001)
In vivo (Mas spec)						✔					(Zhu *et al.*, 2001)
In vivo (COS, LN)	✔	✔	✔		✔	✔	✔		✔		(Gioeli *et al.*, 2002)

1.4.4 Androgen-regulated genes

Androgens can regulate proteins involved in different cellular processes either directly or indirectly. Among the cell-cycle regulatory genes, AR modulates cyclin-dependent kinases (CDK1, CDK2 and CDK4), cyclins (cyclin D3 and cyclin A) and also cell cycle inhibitors such as p21, p27 and p16. Another group of androgen-regulated proteins include genes that are involved in angiogenesis and metastasis. For example; the vascular endothelial growth factor (VEGF) that is enhanced by androgens and maspin, which is known to inhibit tumour invasion and motility and is downregulated by androgens.

Besides genes that positively regulate growth and metastasis, there are also genes that are regulated by androgens and have a negative role on these processes. The enzyme Neutral endo-peptidase 24.11 (NEP) cleaves and inactivates growth-stimulatory neuro peptides on the cell-surface and is upregulated by androgens. Another example is the pro-apoptotic protein Bcl-2 (B-cell lymphomas-2), whose expression inversely correlates with AR expression and androgen exposure (reviewed in Jenster, 1999).

1.5 Mechanisms for AI disease development

Understanding the transition of PCa to the AI state has been the most challenging task in the study of PCa. Studies revealed that even in AI cases the AR pathway remains important in most cancer cells. There are several routes by which alterations of the AR pathway or abnormalities in AR itself can lead to the progression of PCa. Like many other cancers it is possible that prostate tumours initially select genetic changes that increase the likelihood of mutation appearance. This might further lead to the development of mutations that allow the cells to grow independently of androgens. Many studies have found that only a few AR mutations are present in primary PCa, and that AR is frequently mutated in metastatic tumours (Taplin *et al.*, 1999; Marcelli *et al.*, 2000). Additionally, in a study that was done in TRAMP mice (transgenic adenocarcinomas of mouse prostate), more AR mutations were found in the castrated versus the intact mice (Buchanan *et al.*, 2001a; Buchanan *et al.*, 2001b). This finding supports the theory that androgen-ablation therapy provides selective pressure to target the AR signalling pathway. There are several potential mechanisms by which AI cancer can develop, and in some cases these mechanisms can appear together and have an additive or synergistic effect:

1.5.1 The hypersensitive pathway

This mechanism involves an increase in sensitivity to very low levels of androgens. PCa cells that use this mechanism still rely on AR and androgens although with a lowered threshold. There could be various ways by which cells would increase their sensitivity:

1. AR amplification- the AR gene was found amplified in 20-30% of tumours that recur during androgen-ablation therapy, whereas none of the primary tumours from the same patients before the treatment had AR amplification (Visakorpi *et al.*, 1995; Koivisto *et al.*, 1997). This mechanism seems to provide a selective advantage for survival in a low-androgen environment. By having more AR, the chance of the receptor to bind androgens is higher and this might also increase the basal target gene activity due to an increase in incorrectly folded or Hsp-free receptors.

2. Increased AR sensitivity- this results in high-level expression of AR, increased stability and enhanced nuclear localisation of AR in recurrent tumour cells. In addition, the concentration of androgen required for growth stimulation is significantly lower in these cells (Gregory *et al.*, 2001b). This suggests that AR transcriptional activity is strongly related to its level of expression and stability and could be enhanced even in low levels of androgens. One explanation for this mechanism could be the recruitment or amplification of AR coactivators, which can facilitate AR activity.

3. Increased androgen levels- increasing the local production of androgens to compensate for the overall decline in circulating testosterone. PCa can increase the rate of conversion of testosterone to the more potent androgen DHT by increasing 5α-reductase activity. In support of this mechanism is the finding that, after androgen- ablation therapy, serum testosterone levels decrease by 95%, but the DHT concentration decreases only by 60%. Moreover, african men, who have a particularly high incidence of PCa, show the highest incidence of polymorphism in the gene for 5α-reductase that results in higher enzyme activity. It is possible that tumour cells either acquire mutations or are selected for increased expression of 5α-reductase during therapy (reviewed in Feldman and Feldman, 2001).

1.5.2 *The promiscuous pathway*

Most AI tumours express AR protein. However, in some cases, the AR acquires mutations that allow the tumours to bypass the normal growth regulation by androgens and find other mechanisms to proliferate. Genetic changes can be found in many AI tumours. These changes are usually missense mutations in the AR gene that cause a decrease in its ligand specificity and allow cells to continue to proliferate and avoid apoptosis by using circulating steroid hormones and even antiandrogens when the level of androgen is low.

1. AR mutations- AR is located on the X chromosome and is not necessary for survival, therefore germ-line loss-of-function mutations in AR that lead to androgen-insensitivity are frequent. However, there is increasing evidence for somatic mutations in AR in metastatic tumours, especially after androgen-ablation therapy (Marcelli *et al.*, 2000). Many mutations in AR have been described and are generally observed in late-stage disease. The frequency of these mutations varies between 0% and 50% and most of them result in an amino acid substitution. (Culig *et al.*, 1993; Elo *et al.*, 1995; Takahashi *et al.*, 1995; Evans *et al.*, 1996; Suzuki *et al.*, 1996; Paz *et al.*, 1997; Gottlieb *et al.*, 2004) (for a map of AR mutations found in PCa and discussed in this work , see Figure 4) . Generally, these mutations can be classified into 3 groups: 1. mutations that do not affect AR function. 2. mutations that inactivate or inhibit its function. 3. mutations that alter ligand specificity. The third type of AR mutation was first identified in residue 877 in AR of the LNCaP cell line. The T877A mutation in the AR LBD makes the receptor transcriptionally active in response to non-androgen ligands such as estrogens, progestins, adrenal androgens and antiandrogens including cyproterone acetate and hydroxyflutamide (Veldscholte *et al.*, 1990). This mutation was also described in PCa patients (Gaddipati *et al.*, 1994). Similarly, the CWR22 tumour cell line has an H874Y mutation that influences binding of coactivators by affecting the conformation of helix 12 (McDonald *et al.*, 2000). Another mutation was found in the MDA PCa 2a and 2b cell lines, which were established from a patient with bone metastasis. These cells exhibit a double mutation in the AR LBD and they are less sensitive to androgens compared to LNCaP. This mutated AR has the T877A mutation as well as another substitution in amino acid 701 (L701H). L701H alone decreases AR ability's to bind androgens, but enhances the binding to other adrenal corticosteroids, particularly the glucocorticoids cortisol and cortisone. The T877A mutation however, has a

synergistic effect by increasing the affinity of AR for glucocorticoids by 300% more than L701H alone. Therefore, it is likely that physiological levels of circulating cortisol and cortisone are sufficient to promote tumour growth in patients with this double mutation (Zhao *et al.*, 1999; Zhao *et al.*, 2000). The altered receptor mechanism can also explain the clinically observed phenomenon of 'flutamide withdrawal syndrome', in which patients experience worsening of disease symptoms with flutamide, but then improve when it is withdrawn (Small and Srinivas, 1995). Interestingly, the incidence of AR mutation T877A was found to be higher in patients that were treated with flutamide compared to patients that were not treated with flutamide, who had different mutations (Taplin *et al.*, 1999). T877A mutant AR does not have the same response to other antiandrogens. Taken together, these results indicate that AR mutations can occur in response to strong selective pressure from anti-androgens treatment.

2. AR glutamine repeats- studies revealed a correlation between the length of the glutamine repeat located in the N-terminal of AR and the risk of developing PCa as well as the cancer aggressiveness and age of onset of PCa. According to these studies, a shorter glutamine repeat predicts a higher risk, higher grade and earlier onset of disease (Choong and Wilson, 1998; Kantoff *et al.*, 1998). A shortened AR possibly makes the receptor more active, resulting in a growth advantage. Evidence for this link was found in studies on african-americans, who show higher incidence of PCa compared to whites and asians and also have generally shorter glutamine repeat length (Edwards *et al.*, 1992; Irvine *et al.*, 1995).

3. Coregulator alterations- abnormal behaviour of nuclear receptor coregulators has been found to be a mechanism for tumours to progress from steroid hormone dependence to independence in various cancers. For example, SRC-1, is amplified in some breast and ovarian cancers. SRC-1 and TIF2 (transcriptional intermediary factor 2) are overexpressed in recurrent PCa specimens and cell lines (Gregory *et al.*, 2001a). ARA70, the specific AR coactivator, can facilitate the conversion of several androgen antagonists to agonists when it is expressed at an optimal level and thereby enhances AR transcriptional activity (Miyamoto *et al.*, 1998; reviewed in Feldman and Feldman, 2001).

Figure 4- AR mutations found in PCa

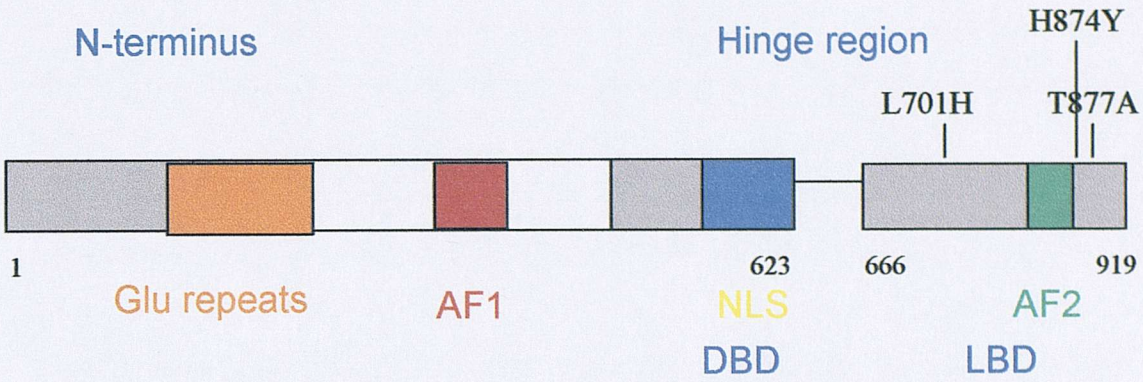


Fig 4. AR mutations found in PCa and discussed in this work. All three mutations were found in the LBD (Ligand binding domain): L701H, was found in the MDA PCa 2a and 2b cell lines, H874Y was discovered in the tumour cell line CWR22 and T877A was first identified in the LNCaP cell line and also found in the MDA PCa 2a and 2b cell lines and in PCa patients.

1.5.3 *The outlaw pathway*

This mechanism refers to cases where AR is activated in an AI manner by the effects of other pathways.

1.Growth factors- certain growth factors like insulin-like growth factor (IGF), keratinocyte growth factor (KGF) and EGF, can activate the AR and thereby induce AR target gene transcription in the absence of androgens. The finding that these growth factors are overexpressed in some PCa supports the theory that AR activity can be altered by other signalling pathways in PCa cells. The effect of growth factors on AR can be either direct or through a downstream molecule that is induced by the receptor tyrosine kinase pathway. IGF-1 induces a fivefold rise in PSA secretion in LNCaP cells. Casodex, an AR antagonist, completely blocks AR activation by IGF-1, suggesting that the AR LBD is necessary for activation by IGF-1 (Culig *et al.*, 1994).

2. Receptor tyrosine kinase pathways- activation of AR by proteins of the receptor tyrosine kinase pathway is also a mechanism that could lead to the transition to AI disease. For example, HER-2/neu, a member of the EGF-receptor family of receptor tyrosine kinases, is constitutively overexpressed in AI cell lines, and AD cell lines can be converted to AI when HER-2/neu is overexpressed. Additionally, HER-2/neu overexpression can activate AR-dependent genes in the absence of androgens (Craft *et al.*, 1999b). However, these effects cannot be blocked by casodex, indicating that the AR LBD is not involved. Similar effects were shown in

studies on breast cancer, suggesting that activation of HER-2/neu is an important mechanism for the transition to hormone-independent disease in both breast and prostate cancer. One of the possibilities is that HER-2/neu activates AR through a MAPK pathway as MAPK can phosphorylate AR *in vitro* and also activate AR activity *in vivo* (Yeh *et al.*, 1999).

3. The Akt pathway- studies on cancer samples have led to an advanced understanding on this mechanism by the discovery of the tumour suppressor PTEN (phosphatase and tensin homology). The latter was found mutated in many types of cancers including glioblastoma, breast cancer and PCa. PTEN is a lipid phosphatase that removes the 3-phosphate from its substrate phosphatidyl inositol (3,4,5)-trisphosphate. When PTEN is inactivated, PKB or Akt becomes activated and phosphorylates and inactivates several pro-apoptotic proteins and also downregulates the cell cycle inhibitor p21 (Medema *et al.*, 2000; Zhou *et al.*, 2000). Therefore, loss of PTEN can lead to enhanced activity of Akt that, in turn, can alter AR signalling directly or indirectly. Studies in LNCaP AD xenograft tumours have shown that overexpressed Akt stimulates tumour growth and downregulates p21 expression (Graff *et al.*, 2000). Additionally, Akt can be activated by HER-2/neu through phosphatidylinositol 3-kinase (PI3K) and in turn phosphorylates AR at S213 and S791, which causes an AI response of the receptor (Wen *et al.*, 2000; reviewed in Feldman and Feldman, 2001).

1.5.4 *The bypass pathway*

Alternative pathways that do not require AR are also capable of developing AI PCa. These pathways are able to facilitate proliferation and inhibit apoptosis in the absence of androgens and AR. Bcl2 is one of the bypass candidate genes that can block apoptosis. In normal prostate epithelial cells Bcl2 is not usually expressed, however, in PIN and AI tumours it is frequently expressed. Furthermore, blocking Bcl2 delays the emergence of AI in LNCaP xenograft tumours. Activation of oncogenes or inactivation of tumour suppressor genes could have a similar bypass role in the development of AI. Another way for prostate epithelial cells to bypass the AR signalling pathway is by producing their own growth factors in an autocrine fashion so they no longer rely on factors secreted by stromal cells. As mentioned earlier, these factors include IGFI and IGFII, EGF, TGFα, KGF and others. It is

likely that androgen-ablation therapy provides a selective pressure that tumours use to escape from the therapy effect by invoking these bypass mechanisms (reviewed in Jenster, 1999; Feldman and Feldman, 2001).

1.5.5 *The lurker cell pathway*

This raises the possibility that androgen-ablation fails because cells that are not dependent on androgen for growth take over and develop into an AI tumour. Various studies have investigated the idea that a subpopulation of AI tumour cells is present prior to the therapy. The candidate cells are the putative epithelial stem cells among the basal cells of the prostate, which are thought to be independent of androgens for their survival (Craft *et al.*, 1999a; Isaacs, 1999). It is believed that PCa tumours contain heterogeneous mixtures of cells that vary in their dependence on androgens for growth, and anti-androgen treatment provides selective pressure that alters the relative frequency of these cells, eventually leading to outgrowth of an AI tumour (reviewed in Feldman and Feldman, 2001).

1.6. The Wnt signalling pathway

1.6.1 The Wnt signalling pathway-an outline

β-catenin, a central component of the Wnt pathway, is thought to be one of the candidates for regulating AR activity. When overexpressed, β-catenin potentiates ligand-dependent AR activity and promotes AR activation by estradiol and the adrenal androgen androstenedione (Truica *et al.*, 2000; Chesire *et al.*, 2002; Yang *et al.*, 2002). The Wnt signalling pathway was initially characterised in Drosophila and has been shown to have roles during fly development (Wingless-type (Wnt) proteins). The Wnt signalling pathway is highly conserved between species from nematode worms to mammals, and is involved in a number of processes including embryonic development, cell polarity, morphology, motility, axis formation, adhesion, apoptosis and tumorigenesis (Akiyama, 2000; Polakis, 2000; Seidensticker and Behrens, 2000; Yamaguchi, 2001). The Wnt signalling pathway provides a network of several separate but interacting cell signalling pathways. In the so-called canonical pathway a specific Wnt (e.g Wnt1, Wnt3A, Wnt8) binds to a frizzled (Fz)/ LDL related protein 5/6 (LRP5/6) receptor complex and activates a series of events that leads to stabilisation of β-catenin, and, as a result, activation of T-cell factor

(Tcf)/lymphoid enhancer factor (Lef) target gene transcription. In Xenopus embryos this results in secondary axis formation (Du *et al.*, 1995). There are other signalling pathways that are activated by noncanonical Wnts (e.g Wnt4, Wnt5A, Wnt11). These are part of the planar-cell-polarity (PCP)-like pathway that is involved in cell morphogenetic movements in gastrulation (Heisenberg *et al.*, 2000) and the Wnt/calcium pathway. The latter leads to increases in intracellular calcium levels and activation of PKC and was discovered in zebrafish and Xenopus (reviewed in Kuhl *et al.*, 2000). Noncanonical Wnts also bind to Fz receptors and can sometime antagonise the canonical pathway (Torres *et al.*, 1996; Kuhl *et al.*, 2001). However, depending on the Wnt receptor expressed and the cell type, some Wnts can have both canonical and noncanonical properties, for example Wnt5A (He *et al.*, 1997).

Several secreted proteins have been identified that are Wnt antagonists and these can be classified into two groups; the secreted frizzled related proteins (sFRPs) that bind to the Wnt ligands and exert antagonistic activity on Wnt signalling by competeing with the Fz receptors. sFRPs bear homology to the Fz receptor gene but lack the seven transmembrane domain (reviewed in Polakis, 2000; Jones and Jomary, 2002). sFRPs may also antagonise signalling by binding directly to Fzs and forming inactive complexes with the receptors (reviewed in Miller *et al.*, 1999; Akiyama, 2000; Polakis, 2000). Although sFRPs are considered as tumour supressor genes, no mutations in sFRPs have been associated with human disease (Jones and Jomary, 2002). This class of antagonists also includes the secreted inhibitors WNT-inhibitory factor-1 (WIF-1) and Cerberus. Like sFRPs, these inhibitors can bind directly to Wnts (Miller *et al.*, 1999). Cerberus also antagonises nodal and bone morphogenetic proteins and therefore has a broader anti-growth role (Miller *et al.*, 1999). The second class of Wnt antagonists includes Dickkopf (Dkk) proteins, which bind to LRP5/6 (Mao *et al.*, 2001a). Dkk-1, is a p53 target gene and sensitises cells to apoptosis by inhibiting the Wnt signalling pathway (Shou *et al.*, 2002). Therefore, in theory, the sFRP class of antagonists can inhibit both canonical and noncanonical pathways, whereas the Dkk class can only inhibit the canonical pathway.

β-catenin regulates cell-cell adhesion (by binding to cadherins) as well as Wnt-mediated transcription (by binding to Tcf/Lef transcription factors) and thus has an important role in embryonic development and tumorigenesis. β-catenin localises in cells to two different pools: in epithelial cells most of it is found in the cell

membrane. There, it provides a link between the cytosolic tail of E-cadherin and α-catenin, which binds to the cytoskeleton, hence it's involvement in cell-cell adhesion (Fodde *et al.*, 2001b). The smaller pool is located in the nucleus and cytoplasm, and this pool is involved in the Wnt signalling pathway. Briefly, in the absence of Wnt ligand, cytoplasmic β-catenin is a target for the ubiquitin-proteasome pathway, and phosphorylation by casein kinase I α (CKIα) and glycogen synthase kinase 3-β (GSK3β) is required for its ubiquitination. Cytoplasmic β-catenin is bound to a multicomponent complex of proteins, which includes Axin and adenomatous polyposis coli (APC) that both bind β-catenin and GSK3β, to facilitate β-catenin phosphorylation followed by its β-transducin repeat-containing protein (β-TrCP)-mediated ubiquitination. Upon Wnt ligand binding Axin translocates to the membrane, the destruction complex is inactivated and unphosphorylated β-catenin accumulates in the cytoplasm and translocates to the nucleus. In the nucleus, β-catenin activates transcription of certain target genes by binding to the nuclear transcription factors Tcf/Lef. The dual functions of β-catenin can influence each other. For example, β-catenin/Tcf signalling can be affected by the loss of E-cadherin expression (Kikuchi, 2003).

1.6.2 The canonical pathway and Wnt-mediated transcription

The Wnt signal begins when Wnts act on target cells by binding to their receptors (for structure of Wnt signalling pathway components, see Figure 5a. for the canonical Wnt signalling pathway outline, see Figure 5b). There are 19 Wnt genes in humans, and they all encode secreted glycoproteins with a conserved sequence of 23-24 cysteine residues (Miller, 2002). The original members of the Wnt gene family are the mouse Wnt-1 and the Drosophila wingless (wg). Mutations in the wg locus result in alteration in the segmental pattern of larve and embryonic lethality (Nusslein-Volhard, 1980). Wnt receptors are members of the frizzled family of transmembrane proteins (Fz). The 10 human Fzs identified have an N-terminal cysteine-rich extracellular domain (CRD) and seven transmembrane domains ending with an intracellular C-terminal domain. Specific Wnts bind to a specific Fz at the extracellular domain, altering the interaction between the extracellular and transmembrane domains (for Fz structure, see Figure 5a). This results in a rearrangement of the cytosloic domain of the receptor and activates a downstream

signal (Miller *et al.*, 1999; Jones and Jomary, 2002). Members of the low-density lipoprotein (LDL) related protein superfamily 5/6, LRP5/6, are also required as receptors for the canonical Wnt signaling pathway (Pinson *et al.*, 2000; Tamai *et al.*, 2000; Wehrli *et al.*, 2000). It is believed that binding of Wnt proteins to their target Fz is involved in the formation of a membrane-located complex that includes Fz and LRP5/6. Despite the evidence that the Fzs and LRP proteins are receptors for the Wnt proteins, it is not fully understood how they transduce signals.

Wnt causes the translocation of Axin to the membrane and enhances the interaction between Axin and LRP5 (Mao *et al.*, 2001b). The activated receptor also transduces a signal to the cytoplasmic protein dishevelled (Dvl), which leads to inhibition of GSK3β phosphorylation of β-catenin in an unkown mechanism. Dvl is a phosphoprotein with several interaction motifs; the N-terminal DIX domain, which is important for homodimerisation, the C-terminal DEP domain that is required for Dvl recruitment to the cell membrane and the central PDZ domain. The DIX and PDZ domains of Dvl are required for β-catenin stabilisation, and both interact with Axin. Studies have shown that casein kinase Iε (CKIε) binds the PDZ domain of Dvl and that CKIε overexpression results in Dvl hyperphosphorylation (Kikuchi, 2000; Seidensticker and Behrens, 2000). Another component that has been suggested to phosphorylate Dvl is casein kinase II (CKII), which also promotes the formation of lymphomas when expressed in transgenic mice (Song *et al.*, 2000). Therefore, one proposed mechanism is that Wnt leads to the phosphorylation of Dvl and this in turn antagonises the action of GSK3β. Dvl is also thought to promote Axin dephosphorylation and activate Jun kinases (Peifer and Polakis, 2000; Polakis, 2000) (for Dvl structure, see Figure 5a).

Free β-catenin accumulates in the cytoplasm and translocates to the nucleus. There are several models in regards to β-catenin translocation to the nucleus. The first one suggests that it can shuttle freely to the nucleus as it is structurally similar to the nuclear import receptor, importin-β, and can therefore interact directly with the nuclear pore complex (Henderson and Fagotto, 2002). It is believed that its localisation is controlled by a balance between its nuclear and cytoplasmic binding partners. The second model speculates that, in non-stimulated cells, APC actively exports β-catenin from the nucleus to the cytoplasm; and, conversely, in Wnt-stimulated cells or when the Wnt pathway is aberrantly activated, β-catenin is

capable of shuttling between the nucleus and the cytoplasm independently of APC (Rosin-Arbesfeld *et al.*, 2000; Henderson and Fagotto, 2002). Lastly, recent studies in Drosophila discovered two proteins, named Legless (Lgs) and Pygopus (Pygo), which play a role in β-catenin nuclear translocation. Lgs contains NLS domain and Pygo is constitutively nuclear. According to this model, Lgs facilitates β-catenin nuclear entry and Pygo serves to retain both Lgs and β-catenin in the nucleus, thereby presenting the latter to Tcf for transcriptional activation (Townsley *et al.*, 2004).

In the nucleus, β-catenin binds to Tcf/Lef transcription factors to facilitate transcription of target genes. The Tcf/Lef family contains four members in mammals; LEF-1, Tcf-1, Tcf-3 and Tcf-4. They contain a high mobility group box (HMG) that has the ability to bind specific sequences of DNA and induce a significant bend that attracts other transcription factors (van Noort and Clevers, 2002) (for Tcf structure, see Figure 5a). Tcf/Lefs can also repress transcription by interacting with the corepressors Groucho, CtBP (C-terminal binding protein), or by binding to CBP. The Groucho proteins bind to the central region of Tcf/Lefs and are able to inhibit gene activation even in the presence of β-catenin (Polakis, 2000; Seidensticker and Behrens, 2000). CtBP binds the C-terminus of Tcf-3 but cannot bind to LEF-1. CBPs are usually coactivators, however, they act as corepressors by acetylating Tcf at a conserved lysine site in the armadillo-binding domain and thus reducing the interaction between Tcf and β-catenin (Akiyama, 2000; Polakis, 2000; Seidensticker and Behrens, 2000). Recently, several regulators were found to be involved in Tcf/β-catenin transactivation activity. For example, Tcf/β-catenin complexes can be phosphorylated by NEMO-like kinase (NLK) and prevented from binding to DNA. Other regulators include SMAD4, Pontin 52, Duplin and retinoids (Akiyama, 2000; Kikuchi, 2000; Polakis, 2000; Seidensticker and Behrens, 2000). Activation of Tcf/β-catenin complexes enables transcription of many different target genes. The subset of genes that is transcribed is influenced by the specific Wnt and Fz combinations and other cellular pathways downstream to the receptor. The best known target genes are c-myc and cyclin D1, which are known to promote cell proliferation. Expression of c-myc and cyclin D1, by activation of β-catenin, promotes G1-S transition and cell cycling (He *et al.*, 1998; Shtutman *et al.*, 1999; Tetsu and McCormick, 1999). Additional target genes include, members of the AP-1

family (c-jun, fra-1), which are immediate early genes and involved in proliferation, differentiation, apoptosis and oncogenic transformation (Mann *et al.*, 1999). Another Wnt-mediated target gene, matrilysin, is an extracellular matrix protease that is thought to be involved in tumour progression (Brabletz *et al.*, 1999; Crawford *et al.*, 1999). Tcf-1, is a β-catenin/Tcf-target gene with tumour suppression function and its epithelial isoform acts as a dominant-negative form as it lacks its β-catenin binding domain (Roose *et al.*, 1999). Develomental genes include siamois and twin (Brannon *et al.*, 1997; Laurent *et al.*, 1997) (for key Wnt-mediated target genes, see table 2).

Figure 5a-Wnt signalling pathway components

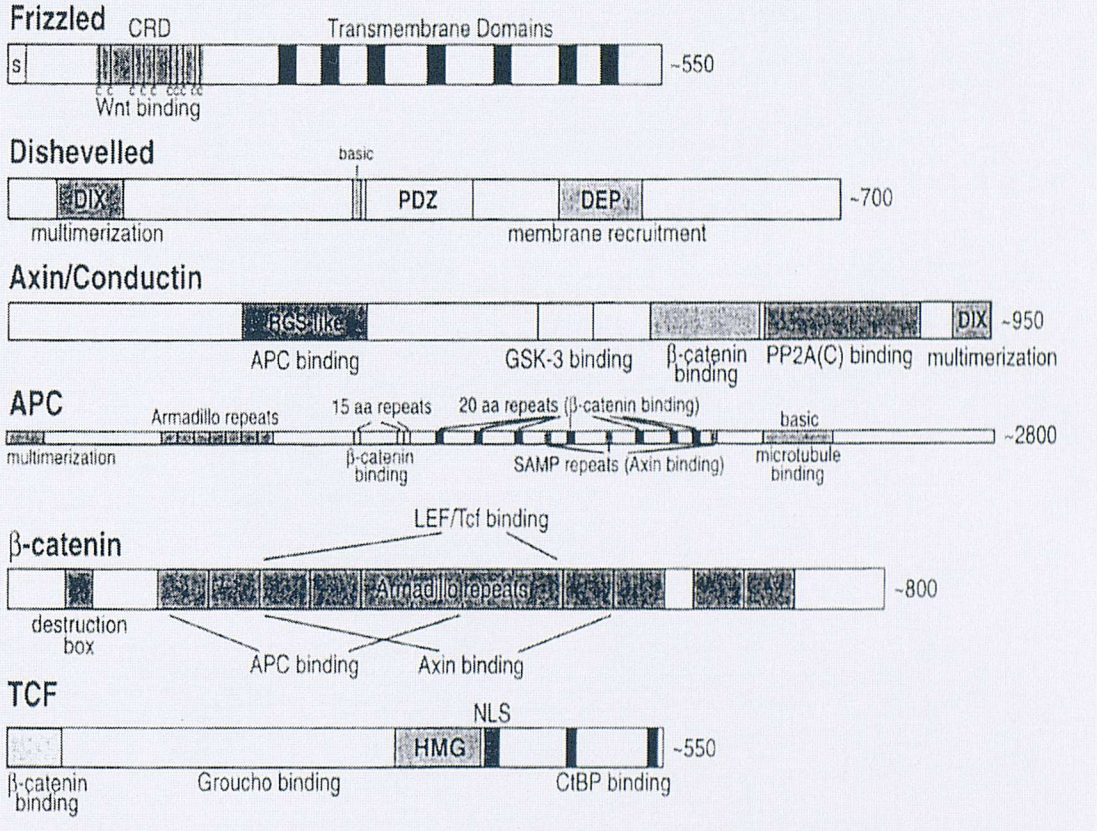


Fig 5a. Schematic representation of several Wnt signalling pathway components. Major domains and interacting proteins are indicated. For Frizzled, c indicates conserved cysteines, s indicates signal sequence. Based on Miller *et al.* review (1999) (Miller *et al.*, 1999)

Figure 5b-The canonical Wnt signalling pathway

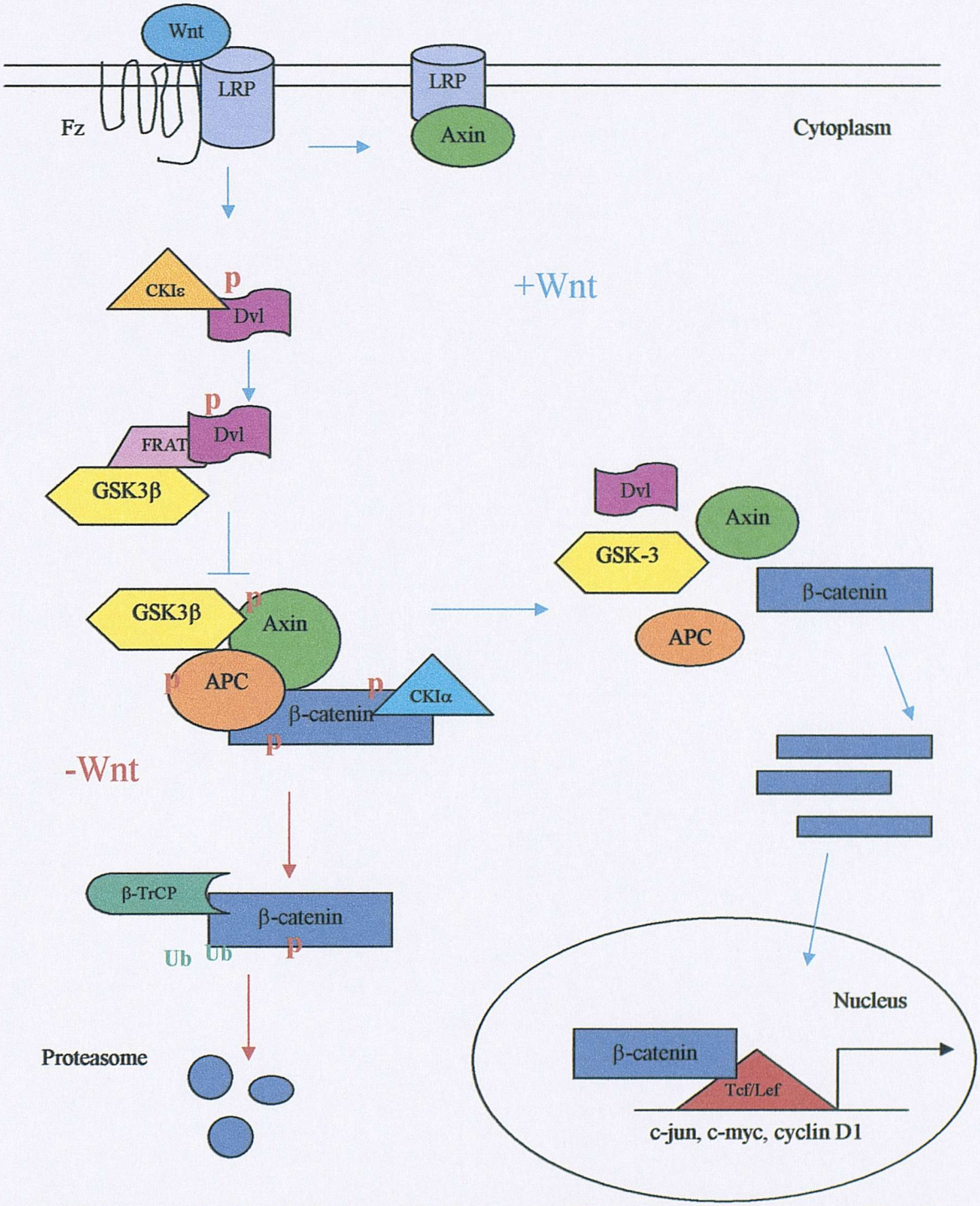


Fig 5b. Diagram showing the key events in the canonical Wnt pathway. In the absence of ligand (-Wnt), demonstrated by red arrows, β-catenin is found in its destruction complex including Axin, GSK3β and APC. β-catenin phosphoryation by CKIα and GSK3β triggers its ubiquitination and degradation in the proteasome. In the presence of the Wnt ligand (+Wnt), demonstrated by blue arrows, Axin is recruited to the plasma membrane and binds to LRP5 (LRP). Dvl is also recruited to the plasma membrane and is being phosphorylated by CK1ε. Phosphorylated Dvl interacts with FRAT, which in turn sequesters GSK3β from the destruction complex. This leads to inhibition of GSK3β phosphorylation of β-catenin, dissociation of β-catenin from the destruction complex followed by its accumulation in the cytoplasm and translocation to the nucleus for initiation of target genes transcription by binding to Tcf/Lef. P indicates phosphorylation, Ub indicates ubiquitination.

Table 2- Wnt-mediated target genes

Key target genes that are up regulated by Wnt, their function and the organism/system they were found in.

Gene	Organism/system	Function	Ref.
c-myc	human colon cancer	Transcription factor	He 1998
Cyclin D	human colon cancer	Cell cycle regulation	Tetsu 1999 Shtutman 1999
c-jun	human colon cancer	Component of the AP-1 transcription factor	Mann B, 1999
fra-1	human colon cancer	Component of the AP-1 transcription factor	Mann B, 1999
matrix metalloproteinase MMP-7	human colon cancer	Zinc endopeptidase	Brabletz 1999 Crawford 1999
Axin-2	human colon cancer	Component of the Wnt signalling pathway	Yan, 2001 Lustig, 2002 Jho, 2002
claudin-1	human colon cancer	Tight junction component	Miwa 2002
matrix metalloproteinase-26	Human carcinoma	Zinc endopeptidase	Marchenko 2002
Id2	human colon cancer	Dominant negative helix-loop-helix protein, regulates proliferation and differentiation	Rockman 2001 Willert 2002
Tcf-1	human colon cancer	Transcription factor	Roose 1999
Siamois	Xenopus	Homebox transcription factor (regulates axis formation)	Brannon 1997
engrailed-2	Xenopus	Homebox transcription factor (regulates neural patterning)	McGrew 1999
twin	Xenopus	Homebox transcription factor (regulates axis formation)	Laurent 1997
nacre	Zebrafish	Pigment cell differentiation	Dorsky, 2000 Saito 2002 Yasumoto 2002
Cdx1	Mouse Wnt-3A	Homebox transcription factor	Lickert 2000

1.7 β-catenin and its destruction complex

β-catenin turnover in the cytoplasm is mediated by a destruction complex that includes Axin, APC and GSK3β. GSK3β is an essential negative regulator of the Wnt signalling as it binds and phosphorylates several proteins in the canonical pathway. Most importantly, GSK3β phosphorylates one or more highly conserved serine/threonine residues near the N-terminus of β-catenin. However, GSK3β is unable to bind β-catenin directly and requires Axin and APC. Phosphorylation of Axin and APC by GSK3β promotes their actions as scaffold proteins that bring together GSK3β and β-catenin in the destruction complex (reviewed in Karim *et al.*, 2004).

1.7.1 β-catenin

Structure: β-catenin consists of 780 amino acids with an N-terminal domain that contains the α-catenin binding site and the GSK3β phosphorylation sites, a central domain that consists of 12 armadillo repeats and a highly acidic C-terminal domain with a transactivation function for target gene activation. The armadillo repeats consist of 42 amino acids (residues 134-671) that interact with APC, E-cadherin and Tcf/Lef factors. Each repeat consists of three α helices (except for repeat 7) which stack upon each other and together form a superhelix with a positively-charged groove that is essential for these interactions (for β-catenin structure, see Figure 5a).

Phosphorylation and regulation: In the absence of Wnt ligand, the cytosolic pool of β-catenin has a short half-life. CKIα primely phosporylates β-catenin on S45 and this triggers β-catenin for phosphorylation on S33, S37 and T41 by GSK3β. Following phosphorylation by GSK3β, β-catenin is recognised by a component of an E3 ubiquitin ligase, β-TrCP, that binds to β-catenin through its WD40 domains. D32 and G34 residues in β-catenin are necessary for the interaction of phosphorylated-β-catenin with β-TrCP. Using its F-box, β-TrCP recruits to the complex other factors such as Skp1 and Cul1, which mediate ubiqiuitin ligation of β-catenin by attachment of a polyubiquitin chain, which is followed by degradation by the 26S-proteasome (reviewed in Polakis, 2000).

β-catenin stability is regulated by other proteins and signalling pathways. Mutations in presenilin, which are associated with Alzheimer's disease, decrease β-

catenin stability in neurons, resulting in increased apoptosis (Zhang *et al.*, 1998). Integrin-linked kinase promotes β-catenin stabilisation and nuclear aaccumulation through downregulation of GSK3β activity (Novak *et al.*, 1998).

β-catenin-interacting proteins can either promote or inhibit its transcriptional activity. Examples of proteins that promote its activity include Teashirt (Tsh), which was found in Drosophila and binds to the C-terminal domain of β-catenin (Gallet *et al.*, 1999) and the chromatin remodelling factor, Brg-1, which binds to β-catenin in the nucleus (Barker *et al.*, 2001). In addition, recent reports suggested that BCL9-2, a homolog of the human proto-oncogene product BCL9 (B cell lymphoma 9), interacts with β-catenin and increases β-catenin-dependent transcription. RNA interference of BCL9-2 in the colon cancer cells, SW480, induced an epithelial phenotype and led to β-catenin translocation from the nucleus to the cell membrane. It is believed that the switch between β-catenin adhesive and transcriptional functions is modulated by phosphorylation on T142 of β-catenin, which favours BCL9-2 binding and precludes interaction with α-catenin (Brembeck *et al.*, 2004; Harris and Peifer, 2005). Examples of proteins that bind to β-catenin and have an inhibitory effect on its transcriptional activity include ICAT, which inhibits the interaction between β-catenin and Tcf (Tago *et al.*, 2000), and the nuclear antagonist of β-catenin, Chibby (Takemaru *et al.*, 2003).

Interestingly, Siah-1 (seven-in-absentia homologue) mediates p53-inducible degradation of β-catenin in a phosphorylation-independent pathway (Liu *et al.*, 2001; Matsuzawa and Reed, 2001). Since Siah-1 downregulates a form of β-catenin in which the phosphorylation sites are mutated, Siah-1 is likely to promote degradation of β-catenin independently of CKIα or GSK3β-mediated phosphorylation. Moreover, Siah-1 binds to the C-terminal domain of APC, and β-catenin downregulation by Siah-1 is dependent on APC.

Mutations and role in cancer: Mutations in the β-catenin gene, CTNNB1, are mainly missense and affect the N-terminal phosporylation sites. Missense mutations in D32 and G34, which are necessary for the interaction with β-TrCP, have also been reported. These mutations abrogate the phosphorylation-dependent interaction of β-catenin with the ubiquitination machinery, resulting in stabilisation of β-catenin (Yost *et al.*, 1996; Akiyama, 2000). In addition to missense mutations, a number of

deletion mutants that remove one or more of the putative GSK3β sites have been isolated. Expression of an amino-terminal truncated form of β-catenin, lacking the GSK3β-phosphorylation sites in the epidermis of transgenic mice leads to a formation of hair follicle-related tumours (Gat *et al.*, 1998). Cell lines harboring activating mutations of β-catenin often express high levels of cytoplasmic and nuclear β-catenin and constitutive activation of Tcf/Lef reporter plasmids (Korinek *et al.*, 1997). These data indicate that the increasing level of β-catenin can be sufficient to promote tumour formation and implicate the amino terminal phosphorylation sites as potential targets for the oncogenic activation of β-catenin. Indeed, β-catenin was found mutated or elevated in a number of human carcinomas (for a list of reported mutations in β-catenin associated with human cancers, see table 3). In colon cancer, the majority of the tumours contain APC mutations and the frequency of mutations in β-catenin is relatively low (Sparks *et al.*, 1998; Samowitz *et al.*, 1999). The exclusivity of β-catenin and APC mutations in colon cancer is also evident from analysis of replication error-positive tumours identified by microsatellite instability. Both the hereditary and sporadic forms of replication error-positive colorectal cancers have a relatively high frequency of β-catenin mutation, whereas APC mutations are relatively rare (Mirabelli-Primdahl *et al.*, 1999; Miyaki *et al.*, 1999).

Several mutations in β-catenin have been found in gastric cancers, which occur with increased incidence in familial adenomatous polyposis (FAP) patients that carry an APC mutation in their germline (Park *et al.*, 1999).

In three separate studies, mutations in β-catenin were identified at high frequency in hepatoblastoma while no APC mutations were found in these tumours (Koch *et al.*, 1999; Jeng *et al.*, 2000; Wei *et al.*, 2000). β-catenin was mutated in 20% of hepatocellular carcinomas (HCC). Moreover, β-catenin was found nuclear in the invasive and intravascular compartments of HCC tumours (Huang *et al.*, 1999; Nhieu *et al.*, 1999). Interestingly, the β-catenin target genes, c-myc and cyclin D1, were found amplified in a subset of HCC (Tetsu and McCormick, 1999). β-catenin mutations might occur as a second "hit" in HCC tumour progression in a cancer pathway initiated by c-myc (de La Coste *et al.*, 1998).

In medulloblastoma, β-catenin was found mutated in a small percentage of tumours where mutations in APC have not been detected (Zurawel *et al.*, 1998).

Other cancers, such as endometrial and ovarian tumours, contain activating mutations in β-catenin and these do not occur with increased incidence in patients with FAP (Gamallo *et al.*, 1999; Wright *et al.*, 1999). The β-catenin mutations associated with ovarian cancer appeared to be confined to the endometrioid subtype. Additionally, in this tissue, cancers with activated β-catenin signalling are less aggressive than their nonactivated counterparts (Wright *et al.*, 1999).

Another type of cancer with β-catenin mutations, albeit at low frequency, is melanoma. Although only one of sixty-five melanomas contained β-catenin gene mutations, β-catenin protein was found to be nuclear in one-third of tumours (Rimm *et al.*, 1999). Therefore, additional mechanisms for β-catenin activation are likely to occur in these tumours.

The highest percentage of β-catenin mutations, with at least 75% of mutations in the N-terminal region of CTNNB1, was found in a common skin tumour known as pilomatricoma (Chan *et al.*, 1999). Studies on transgenic mice expressing mutant β-catenin in the skin indicate that these tumours originate from the hair follicle, which is consistent with the lack of hair in mice harbouring mutations in Lef (van Genderen *et al.*, 1994; Gat *et al.*, 1998).

Table 3-β catenin mutations found in human cancers

Amino acinds affected by mutations in CTNNB1 gene are indicated. The frequency of mutations (freq.) in each tumour is represented as the number of tumours with mutations/total number of tumours examined. The amino acid affected by the mutation and the number of mutations found at each position are listed. Δ indicates interstitial deletion. In some cases, more than one mutation was found in a single tumour. Based on Polakis *et al.* review (2000)(Polakis, 2000)

tissue	freq.	S29	Y30	L31	D32	S33	G34	I35	H36	S37	G38	A39	T40	T41	T42	A43	P44	S45	L46	S47	G48	K49	Δ	reference
colorectal	9/202						1							3				5					0	Samowitz 1999
colorectal	2/92													1				1						Kitaeva 1997
colorectal-w/o APC mutation	7/58																						7	Iwao 1998
colorectal-w/o APC mutation	13/27					2	1							3				5					1	Sparks 1998
colorectal HNPCC	12/28				2		2			1				2				5						Miyaki 1999
colorectal w/ MSI	13/53																	6						Mirabelli-primdahl 1999
colorectal w/o MSI	0/27																							Mirabelli-primdahl 1999
desmoid, sporadic	1/1													1									0	Shitoh 1999
desmoid, sporadic	22/42													10				12						Tejpar 1999
endometrial w/ MSI	3/9				2	1																		Mirabelli-primdahl 1999
endometrial w/o MSI	10/20				3	1	2			3				1										Mirabelli-primdahl 1999
gastric, intestinal-type	7/26	2			5																			Park 1999
gastric, diffuse-type	0/17																							Park 1999
hepatocellular w/HCV	9/22				3	1				3				1				2						Huang 1999
hepatocellular	12/35				1	1	2	1	1				1	2				2		1			2	Van Nhieu 1999
hepatocellular	6/26				2		1			1								1					1	de la Coste 1998
hepatocellular	14/75				5	1	1							1				4					2	Miyoshi 1998
hepatocellular	21/119				3	3	1	1		2				4				8					2	Legoix 1999
hepatoblastoma, sporadic	8/9						2			1								1					4	Jeng 2000
hepatoblastoma, sporadic	27/52				2		3			1				5									16	Koch 1999
hepatoblastoma	12/18				2		1							1				1					7	Wei 2000
kidney, Wilm's tumor	6/40													1				2					3	Koesters 1999
medulloblastoma, sporaic	3/67					2				1														Zurawel 1998
melanoma	1/65																	1						Garcia-Rostan 1999
ovarian, endometriod	7/13				3	1				2				1									0	Gamallo 1999
ovarian, endometriod	3/11									2				1										Palacios 1998
ovarian, endometriod	10/63					2	2			6														Wright 1999
pancreatic tumors	0/111																							Gerdes 1999
pilomatricoma	12/16				2	4	3			2				1										Chan 1999
prostate cancer	5/104				1	2								1				1						Voeller 1998
thyroid, anaplastic	19/31					1			1	3	1		8	2	1	1	4	2	1	2		9	0	Garcia-rostan 1999

1.7.2 APC

Structure: APC encodes a 300 KDa protein; the N-terminal domain contains an oligomerisation domain followed by seven repeats of an armadillo motif. The armadillo motif binds to KIF3 (kinesin super-family 3), a protein that regulate vesicle transport on microtubules, and to Asef, which is involved in cell migration. Additionally, PP2A (protein phosphatase 2A) binds to the N-terminal domain of APC. The middle portion contains three binding repeats and seven repeats more C-terminal to these (Fodde *et al.*, 2001b). Both types of repeats are able to bind β-catenin in its armadillo repeat region, which is also the binding-region for E-cadherin, Tcf and Axin. Therefore, the interactions of β-catenin with all these proteins are mutually exclusive (Morin, 1999; Kikuchi, 2000). The RGS (regulator of G protein signalling) domain of Axin interacts with the SAMP (Ser-Ala-Met-Pro) repeats which are located in between the C-terminal repeats that bind β-catenin (Kikuchi, 2000). Following the seven-20 aa repeats is a basic amino acid cluster region, which directly binds to microtubules (Smith *et al.*, 1994) and another microtubule-binding protein EB1 (Su *et al.*, 1995). The C-terminal domain also has an S/TXV motif that interacts with human Dlg (Drosophila discs large tumour suppressor protein) homolog, which is believed to have a role in cell cycle progression and neuronal function (Matsumine *et al.*, 1996) (for APC structure, see Figure 5a).

Regulation of β-catenin and the cytoskeleton: APC is recognised as a tumour suppressor and mediates β-catenin downregulation by providing proximity between GSK3β and β-catenin. APC is ubiquitously expressed and has been found both in the cytoplasm and in the nucleus (Akiyama, 2000). APC also participates in several cellular processes including cell adhesion, cell migration, signal transduction, microtubule assembly and chromosome segregation and apoptosis (Morin, 1999; Fodde, 2002). The subcellular localisation of APC was found to be important in maintaining the structural integrity of the cytoskeleton (Fodde, 2002) and it provides chromosomal stability.

In the destruction complex of β-catenin GSK3β phosphorylates APC and thereby facilitates its binding to β-catenin and decreases APC binding to microtubules (Zumbrunn *et al.*, 2001). GSK3β preferentially binds to APC/β-catenin complexes rather than β-catenin alone in cancer cells (Morin, 1999).

A recent study showed that APC binds to CtBP and acts as an adaptor between β-catenin and CtBP. This results in APC/β-catenin complexes being sequestered from Tcf, thus lowering the availability of nuclear β-catenin (Hamada and Bienz, 2004).

APC localises to clusters at microtubule ends (Nathke *et al.*, 1996; Mimori-Kiyosue *et al.*, 2000). The clusters result from movement of APC along the microtubules towards the growing ends. APC also interacts with a microtubule plus-end-directed motor protein, KIF3, through an association with the kinesin superfamily-associated protein KAP3 (Jimbo *et al.*, 2002). The interaction between APC and KAP3 is required for the accumulation of APC in clusters. Therefore, a C-terminal truncated form of APC does not accumulate in clusters.

APC also localises to the external face of kinetochores where microtubules attach to chromosomes (Fodde *et al.*, 2001b). Cells that express a mutant APC that lacks the C-terminal region are prone to errors in chromosome segregation.

In addition to association with microtubules, APC is likely to connect to actin filaments. APC interacts with Asef, activates its ability to stimulate the GDP/GTP exchange of Rac and induces lamellipodia formation and ruffling of the plasma membrane, thereby stimulating cell migration (Kawasaki *et al.*, 2000). Although APC localises mainly to microtubules, when this interaction is disrupted it associates with the actin filaments.

Mutations and role in cancer: APC was identified on the gene responsible for FAP, an autosomal inherited disease that leads to the development of multiple tumours, especially to colorectal carcinomas. Mutations of APC are frequently identified in FAP and colorectal cancers, but are rare in other cancers (Polakis, 2000; Fodde *et al.*, 2001b). In FAP, germ-line mutations are scattered throughout the 5' half of the APC gene. APC is mutated in 85% of colon tumours, and the majority of the sporadic mutations located on the central region of APC, which typically result in C-terminal truncation of the gene. These truncated proteins can still bind to β-catenin but not to Axin, suggesting that the APC-Axin interaction is required for APC tumour suppression function (Akiyama, 2000; Seidensticker and Behrens, 2000). Moreover, mice lacking w.t APC but expressing a truncated mutant APC retaining a single Axin binding site are viable and do not develop intestinal neoplasia (Smits *et al.*, 1999). Selective pressure is directed against the presence of Axin-

binding sites, because these sites are critical in the regulation of β-catenin. The remaining N-terminal truncated form of APC may affect cell migration by activating Asef, leading to metastasis. Moreover, since APC has a role in microtubule attachment and stabilisation of kinetochores, truncated mutation in APC may be linked to the genetic instability, which is another early event in colorectal cancer.

The mutation cluster region (MCR) in APC is located between codons 1250-1500. A correlation between the presence of a germ-line mutation in the MCR and the severity of polyposis has been noted (Wu *et al.*, 1998; Ficari *et al.*, 2000). However, the enhanced severity of polyposis suggests there should also be selective pressure for somatic mutations in the MCR. Indeed, somatic mutations were found in the MCR and their appearance in this region seems to be relative to the position of the germ-line mutations in FAP (Lamlum *et al.*, 1999). In whichever case, the degradation of β-catenin is impaired when APC is mutated, leading to stabilisation of β-catenin and activation of target gene transcription.

1.7.3 Axin

Structure: Axin is a scaffold protein that provides proximity for physical interaction between several binding proteins. Axin consists of an N-terminal RGS (regulator of G protein signalling) domain that binds to APC, a central domain for binding to GSK3β, β-catenin and CKIα, and the DIX domain in the C-terminus (for Axin structure, see Figure 5a). The DIX domain is crucial for binding to Dvl and for oligomerisation of Axin itself. Oligomerisation of Axin is essential for its role as a negative regulator in the Wnt pathway as C-terminal mutants are unable to inhibit transcription (Akiyama, 2000; Seidensticker and Behrens, 2000). The site phosphorylated by GSK3β is adjacent to the N-terminal region of the GSK3β-binding site (Ikeda *et al.*, 1998).

Phosphorylation and regulation: Phosphorylation of Axin by GSK3β increases its stability and binding to β-catenin, which in turn facilitates the interaction between GSK3β and β-catenin and phosphorylation of the latter by GSK3β (Yamamoto *et al.*, 1999). Expression of Axin induces the downregulation and inactivation of β-catenin in several cell lines (Kishida *et al.*, 1998; Ross *et al.*, 2000; Kishida *et al.*, 2001; Reya *et al.*, 2003). Studies using the mouse Fused locus encoding Axin and xenopus embryos injected with Axin mRNA indicated that Axin inhibits dorsal axis formation

by interfering with signalling through the Wnt pathway (Zeng *et al.*, 1997). This suggests that Axin regulates an early step in embryonic axis formation in mammals and amphibians. One mechanism for β-catenin dissociation from the destruction complex involves interaction between Dvl/FRAT-1/PP2A, degradation and dephosphorylation of Axin (Kikuchi, 2000). FRAT-1 (frequently rearranged in advanced T-cell lymphomas) is a GSK3-binding protein and it can bind to GSK3β, the PDZ domain of Dvl and also complex with Axin (Seidensticker and Behrens, 2000). Wnt-mediated phosphorylation of Dvl by CKIε increases Dvl affinity for FRAT-1, which binds to and inhibits GSK3β (Kishida *et al.*, 2001; Lee *et al.*, 2001). Therefore, the interaction between FRAT-1 and Dvl is believed to be involved in the dissociation of the β-catenin destruction complex. PP2A is a serine/threonine protein phosphatase and *in vitro* studies indicate it is one of the major candidates for Axin dephosphorylation. Axin binds directly to the PP2A catalytic subunit (Hsu *et al.*, 1999). PP2A mutations have been found in primary colon tumours and the B56 subunit of PP2A interacts with APC to downregulate β-catenin (Peifer and Polakis, 2000; Polakis, 2000). Dephosphorylation of Axin during Wnt signalling leads to Axin instability, which weakens its binding to β-catenin and may also prevent GSK3β from phosphorylating β-catenin. The interaction between Axin and APC is important for tumour suppression although mutants of Axin lacking the APC binding domain can still downregulate β-catenin (Akiyama, 2000).

The binding between Axin and LRP5 is believed to be important for the Wnt signalling pathway. Wnts induce Axin translocation to the plasma membrane where it binds to LRP5, and the LRP5 sequences involved in binding to Axin are required for Lef activation (Mao *et al.*, 2001b).

Dvl and Axin have the DIX domain that is important for homodimerisation and necessary for binding to intracellular vesicles and actin filaments (Capelluto *et al.*, 2002). This might suggest that Axin and Dvl are involved in regulation of receptor-mediated endocytosis of components of the Wnt signalling pathway.

Two additional Axin-interacting proteins have been identified: Diversin, which can also bind and recruit CKIε to the destruction complex of β-catenin (Schwarz-Romond *et al.*, 2002) and I-mfa (inhibitor of myogenic basic helix-loop-helix transcriptional factor)-domain containing protein, which interacts with Axin and decreases the inhibitory effect of Axin on β-catenin (Kusano and Raab-Traub, 2002).

Mutations and role in cancer: Based on its ability to downregulate β-catenin, Axin is expected to be a tumour suppressor. Indeed, studies have reported biallelic inactivation of Axin in HCC's (Satoh *et al.*, 2000). These mutations were found in carcinomas with intact genes of β-catenin and APC. All of these mutations generated truncated forms of Axin that lacked the β-catenin-binding site.

An Axin-related protein in mice, named conductin or Axin 2, has similar biochemical characteristics to Axin and is also able to downregulate β-catenin when it is overexpressed (Behrens *et al.*, 1998). However, conductin is not able to suppress Axin mutations in HCC. More recent reports suggested that Axin 2 is a direct β-catenin/Tcf target gene, which is upregulated by Wnt and provide a mechanism for suppressing the Wnt signal (Yan *et al.*, 2001; Jho *et al.*, 2002; Lustig *et al.*, 2002).

1.8 Glycogen Synthase Kinase-3

Glycogen Synthase Kinase 3 (GSK-3) is a multifunctional serine/threonine kinase highly abundant in brain and found in all eukaryotes. It was first discovered as the enzyme capable of phosphorylating and inactivating glycogen synthase and thus preventing glycogen synthesis (Woodgett and Cohen, 1984). Apart from its involvement in glycogen metabolism, GSK-3 is also a key component of other signalling pathways such as those regulated by receptor tyrosine kinases and G-protein coupled receptors, and also the Wnts. GSK-3 is known to be involved in different cellular processes including cell-cycle regulation, cell adhesion, proliferation and apoptosis. Given its contribution to many cellular processes, it is not surprising that GSK-3 has been implicated in several human diseases including diabetes, Alzheimer's disease, bipolar disorder and cancer (reviewed in Doble and Woodgett, 2003).

1.8.1 GSK-3 structure and phosphorylation sites

GSK-3 found in mammalian cells exists in two isoforms, α and β that are encoded by distinct genes. GSK3α has a mass of 51 KDa and GSK3β is 47 KDa. Both isoforms share 98% homology in their kinase domains and 36% identity in their C-termini (Woodgett, 1990) (for GSK-3 structure, see Figure 6a). Although structurally similar, they exhibit different functions and cannot always compensate for each

other. For example, GSK3α is unable to rescue the degenerative liver phenotype caused by the loss of GSK3β in mice (Hoeflich *et al.*, 2000). GSK3β is better characterised than GSK3α, and GSK3β has been studied in more detail in the context of the Wnt signalling pathway. Here, GSK3α and GSK3β will be referred to as GSK-3 when results apply to both forms.

GSK-3 has a preference for target proteins that are pre-phosphorylated at a 'priming' residue located C-terminal to the GSK-3-phosphorylation site (Fiol *et al.*, 1987). The consensus sequence for a GSK-3 substrate is Ser/Thr-X-X-X-Ser/Thr-P, where the first Ser/Thr is the target residue and the last is the priming site (X=any amino acid, but often proline). The efficiency of phosphorylation of primed substrates is 100-1000 fold higher (Thomas *et al.*, 1999). The pre-phosphorylated residue is believed to bind to a positively charged pocket in GSK-3 that both optimises the orientation of the kinase domain and places the substrate at the correct position for phosphorylation in the catalytic groove. Mutation of arginine 96 to alanine (R96A), in GSK3β disrupts the positively charged pocket so that primed substrates can no longer bind, although the enzyme retains its activity towards unprimed substrates (Frame *et al.*, 2001).

Studies of the crystal structure of GSK-3 have revealed that the activation loop (the T-loop) is tyrosine-phosphorylated at Y216 and Y279 in GSK3β and GSK3α, respectively. This could play a role in forcing open the substrate-binding site (Dajani *et al.*, 2001) (for GSK-3 phosphorylation sites, see Figure 6a). Although the kinase responsible for this phosphorylation has not been identified in mammalian cells, one candidate, named Zak1, was found to phosphorylate the Y216 equivalent in the slime mould Dictyostelium discoideum (Kim *et al.*, 1999). It is also possible that tyrosine phosphorylation in GSK-3 is an autocatalytic event (Wang *et al.*, 1994). Apoptotic stimuli such as staurosporine treatment or neurotrophic factor withdrawl increase GSK-3 activity and enhance tyrosine phosphorylation in certain neuronal cells (Bhat *et al.*, 2000; Bijur and Jope, 2001). In addition, LPA (lysophosphatidic acid), which is known to cause apoptosis in adult neurons, also leads to an increase in GSK-3 tyrosine phosphorylation (Sayas *et al.*, 2002). These findings suggest a role for tyrosine phosphorylation of GSK-3 in apoptosis.

Another major phosphorylation site in GSK3β and GSK3α is inhibitory and occurs on Serine 9 and Serine 21, respectively. Stimulation of cells with insulin

causes inactivation of GSK-3. This occurs via PI3K-induced activation of PKB, which then phosphorylates and inactivates GSK-3 (Cross *et al.*, 1995). Other stimuli that lead to inactivation of GSK-3 through serine phosphorylation include the growth factors EGF and PDGF, which stimulate the GSK3β-inactivating kinase called MAPK-activated protein kinase-1 (MAPKAP-K1) (Brady *et al.*, 1998), PKC activators (Fang *et al.*, 2002), PKA activators (Li *et al.*, 2000) and p70 ribosomal S6 kinase-1 (S6K1) activators (Armstrong *et al.*, 2001). Phosphorylation of S9/S21 creates a primed pseudosubstrate in GSK-3 that binds to the positively charged pocket. The N-terminal domain folds back onto arginine 96 in the catalytic domain, thus acting as a competitive inhibitor for true substrates (primed or unprimed) (reviewed in Doble and Woodgett, 2003).

Figure 6a- GSK-3 structure and phosphorylation sites

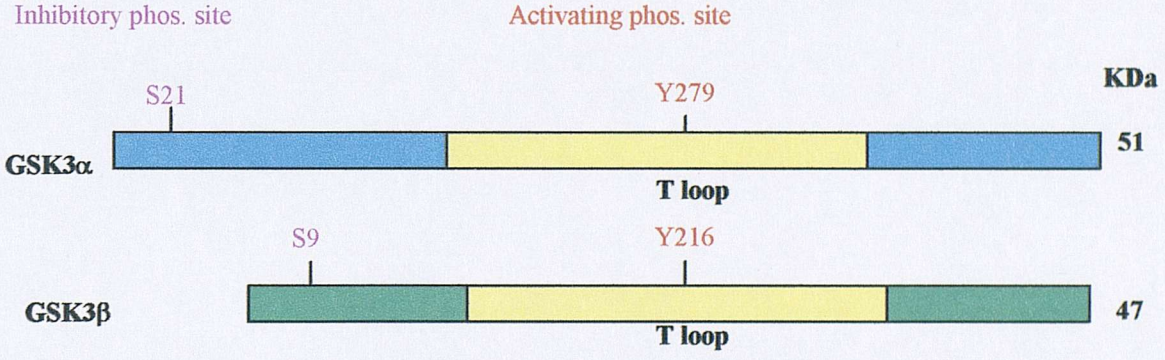


Fig 6a. Schematic representation of the main domains in GSK3α and GSK3β. Inhibitory phosphorylation site on serine are indicated in pink and activating phosphorylation site on tyrosine are indicated in red. Kinase domain (in yellow) with the T-loop and their mass (KDa) are also indicated.

1.8.2 GSK3β in the Wnt signalling pathway

GSK3β phosphorylates β-catenin on serine and threonine residues in the N–terminal region, and this targets for ubiquitination and degradation by the proteasome. Several reports have determined that β-catenin is a primed substrate for GSK3β as it gets phosphorylated by CKIα on S45 (Amit *et al.*, 2002; Hagen and Vidal-Puig, 2002; Liu *et al.*, 2002; Yanagawa *et al.*, 2002).

Expression of FRAT-1 stimulates β-catenin/Tcf signalling because it sequesters GSK3β from the β-catenin-degradation complex (Li *et al.*, 1999). A small peptide derived from FRAT called FRAT-tide is sufficient to prevent Axin binding to

GSK3β and phosphorylation of Axin and β-catenin due to an overlap in the binding sites for FRAT and Axin in GSK3β (Thomas *et al.*, 1999; Bax *et al.*, 2001). A mutant of GSK3β that cannot bind to FRAT accumulates in the nucleus, suggesting that FRAT is also involved in the nuclear export of GSK3β (Franca-Koh *et al.*, 2002). Smalley *et al.* (1999) found that expression of a domain of Axin that interacts with GSK3β but not with β-catenin, activated β–catenin/Tcf signaling in a similar manner to FRAT (Smalley *et al.*, 1999). Surprisingly, FRAT homologues have not been identified in Drosophila or C. eleganc. Moreover, the targeted inactivation of all three FRAT genes in mice has no phenotype, indicating that FRAT is not a core component of the Wnt pathway.

Interestingly, GSK3β bound to Axin does not become phosphorylated on S9 in response to insulin, restricting the effects of insulin to the pool of GSK3β, which is not bound to Axin. Conversely, Wnt signalling does not appear to affect insulin signalling (Ding *et al.*, 2000). This indicates that GSK3β function in the Wnt signalling pathway is insulated from GSK3β regulators that are not part of the Wnt pathway, possibly due to an effective sequestration of GSK3β by Axin in the destruction complex (reviewed in Doble and Woodgett, 2003).

1.8.3 Other pathways and substrates to GSK-3

GSK-3 plays a role in the regulation of various pathways such as glycogen synthesis, nuclear export of cyclin D1 and STAT (signal transducer and activator of transcription), and the hedgehog pathway. Phosphorylation by GSK-3 often has an inhibitory effect on its substrates. Consistent with this, GSK-3 activity tends to be negatively-regulated by ligands such as insulin, EGF and Wnt. Signal transduction initiated by Wnt, for example, leads to GSK3β inactivation and hence activation of Wnt/β-catenin-mediated transcription (reviewed in Doble and Woodgett, 2003).

Glycogen metabolism- Insulin stimulates the dephosphorylation and activation of glycogen synthase, the rate-limiting enzyme of glycogen synthesis. This occurs by a combination of events, including regulation of phosphatase activity, activation of PI3K and subsequent PKB phosphorylation and inactivation of GSK-3. Moreover, insulin, by inhibiting GSK-3, also stimulates the dephosphorylation and activation of the initiation factor 2B, leading to an increased rate of protein synthesis (Welsh and Proud, 1993; Welsh *et al.*, 1998; Frame and Cohen, 2001).

The hedgehog pathway (Hh)- Recent studies in Drosophila discovered another function for GSK-3 in the Hh pathway. Interestingly, the Hh and the Wnt signalling pathways are somewhat similar (Jia *et al.*, 2002; Price and Kalderon, 2002). First, both Wnt and Hh are secreted proteins involved in embryonic patterning. Second, rather than β–catenin, the Hh pathway uses the protein Cubitus interruptus (Ci) to transduce its signal to the nucleus, and, in the absence of ligand, Ci is targeted for partial proteolysis (Ingham and McMahon, 2001). GSK-3, in combination with CKI and PKA, phosphorylates Ci155, to target it for proteolysis in the absence of a Hh signal.

Cell adhesion- GSK-3 plays an important role in cell adhesion by regulating the epithelial-mesenchymal transition (EMT) that occurs during embryonic development and tumour progression. GSK3β binds and phosphorylates the zinc-finger transcription factor Snail, which is a repressor of E-cadherin and an inducer of the EMT (Zhou *et al.*, 2004). GSK3β phosphorylates Snail at two sites; the first site regulates its β-TrCP-mediated ubiquitination and the second controls its cellular localisation. Inhibition of GSK-3 results in the upregulation of Snail and downregulation of E-cadherin *in vivo*. Recently, another study demonstrated that GSK-3 inhibits the transcription of Snail and thereby maintains E-cadherin expression (Bachelder *et al.*, 2005).

Cell cycle- GSK-3 phosphorylates cyclin D1 on T286, which triggers its nuclear export and subsequent proteolysis (Alt *et al.*, 2000). This phosphorylation is enhanced if cyclin D1 is bound to CDK4, thus inhibiting entry into S-phase (Diehl *et al.*, 1998). Treatment of rat intestinal epithelial cells with the GSK-3 inhibitor LiCl, increases cyclin D1 protein levels (Shao *et al.*, 2000).

Transcriptional control- GSK-3 phosphorylates and regulates the activity of several transcription factors. For example, GSK-3 phosphorylates c-Jun at T239, S243 and S249 in a region proximal to the DNA-binding domain, which results in decreased binding of c-Jun to DNA and activation of transcription. The same phosphorylation sites are conserved in the JunB and JunD isoforms as well as in the oncogenic form v-Jun (Boyle *et al.*, 1991; de Groot *et al.*, 1993; Nikolakaki *et al.*, 1993).

Another factor phosphorylated by GSK-3 is C/EBPα (CCAAT enhancer binding protein α), a transcription factor that controls the expression of many genes and is

required for preadipocyte differentiation. GSK-3 phosphorylates C/EBPα on two threonine residues (222 and 226). An unidentified kinase may prime phosphorylates on T226 by phosphorylating T230. In 3T3-L1 preadipocytes, C/EBPα becomes dephosphorylated in response to insulin and LiCl (Ross *et al.*, 1999).

C-myc, the immediate early gene transcription factor that plays important roles in the regulation of cell proliferation, differentiation and apoptosis, can be phosphorylated both by ERK2 (extracellular signal-regulated kinase 2) and GSK-3. ERK2 is the priming kinase phosphorylating c-myc on S62 and this in turn triggers GSK-3 phosphorylation on T58, which targets c-myc for ubiquitin-mediated degradation (Sears *et al.*, 2000).

The nuclear factor of activated T-cells c (NFATc) is involved in the regulation of interleukin-2 production and T-cell proliferation when it is not phosphorylated. NFATc can be phosphorylated at several sites clustered in the NFAT homology domain. This phosphorylation is inhibitory and prevents its binding to DNA. At least some of these sites can be phosphorylated by GSK-3 *in vitro*, and overexpression of GSK-3 promotes NFATc nuclear export (Beals *et al.*, 1997).

Heat-shock factor-1 (HSF-1) is a key regulator of heat-shock genes. In resting conditions its activity is suppressed by phosphorylation on S303 by GSK-3, which is dependent on prior phosphorylation on S307 by ERK1 (Chu *et al.*, 1998). In Xenopus oocytes, 25 mM LiCl elevates heat-shock-induced DNA binding of HSF-1 and delays attenuation of DNA binding during recovery from heat shock (Xavier *et al.*, 2000).

Apoptosis and inflammation –GSK-3 plays an important role in apoptosis. It was first discovered to induce apoptosis in pheochromocytoma (PC12) cells and Rat-1 fibroblasts. In neuroblastoma cells, LiCl treatment protects cells from apoptosis induced by heat shock, whereas overexpression of GSK-3 has the opposite effect (Bijur *et al.*, 2000). Similarly, LiCl and two other GSK-3 inhibitors (SB216763 and SB415286) protect cerebellar granule neurons from apoptosis (Cross *et al.*, 2001). The mechanism by which the inhibition of GSK-3 suppresses apoptosis is not fully understood, but it involves the PI3K cell survival pathway. It is known that survival factors that inhibit GSK-3 via PI3K and PKB do not stabilise β-catenin and β-catenin/Tcf-dependent gene transcription. However, it is possible that β-catenin/Tcf target genes are involved in apoptosis regulation, since GSK-3 inhibition via Wnt1

can inhibit apoptosis by activating β-catenin/Tcf-mediated transcription in Rat-1 fibroblasts (Chen *et al.*, 2001). In addition, it has been shown that the tumour suppressor p53 binds and activates GSK-3 in the nucleus of neuroblastoma cells treated with an inhibitor that induces DNA damage and stimulates p53 response (Watcharasit *et al.*, 2002).

In contrast with the pro-apoptotic effect of GSK-3 in neuronal cells, mice that do not express GSK3β die in utero as a result of increasing apoptosis in the liver (Hoeflich *et al.*, 2000). Possibly this is caused by an imbalance in the anti-apoptotic signalling induced by tumour necrosis factor-α (TNF-α) as a similar phenotype has been observed in mice harbouring defects in the NF-kB pathway (Davis *et al.*, 2001). This suggests that GSK3β is required for the nuclear function of NF-kB, and implies that inhibition of GSK3β is important in inflammatory responses. In summary, GSK-3 seems to play an important, but tissue-specific, role in apoptosis.

1.8.4 GSK-3 in human disease

Many of the pathways that GSK-3 is involved in are linked to human diseases. Theoretically, GSK-3 would seem to be a potential tumour suppressor, however, no mutations or deletions in its coding gene have been found in human tumours (reviewed in Polakis, 2000). The Wnt and Hedgehog pathways are both involved in several forms of human cancer. Additionally, GSK-3 is linked to Alzheimer's disease, since GSK-3-mediated hyperphosphorylation of tau, which is associated with neurofibrillary tangles, is one of the hallmarks of this disease. In normal brain, tau is a soluble protein that stabilises microtubules and promotes their polymerisation. Hyperphosphorylation of tau leads to its assembly into filaments and inability to bind to microtubules (Yoshida and Ihara, 1993; Leclerc *et al.*, 2001). These events are believed to underlie the degeneration of nerve cells. Many kinases, including GSK-3, have been shown to phosphorylate tau *in vitro*, and several lines of evidence have shown that GSK-3 can also phosphorylate tau *in vivo* (Hanger *et al.*, 1992; Mandelkow *et al.*, 1992). For example, LiCl induces partial dephosphorylation of tau in the brain and affects microtubule stability (Munoz-Montano *et al.*, 1997). Morover, insulin and IGF-1 induce tau dephosphorylation in human neuronal NT2N cells via PI3K, suggesting that tau phophorylation is GSK-3 dependent (Bramblett *et al.*, 1993). Several genes have been genetically associated with Alzheimer's. One of

them is Presenilin1, which was found to associate with β-catenin independently of the Wnt pathway (Kosik, 1998). However, the effects of this interaction are controversial, with one report suggesting destabilisation of β-catenin and induction of neuronal apoptosis (Zhang *et al.*, 1998) and the other indicating no effect.

The use of LiCl revealed an implication for GSK-3 in other neurodegenerative diseases such as bipolar disorder, and epilepsy (Chen 1999, Manji 1999)(Chen *et al.*, 1999).

Apart from a role in neurological pathologies, GSK-3 also plays a role in the development of non-insulin-dependent diabetes mellitus (NIDDM/ diabetes type II). The initial stages of the disease are characterised by resistance to insulin in peripheral tissues, which later leads to inability of the pancreas to produce enough insulin. Levels and activity of GSK-3 are moderately elevated in diabetic mice (Eldar-Finkelman *et al.*, 1999). Selective maleimide-based GSK-3 inhibitors, named SB415286 and SB216763, have a great therapeutic potential for the treatment of the disease. In liver cells, these inhibitors promote the dephosphorylation and activation of glycogen synthase, facilitating the conversion from glucose to glycogen (Coghlan *et al.*, 2000). In addition, these compounds were able to repress the expression of genes encoding the enzymes that control hepatic gluconeogenesis (Lochhead *et al.*, 2001). This suggests that GSK-3 inhibitors might be able to suppress the production of glucose by the liver, as well as enhance its conversion into glycogen. Recently, several other potent inhibitors of GSK-3 were studied. CHIR 99021, and the closely related compounds, CHIR 98023 and CHIR 98014, also promoted the activation of glycogen synthase and stimulated glycogen deposition in the liver. Interestingly, in zucker diabetic fatty (ZDF) rats, these compounds were found effective in lowering the concentrations of glucose and insulin, reflecting improved sensitivity to insulin (Cline *et al.*, 2002; Ring *et al.*, 2003).

1.8.5 Inhibition of GSK-3

Given the link between abnormal GSK-3 activity and various human diseases, interest in finding appropriate inhibitors of GSK-3 has increased in the last few years. LiCl, the best characterised GSK-3 inhibitor, primarily used as a therapeutic agent for bipolar disorder, is also used in Alzheimer's disease, epilepsy and studies on diabetes type II. LiCl inhibits through competing with magnesium (Mg^{2+}), but not

with the substrate or ATP (Klein and Melton, 1996; Ryves and Harwood, 2001). LiCl inhibition of GSK-3 also occurs indirectly by inceasing of the inhibitory serine phosphorylation site in GSK-3 (Klein and Melton, 1996). However, LiCl is not a selective inhibitor of GSK-3 and it is known to affect other enzymes and pathways that complicate interpretation of any results obtained. These include inhibition of polyphosphate 1-phosphatase, inositol monophosphatase, casein kinase-II (CKII), MAP kinase-activated protein kinase-2 (MAPKAP-K2) and p38-regulated/activated kinase (PRAK) (Berridge *et al.*, 1989; Davies *et al.*, 2000), as well as activation of PKB and JNK in cellular pathways (Chalecka-Franaszek and Chuang, 1999; Yuan *et al.*, 1999). Several new GSK-3 inhibitors have been recently developed, most of which are ATP competitive. SB415286 and SB216763, developed by Glaxo SmithKline (GSK), are small, highly potent and very selective for GSK-3 (Coghlan *et al.*, 2000) (Smith *et al.*, 2001) (for chemical structures of SB415286 and SB216763, see Figure 6b). They were found to be neuroprotective in primary neurons induced to die by inhibition of the PI3K pathway (Cross *et al.*, 2001). Interestingly, treatment with these compounds was found to be more potent than FRAT-1 overexpression in mammalian cells in terms of neuroprotection (Culbert *et al.*, 2001). Moreover, Coghlan *et al.* (2000) showed that these compounds are able to induce β-catenin/Tcf-mediated transcription in HEK 293 cells in a dose-dependent manner (maximal effective concentration of 5 μM and 30 μM for SB216763 and SB415286, respectively). Although LiCl also induces β-catenin/Tcf-mediated transcription in this assay, it also stimulates the response of a control β-catenin-independent reporter (Coghlan *et al.*, 2000).

Recently, studies on new GSK-3 inhibitors, named CHIR 99021 (Cline *et al.*, 2002; Ring *et al.*, 2003), AR-A014418 (Bhat *et al.*, 2003) and bis-7-indolylmaleimide (Kuo *et al.*, 2003) revealed they are highly potent and very specific. As mentioned earlier, CHIR 99021, and closely related compounds, were studied in rodent models of type II diabetes and found very efficient.

Interestingly, the new natural product 6-bromoindirubin and its synthetic, cell-permeable derivative, 6-bromoindirubin-3'-oxime (BIO), were recently discovered to have selective inhibitory effects for GSK-3. Cocrystal studies showed that BIO interacts with the ATP binding pocket of GSK3β. BIO inhibits the phosphorylation

on Y276 in GSK3α and Y216 in GSK3β (Meijer *et al.*, 2003), and is also able to activate the Wnt pathway in studies on embryonic stem cells (Sato *et al.*, 2004).

Other GSK-3 inhibitors include the bivalent form of zinc, which mimics the action of insulin (Ilouz *et al.*, 2002), the paullone type compounds (for example, kenpaullone), which are ATP competitive and also inhibit CDK's (Schultz *et al.*, 1999), beryllium (Be^{2+}), another metal ion, which competes for Mg^{2+} and ATP and can inhibit GSK-3 to half its maximal activity *in vitro* (Ryves *et al.*, 2002). Additionally, small thiadiazolidinones (TDZD) derivatives, are non-ATP competitive GSK-3 inhibitors and are believed to interact with the primed phosphate substrate binding site of GSK-3 and thereby inhibit its function (Martinez *et al.*, 2002).

Figure 6b- The chemical structure of SB415286 and SB216763

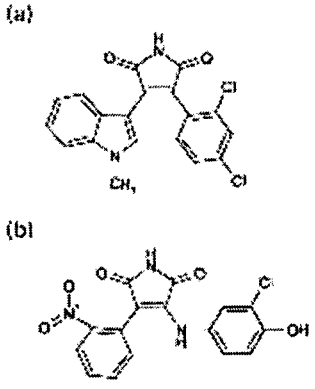


Fig 6b. SB216763 (a) and SB415286 (b) are maleimide-based inhibitors.

1.8.6 GSK3β regulation of AR and PCa

Several studies have suggested a potential link between AR and GSK3β signalling pathways. First, testosterone, but not estrogen, prevents the heat-shock-induced overexpression of GSK3β, suggesting a role for androgen as a neuroprotective agent (Papasozomenos and Shanavas, 2002). Second, GSK3β inactivation results in increased nuclear levels of β-catenin, which is believed to augment AR activity in PCa cells (Sharma *et al.*, 2002). However, several reports on the role of GSK3β in the regulation of AR activity and growth of PCa cells contribute contradicting results to this research. Salas *et al.* (2004) showed that GSK3β phosphorylates AR and inhibits its transcriptional activity directly when the Akt pathway is bypassed (Salas

et al., 2004). The PI3K inhibitor, LY294002, causes an increase in AR phosphorylation while LiCl leads to a reduction in AR phosphorylation in PCa cells stably expressing a constitutively-active construct of GSK3β. Another group supported these results by showing that GSK3β phosphorylates and binds the AR N-terminal domain *in vitro* and inactivates it in COS-1 and LNCaP cells (Wang *et al.*, 2004). Moreover, Wang *et al.* (2004) also showed that treatment with LiCl blocks the inhibitory effect of GSK-3 on AR in COS-1 cells, and a constitutively-active construct of GSK3β (S9A) leads to a growth arrest in the PCa cell line CWR22R. According to this group, GSK3β directly influences AR activity, since GSK3β affects the AF-1 activity of AR and not AF-2, which is involved in the regulation of AR by β-catenin. In contrast, a third group found that GSK3β is required for androgen-stimulated expression of PSA promotor activity and they observed a reduction in AR activity in PCa cells in the presence of GSK-3 chemical inhibitors or GSK3β siRNA (Liao *et al.*, 2004). Additionally the latter group also showed an increase in GSK3β Y216 phosphorylation in androgen-stimulated LNCaP cells. This was suppressed by PI3K inhibitors, however, further experiments suggested that Akt is not involved in this regulation.

1.9 The Wnt signalling pathway in human cancer

The Wnt signalling pathway and its components are involved in many human carcinomas and the development of neoplastic processes from initiation, proliferation and progression to mutation accumulation. The interest in Wnt gene involvement in human cancer increased with the identification of Wnt-1 as an oncogene in mouse mammary tumours. However, to date, mutations or amplification of genes encoding Wnt ligands or receptors have not been reported in cancer (reviewed in Miller *et al.*, 1999; Polakis, 2000). On the other hand, several intracellular components of the Wnt pathway including APC, Axin and β-catenin, have been implicated in human cancers (for details on mutations and role of β-catenin, APC and Axin in cancer, see section 1.7). The Wnt signalling pathway is aberrantly activated in tumours of many tissues, including the prostate. The best studied carcinoma, in which the canonical Wnt signalling pathway is activated, is colon cancer (Morin, 1999; Seidensticker and Behrens, 2000). Tumours with mutations in the tumour suppressor APC have

increased level of free β-catenin. Interestingly, in 50% of colon carcinomas with intact APC, the β-catenin gene (CTNNB1) is mutated (Morin, 1999; Peifer and Polakis, 2000; Fodde, 2002). Some tumours were found to have mutations outside the regulatory region in CTNNB1 and these are believed to affect cell adhesion (Morin, 1999). Another common tumour that has mutations in genes involved in the Wnt pathway is hepatocellular carcinoma (HCC). Missense mutations and interstitial deletions were found in β-catenin in HCC tumours. In addition, Axin was found mutated in 10% of HCC tumours when β-catenin was not mutated (Buendia, 2000; Polakis, 2000; Tannapfel and Wittekind, 2002). In gastric cancer, apart from mutations in the CTNNB1 gene, tumours also have germline mutations in E-cadherin, APC promotor hypermethylation, Fz upregulation and down regulation of the Wnt antagonist sFRP (Groden, 2000; To *et al.*, 2001). Other human tumours that show accumulation of β-catenin protein or mutations in the β-catenin gene include melanoma, ovarian cancer, cervical cancer, medullublastoma and lung adenocarcinoma (reviewed in Polakis, 2000; Karim *et al.*, 2004).

1.9.1 Evidence for β-catenin/Wnt signalling involvement in PCa

Given the oncogenic activity of β-catenin and its connection to human cancers, interest in its expression and function in PCa has increased in the last few years. About 5% of primary prostate tumours have activating mutations in β-catenin, most of which consist of missense changes in the conserved residues important for its phosphorylation-dependent degradation (Voeller *et al.*, 1998; Chesire *et al.*, 2000; Gerstein *et al.*, 2002; de la Taille *et al.*, 2003) (for a summary of β-catenin mutations found in PCa, see Figure 7). In addition, the mutations are present focally suggesting they occur during tumour progression. In another report a similar rate of mutation was found when screening the same region of β-catenin in primary prostate tumours and additionally, the mutation-positive tumours accumulated β-catenin in the nucleus (Chesire *et al.*, 2000). In all the studies that examined β-catenin mutations in PCa, only one alteration, which was a 23 codon (Δ24-47) deletion of the entire GSK3β phosphorylation site, was found in advanced cases. A similar observation was reported in colon cancer studies where more β-catenin missense changes were found in small colorectal adenomas compared with in adenocarcinomas, and interstitial

deletions were exclusively found in adenocarcinomas, suggesting that deletions in β-catenin are associated with more aggressive types of cancer (Iwao *et al.*, 1998; Samowitz *et al.*, 1999). The detection of β-catenin missense alterations in primary PCa tumours possibly indicates that β-catenin contributes to early tumour formation and/or less aggressive cancer. One interesting *in vivo* study that was carried out in normal prostate using transgenic mice shed a different light on these conclusions. Gounari *et al.* (2002) engineered transgenic mice to express mutant β-catenin that lacks the entire GSK3β phosphorylation domain (Catnb +/lox(ex3)). These mice developed PIN-like lesions, which are associated with early disease that can progress to PCa, indicating that stabilised β-catenin induces prostate growth. However, these PIN-like lesions did not progress to an advanced prostate carcinoma. This suggests that β-catenin deregulation might not be sufficient to promote malignancy but have a role in the development of early tumour formation (Gounari *et al.*, 2002).

Importantly, Chesire *et al.* (Chesire *et al.*, 2002) showed that 25% of metastatic tumours contained nuclear β-catenin, but that the β-catenin gene was normal in all but one of the tumours examined. Another report on β-catenin localisation showed that it was aberrantly localised in the cytoplasm and/or nucleus of 23% and 39% of primary and advanced tumours, respectively (de la Taille *et al.*, 2003). A study by Gerstein *et al.* (2002) reported a loss of APC in a small number of AI lesions, raising the possibility that this could be the cause for the aberrant β-catenin localisation reported by de la Taille *et al.* (2003). In addition to gene loss, altered expression of APC in advanced PCa might be related to promotor hypermethylation (Maruyama *et al.*, 2002). However, APC is involved in other processes in addition to control of β-catenin. For example, APC has a positive role in chromosomal stability (Fodde *et al.*, 2001a; Kaplan *et al.*, 2001). Another member of the Wnt pathway that was found altered in PCa is the E3 ubiquitin ligase component, β-TrCP, which binds the phosphorylated β-catenin and activates its ubiquitination-dependent degradation (Gerstein *et al.*, 2002). E-cadherin, which is involved in cell-cell adhesion and also binds to β-catenin, was reported to have altered expression in 50% of PCa tumours (Umbas *et al.*, 1992). One hypothesis suggests that altered E-cadherin expression promotes tumour progression not only by allowing loss of adhesion but also by affecting β-catenin intracellular balance, which, in turn, might lead to an increase in β-catenin/Tcf transcriptional activity. Taken together, these results suggest that

processes other than mutations in β-catenin alone might play an important role in the progression of PCa.

Truica *et al.* (2000) were the first to report that β-catenin, especially an active form of β-catenin, enhances AR-dependent transcription in an AD manner in the LNCaP PCa cell line (Truica *et al.*, 2000). Using the immunoprecipitation technique in LNCaP cells they also demonstrated that AR and β-catenin form a complex. The interaction between AR and β-catenin was detected in the absence and presence of androgens, however, addition of hormone increased the amount of complex. Their results also suggested a role for β-catenin in the transition to the AI state by showing that β-catenin can relieve the suppression of the anti-androgen bicalutamide on AD transcription and change the sensitivity of AR to ligand such that it responds to weak adrenal androgens (for example, androstenedione) and estradiol. Another study demonstrated a ligand-dependent protein-protein interaction between AR and β-catenin using the Yeast Two Hybrid System. This requires the LBD of AR, and the first six armadillo repeats of β-catenin (Yang *et al.*, 2002). Interestingly, although LXXLL motifs, which are known to be important in most coactivators for binding and modulating nuclear receptor activity, are present in β-catenin, they do not seem to be involved in the regulation of AR transactivation (Yang *et al.*, 2002; Song *et al.*, 2003). Immunolocalisation assays using overexpressed β-catenin and AR revealed that β-catenin is predominantly cytoplasmic in the absence of androgens and upon androgen treatment, translocates to the nucleus with the agonist-bound AR to activate AR-mediated transcription (Mulholland *et al.*, 2002; Pawlowski *et al.*, 2002; Yang *et al.*, 2002). Although Chesire *et al.* (2002) were unable to detect β-catenin ligand-mediated nuclear localisation in PCa cells, they did show that β-catenin localises to the nucleus during testosterone-induced prostate gland re-growth in a castrated rat model (Chesire *et al.*, 2002). Moreover, ectopic expression of E-cadherin in E-cadherin-negative cells leads to redistribution of β-catenin to the cell membrane and a reduction in AR activity (Yang *et al.*, 2002). Interestingly, a recent report indicated that Wnt3A-conditioned medium induces the formation of AR/β-catenin complexes in the nucleus (Verras *et al.*, 2004). Moreover, in support of the other reports, they also documented that these complexes were more abundant in LNCaP cells in the presence of androgens.

Several reports have investigated the possibility that β-catenin also binds and regulates the transactivation of other nuclear receptors. Their results demonstrated that, similar to enhancement of AR transcriptional activity, β-catenin also coactivates RAR and VDR (Easwaran *et al.*, 1999; Palmer *et al.*, 2001; Pawlowski *et al.*, 2002; Yang *et al.*, 2002). Interestingly, although the AR LBD shares a high degree of conservation with the LBD of ER, PR and GR and much less homology with RAR and VDR, a cross-regulation between β-catenin and the former group of receptors was not detected.

There is increasing evidence that activation of AR-dependent transcription has an inhibitory effect on Wnt-dependent transcription due to a competition between AR and Tcf/Lef transcription factors over binding to β-catenin (Chesire and Isaacs, 2002; Pawlowski *et al.*, 2002; Mulholland *et al.*, 2003; Song *et al.*, 2003). Using transient transfections in CV-1 and GnRH neuronal cells, two separate studies have shown that liganded AR represses β-catenin/Tcf-responsive promotor activity and Tcf inhibits AR independently of β-catenin (Pawlowski *et al.*, 2002; Song *et al.*, 2003). Mullholand et al. (2003) used GFP and HcRed-fused proteins to quantify the reciprocal balance of β-catenin between Tcf-4 and AR using SW480 cells transfected with AR. This group reported that the same amount of protein colocalisation of β-catenin and AR or β-catenin and Tcf-4 was found in cells that were treated with DHT or in untreaed cells, respectively. They also showed that the anti-androgen, casodex, reduces the AR-mediated inhibition of Tcf-4-dependent transcription (Mulholland *et al.*, 2003).

In summary, there is increasing evidence that components of the Wnt pathway are involved in the progression of PCa. The published data support the hypothesis that processes other than mutations in β-catenin lead to its aberrant localisation in advanced PCa. The discoveries that β-catenin is able to bind AR *in vivo* and *in vitro* and is found nuclear in prostate tumours suggest a mechanism by which β-catenin regulates AR activity in PCa. Moreover, reports demonstrating the ability of β-catenin to alter AR ligand specificity suggest that β-catenin and possibly the Wnt pathway have a role in the transition from AD to AI disease. However, this possibility requires further investigation.

The majority of the experiments described above were carried out using non-prostate cell lines and/or by overexpressing mutant 'activated' forms of β-catenin

using strong promoters. However, most advanced prostate tumours have w.t endogenous nuclear β-catenin (Chesire *et al.*, 2002). Moreover, artificial overexpression of β-catenin can also affect signalling pathways that are not regulated by endogenous β-catenin, for example, activation of p53 (Damalas *et al.*, 1999; Oren *et al.*, 2002). One of the goals of my work was to determine the importance of endogenous β-catenin and the involvement of the canonical Wnt signalling pathway in the regulation of AR activity in PCa cells.

Figure 7- A summary of β-catenin mutations found in PCa

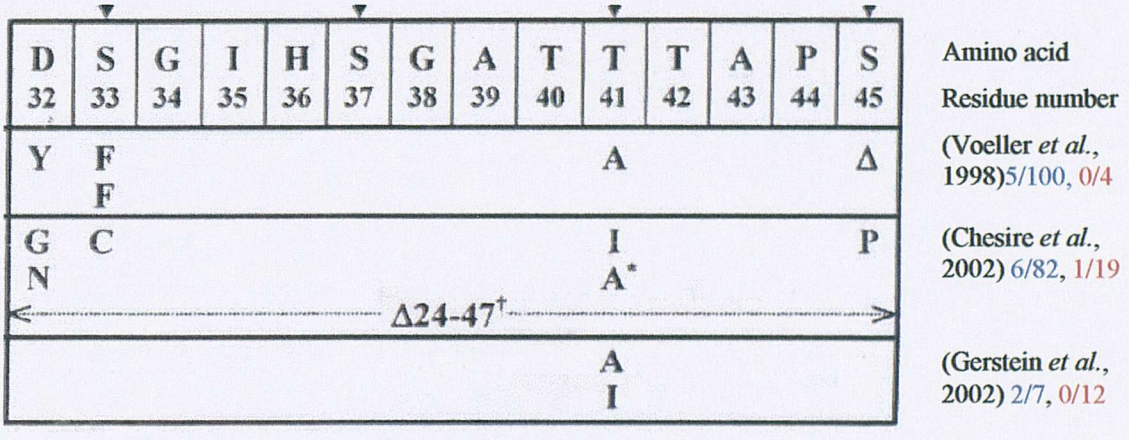


Fig 7. β-catenin regulatory domain mutations in PCa. β-catenin phosphorylation sites are indicated by arrow heads above the amino acids. References of studies done on β-catenin alteration in PCa are shown and below listed the number of changes detected per cases tested (localised (in blue) and advanced PCa (in red)). Amino acid changes or deletion are noted. Except for Δ24-47 mutation, all mutations were found in primary tumours. Δ indicates deleted codons, * indicates mutation occured in a xenograft, † indicates mutation observed in hormonal failure cases. Based on Chesire *et al.* review (2003)(Chesire and Isaacs, 2003)

1.10 PCa cell lines studies

One of the major problems in studying PCa and defining its molecular basis has been the shortage of model systems. This is due to a limitation in availability of tissue, heterogeneity within surgical prostatectomy samples, difficulties in growing explants *in vitro,* and also a limited number of immortalised cell lines. A few *in vitro* transplantable human PCa cell lines have been characterised. The most studied cell lines are DU145, PC3 and LNCaP. The DU145 cell line was isolated from a human brain metastasis and identified as a moderately differentiated adenocarcinoma with foci of poorly differentiated cells (Mickey *et al.*, 1977; Stone *et al.*, 1978). The PC3

cell line was isolated from a bone metastasis of an undifferentiated adenocarcinoma of the prostate (Kaighn *et al.*, 1979). Both DU145 and PC3 cells have lost their AR expression and exhibit hormone resistance characteristics. The androgen-responsive cell line, LNCaP was derived from a needle aspiration biopsy of a metastatic lesion in the lymph node. LNCaP cells grow faster in a medium containing fetal bovine serum compared to a medium containing hormone-depleted serum (Horoszewicz *et al.*, 1980; Horoszewicz *et al.*, 1983). These cells express AR, although it contains a single-point mutation that results in a substitution in the LBD that alters the receptor ligand-specificity (T877A) (for AR mutations see section 1.5.2-1) (Veldscholte *et al.*, 1992a; Veldscholte *et al.*, 1992b). LNCaP-r is a hormone resistant cell line that originated from LNCaP cells that were subcultured in low levels of androgens (0.04 nmol/l testosterone), mimicking the development of hormone-resistant carcinomas seen in patients (Pousette *et al.*, 1997). The model system of LNCaP/LNCaP-r has been very useful for studying the differences in PCa markers between AD and AI cell lines and the way AD cells progress to the hormone resistance stage.

Recently, a number of prostate cancer xenografts, grown in severe combined immuno-deficient mice (SCID), have been established (Klein *et al.*, 1997; Craft *et al.*, 1999a; Laitinen *et al.*, 2002) and cell lines derived from them are widely used in PCa research. Using xenograft models allows the expansion of a small amount of tissue for analysis and the enrichment of a relatively homogenous tumour cell population from a heterogeneous biopsy sample. In addition, the lines derived from xenografts originated from patients at different stages of the disease and therefore represent both AD and AI carcinomas. In this study, two such cell lines were used, LAPC-4, and CWR-R1. LAPC-4 cells are derived from an androgen-responsive xenograft and retain all the tumour characteristics such as expression of PSA, w.t AR and androgen-sensitivity (Klein *et al.*, 1997). The CWR-R1 cell line was derived from the xenograft CWR22 model, which was initially dependent on androgens and following castration became an AI xenograft. Like the recurrent parental CWR22 xenograft, CWR-R1 cells express a functional but mutant AR (H874Y). The receptor is highly stable in the absence of androgens and can respond better to ligands such as estradiol, adrenal androgens, and the anti-androgen hydroxyflutamide (Wainstein *et al.*, 1994; Tan *et al.*, 1997; Gregory *et al.*, 2001b). 22Rv1 is derived from the AI xenograft CWR22R and like it also express AR and PSA (Nagabhushan *et al.*, 1996; Sramkoski *et al.*, 1999).

1.11 The working hypothesis

The Wnt signalling pathway is activated during PCa progression. This does not involve mutations in β-catenin or APC, however, this activation promotes AR signalling and possibly the transition to androgen- independence. If this proves to be true, inhibitors of the Wnt pathway could be used to prevent progression to AI PCa.

1.12 Aims

(1) Define the role of the Wnt signalling pathway and its components in the regulation of AR signalling.

(2) Determine the role of endogenous β-catenin in the regulation of AR activity in PCa cells.

(3) Design inhibitors that affect AR activity specifically and thereby repress PCa growth and/or the transition to androgen-independence.

2. Materials and Methods

2.1 Transformation

Subcloning efficiency DH5α competent cells (Invitrogen) were used for all transformations except after site-directed mutagenesis (for Mutagenesis see section 2.4). DNA was added to 50 µl competent cells and incubated on ice for 10 minutes. The cells were heat-shocked at 37°C for 20 seconds and then placed on ice for a further 2 minutes. 500 µl S.O.C medium (10 mM magnesium sulphate, 2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 20 mM glucose) (Invitrogen), was added. The transformation mixture was then shaken at 200 rpm for 45 minutes at 37°C, after which 250 µl was spread on selective agar plates.

2.2 Minipreps

Minipreps were carried out using the Ultra Clean Miniplasmid prep kit (Mo Bio Laboratories). The concentration of solutions 1-4 in this kit are protected information. All steps were carried out at room temperature and centrifugations were done at 10,000 g. 1.5 ml of LB (Luria-Bertani) (recipe for 1000 ml, pH 7.0-10 g NaCl, 10 g Bactotryptone, 5 g yeast extract) containing carbenicillin (at 50 µg/1 ml) was inoculated with a single colony of transformed bacteria and grown for 16 hours at 37°C in a shaking incubator at 200 rpm. The culture was centrifuged for one minute, supernatant was discarded and then the pellet was centrifuged for a further 30 seconds. All traces of supernatant were removed and the pellet was resuspended in 50 µl solution 1 (Tris, EDTA (Ethylenediamine-Tetraacetic Acid), RNase A). The cells were lysed by adding 100 µl solution 2 (sodium dodecyl sulphate (SDS), sodium hydroxide), and inverting the tube once to mix. After addition of 325 µl solution 3 (potassium acetate, binding salt), the tube was centrifuged for one minute and the supernatant transferred to a spin filter. The spin filter was centrifuged for one minute and, after discarding the supernatant, the filter was washed with 300 µl solution 4 (ethanol, Tris, sodium chloride), followed by centrifugation for one minute. The filter was transferred to a new 2 ml collection tube and the DNA was eluted by adding 50 µl of solution 5 to the centre of the spin filter for specific elution.

After incubation for 5 minutes and centrifugation for one minute, the eluted DNA plasmid was ready to use.

2.3 Maxipreps

Maxipreps were carried out using the Endofree Plasmid kit (Qiagen). A single colony of transformed bacteria was used to inoculate a culture of 200 ml LB with selective antibiotic, grown at 37°C with shaking at 200 rpm for 16 hours. The bacterial cells were then centrifuged at 6000 g for 15 minutes, and the pellet was resuspended in 10 ml Buffer P1 (resuspension buffer: 50 mM Tris-Cl pH 8, 10 mM EDTA, 100 μg/ml RNase A). Lysis was carried out by addition of 10 ml Buffer P2 (lysis buffer: 200 mM sodium hydroxyide, 1% SDS) and incubation for 5 minutes at room temperature. For neutralising, 10 ml cold Buffer P3 (neutralisation buffer: 3M potassium acetate pH 5.5) was added, and the mix was poured into a barrel of a QIAfilter Cartridge. After incubation for 10 minutes at room temperature, the lysate was filtered through the cartridge and 2.5 ml of Buffer ER (endotoxin removal buffer; the composition is protected information) was added. The lysate was incubated on ice for 30 minutes and during this incubation, a QIAGEN-tip (containing an anion-exchange resin to bind DNA) was equilibrated by addition of 10 ml Buffer QBT (equilibration buffer: 750 mM sodium chloride, 50 mM MOPS pH 7, 15% isopropanol, 0.15% Triton X-100). Once the 30 minute incubation was finished, the filtrate was passed through the equilibrated column. The column was then washed twice with 30 ml Buffer QC (wash buffer: 1M sodium chloride, 50 mM MOPS pH 7, 15% isopropanol), and the DNA was eluted from the column by addition of 15 ml Buffer QN (elution buffer: 1.6 M sodium chloride, 50 mM MOPS pH 7, 15% isopropanol). 10.5 ml of isopropanol was added to the eluate to precipitate the DNA, and immediately after that a 30 minute centrifugation at 15,000 g and 4°C was carried out. The supernatant was discarded and the pellet was washed with 5 ml endotoxin-free 70% ethanol. After further 10 minutes centrifugation at 15,000 g and 4°C, the pellet was air-dried and dissolved in 500 μl Buffer TE (10 mM Tris-Cl pH 8, 1 mM EDTA). DNA concentration was determined by measuring the UV absorbance at 260 nm.

2.4 Mutagenesis

The QuikChange™ II site-directed mutagenesis kit (Stratagene) was used to make the single mutations S81A, S94A, and S650A. Using this kit, double-stranded DNA is used as a template and mutagenic primers are extended during temperature cycling by *PfuUltra* HF DNA polymerase for generation of mutated plasmids. Templates for S94A and S650A were pSV w.t and H874Y AR, and pSV H874Y AR was used as a template for the S81A. Oligonucleotide primers, complementary to opposite strands of the template plasmid were designed both to incorporate the desired amino acid changes and to include a restriction enzyme site (for oligonucleotide primer sequences, see section 2.5). PCR reactions were set up in a total volume of 50 µl, using the reaction buffer provided, 50 ng DNA template, 125 ng of each oligonucleotide primer, 1 µl of dNTP mix and 2.5 U DNA polymerase. Reactions were heated to 95°C for 30 seconds, followed by 16 cycles of 30 seconds at 95°C, 1 minute at 55°C and 6 minutes at 68°C. After temperature cycling, samples were cooled to 37°C and 10U *Dpn* I endonuclease, which digests methylated and hemimethylated DNA was added in order to digest the parental DNA template and to select for mutation-containing synthesised DNA (at 37°C for 1 hour). Since polymerisation by *PfuUltra* HF DNA polymerase results in nicked circular strands, only the non-mutated template DNA is digested. 1 µl of the nicked vector DNA containing the desired mutations was then transformed into 50 µl XL1-Blue supercompetent cells. The DNA was incubated on ice for 30 minutes, followed by a 45 second heat-pulse at 42°C and a further 2 minute incubation on ice. 0.5 ml LB was then added to the reactions and after one hour incubation on the shaker at 37°C and 200 rpm, the entire mixture was spread on selective plates. After amplification of DNA by Miniprep, samples were digested using the appropriate restriction enzymes (Alw44I for S94A, BlnI for S81A, BsrBI for S650A) to verify that mutations had been introduced into the plasmids. Each reaction was set up in 20 µl (10 µl DNA, 2 µl 10x buffer, 1 µl restriction enzyme and water) and incubated for 1 hour at 37°C. The integrity of all mutations was subsequently confirmed by sequence analysis.

2.5 DNA plasmid constructs

pMMTV-luciferase reporter, pSG5 AR, pSV (w.t and H874Y) AR and the β-galactosidase reporter pDM-β-Gal were provided by Charlotte Bevan (Imperial

College, London). The β-catenin/Tcf reporter plasmid, pOT-luciferase, was kindly provided by Bert Vogelstein (Johns Hopkins University, Baltimore). The β-galactosidase reporter, RSV-β-Gal, was provided by Maria Vivanco (ICR, London). β-catenin (pMT23-β-SA), is described in Giannini *et al.* (Giannini *et al.*, 2000), and Tcf-4 cDNA was a gift from Marc van de Wetering and Hans Clevers (Utrecht University, Utrecht, The Netherlands). Rat Axin and Axin deletion mutants were provided by Akira Kikuchi. These are described in Ikeda *et al.* (Ikeda *et al.*, 1998) and the GFP-Axin constructs are described in Orme *et al.* (Orme *et al.*, 2003) (see also Figure 21). Wnt1 was from Tony Brown (Cornell) and was cloned into pMT23 by Robert Kypta. pGK and pGK-Wnt3A, (Shibamoto *et al.*, 1998), were from Shinji Takada. LRP C2 and C6, described in Mao *et al.* (Mao *et al.*, 2001b), were from Dianqing Wu (Connecticut). pTER and pTERβi, (van de Wetering *et al.*, 2003), were from Marc van de Wetering. pTER control 1 and control 2 siRNA plasmids are described in Mazor *et al.* (2004) (Mazor *et al.*, 2004). GSK3β S9A, (Sperber *et al.*, 1995), was from Virginia Lee (Philadelphia). AX2 (FlagAx-(501-560)), AX2P, FRAT, FRATΔC and GSK3β constructs (w.t and K216R GSK3β) (Smalley *et al.*, 1999; Franca-Koh *et al.*, 2002; Fraser *et al.*, 2002) were generously provided by Trevor Dale (Cardiff School of Biosciences, UK). W.t AR mutants (S94A and S650A), H874Y AR mutants (S81A, S94A and S650A) were generated using the QuikChange™ II site-directed mutagenesis kit (Stratagene) (for Mutagenesis, see section 2.4). Residues 94 and 650 were mutated from serines to alanines in w.t and H874Y AR and residue 81 was mutated from serine to alanine in H874Y AR. For the creation of the S81A mutation, a pair of complementary primers were designed to incorporate two point mutations for the single mutation from serine to alanine, and together with another point mutation created a restriction site for BlnI such that the mutated plasmid could be distinguished from the non-mutated H874Y AR; The following oligonucleodides were used as primers (mutated bases are shown in bold):

5' -GCAGCAAGAGACT**GC**CCC**T**AGGCAGCAGCAGC-3'

3' -CGTCGTTCTCTGA**CG**GGG**A**TCCGTCGTCGTCG-5'

For the creation of the S94A mutation, a pair of complementary primers were designed to incorporate two point mutations for the single mutation from serine to alanine, and thereby also introducing a restriction site for Alw44I such that the mutated plasmid could be distinguished from the original non-mutated plasmid (w.t

or H874Y AR); The following oligonucleodides were used as primers (mutated bases are shown in bold):

5'-GGTGAGGATGGT**GCA**CCCCAAGCCCATCG-3'

3'-CCACTCCTACCA**CGT**GGGGTTCGGGTAGC-5'

For the creation of S650A mutation, a pair of complementary primers were designed to incorporate three point mutations for the single mutation from serine to alanine, and thereby also introducing a restriction site for BSrBI such that the mutated plasmid could be distinguished from the original non-mutated plasmid (w.t or H874Y AR); The following oligonucleodides were used as primers (mutated bases are shown in bold):

5'-GCTTCCAGCACCACC**GCT**CCCACTGAGGAG-3'

3'-CGAAGGTCGTGGTGG**CGA**GGGTGACTCCTC-5'

All mutations were confirmed by sequencing analysis. Primers for sequencing reactions were as follows:

for S81A and S94A mutants- 5'-ATGGAAGTGCAGTTAGGG-3';

for S650A mutant- 3'-GTACTCGGGGTAGGT-5'

2.6 Cell lines, cell culture and growth assays

In this study, initially several PCa lines were examined and later CWR-R1, 22Rv1 and PC3 cells, which were the most relevant lines for the study, were used. Table 4 is a summary of the PCa cell lines used in this study. Another cell line that was used as control is HCT116, which is a colon cancer cell line that has high levels of β-catenin as a result of mutated β-catenin. The fibroblast cell lines HEK 293 and COS7 were used as AR-negative controls and cell lines with high transfection efficiency.

2.6.1 Cell culture

The PCa cell lines used were from the American Type Culture Collection (Rockville, MD), except for LAPC-4 cells, which were provided by Charles Sawyers (University of California Los Angeles, CA), CWR-R1 cells, which were provided by Christopher Gregory (University of North Carolina at Chapel Hill, NC), and LNCaP-r cells which were provided by El-Nasir Lalani (Imperial College, London). HCT116, which is a colon cancer cell line that has high levels of β-catenin as a result of a stabilising mutation in the coding region of the β-catenin gene was used as a control cell line. The cell lines HEK 293 and COS7 are fibroblast cell lines that are also AR negative.

All cells were cultured at conditions of 37°C, 5% CO_2. LNCaP, LNCaP-r, PC3 and DU145 cells were grown in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen) and 1% antibiotics (100 U/ml Penicillin, 100 µg/ml Streptomycin, Sigma). COS7, HEK 293 and HCT116 cells were grown in Dulbecco's Modified Eagles Medium (DMEM, Invitrogen) containing 10% FBS and 1% penicillin-streptomycin solution. DMEM medium for COS7 cells contained higher glucose levels (4500 mg/l). CWR-R1 cells were grown in Richter's Improved MEM, Zn option, medium (IMEM, Invitrogen) containing 20 ng/ml epidermal growth factor (EGF, Merck Biosciences), 10 mM Nicotinamide (Calbiochem), 5 µg/ml insulin (Roche diagnostics), 5 µg/ml transferrin (Roche diagnostics), 1% penicillin-streptomycin solution and 2% FBS. 22Rv1 cells (Sramkoski *et al.*, 1999) were grown in 1:1 RPMI/DMEM (high glucose) with 20% FBS. LAPC-4 cells were grown in Iscove's Modified DMEM medium (IMDM, Cambrex Bio Science) containing 15% FBS, 1% penicillin-streptomycin solution and 10 nM R1881 (synthetic androgen, methyltrienolone, purchased from DuPont-NEN). For all hormone treatments, cells were incubated in medium containing Charcoal-Stripped Serum (CSS, First Link (UK) Ltd) prior to hormone treatment; phenol red free RPM1-1640 and DMEM (Invitrogen) were supplemented with 5% CSS, IMEM and phenol red free IMDM were supplemented with 2% and 7.5% CSS, respectively. Hormone treatment was of 10 nM R1881 for all cell lines except 22Rv1, which were treated with 1 nM R1881. LNCaP cells were treated with either 1 or 10 nM R1881. AR mutant analysis in PC3 cells was carried out in the presence of 0.15 nM R1881. Control cultures always received an equal volume of carrier (ethanol).

***2.6.2** Cell growth assays*

Cell growth assays were conducted according to Gregory *et al.* (Gregory *et al.*, 2001b). Briefly, cells (1.5 X 10^5/well) were plated in 12-well plates (three wells were used for each condition) and allowed to attach overnight. Carrier or GSK-3 inhibitors were then added, and, when indicated, R1881 (or carrier) was added 30 minutes later in order to allow the inhibitors to work before the hormone binds to the AR. Cells were collected by trypsinisation at the indicated times and were counted using an automated counter (Coulter Counter) or using a haemocytometer (Hausser Scientific).

Table 4. PCa cell lines used in this study

Cell lines	Source	AR	Hormon-dep. growth
LAPC4	LN met	Wt	AD
LNCaP	LN met	Mutated	AD
LNCaP-r	LNCaP line	Mutated	AI
CWR-R1	Primary	Mutated	AI
22Rv1	Primary	Mutated	AI
PC3	Bone met	-	AI
DU145	Brain met	-	AI

LN met= Lymph Node metastasis. AD/AI=Androgen dependent/independent.

2.7 Transient transfections

All cells were transfected in triplicate in 6-well tissue-culture plates when they were approximately 70% confluent. All cell types were washed with Optimem1 medium, a serum-free medium (Invitrogen), prior to transfection. Transfections of LNCaP and LNCaP-r cells were performed using 5 μl Lipofectin reagent (Invitrogen), according to the manufacturer's instructions, and 1 μg of DNA. PC3 cells were transfected using 5 μl Fugene6 (Roche Diagnostics), following the manufacturer's instructions, and 1 μg of DNA. All other cell lines were transfected with Lipofectamine Plus (Invitrogen) using 3.5 μl plus reagent, 2 μl of Lipofectamine and 1 μg DNA, following the instructions provided. For transcription assays, each well of a 6-well plate was transfected with either RSV promoter-driven or pDM-driven β-galactosidase (20 ng for HCT116, COS7, 22Rv1 and HEK 293 cells, 200 ng for all other cell lines), 300 ng pOT-luciferase (to measure β-catenin/Tcf activity) or 400 ng pMMTV-luciferase (to measure AR activity). After addition of various plasmids the total amount of DNA was brought up to 1 μg with empty vector. Amounts of DNA transfected per well were as follows: 500 ng of Wnt or empty vector pMT23 (for Wnt1) or pGK (for Wnt3A), 500 ng of β-catenin, 300 ng Tcf-4 or the empty vector

pMT23, 200 ng of C2 or C6 with the empty vector pMT23, 200 ng of AR or the empty vector pSG5, 100 ng of GFP-tagged Axin, its mutants, or GFP as control, 600 ng of the FLAG-tagged AX2, AX2P, or the myc-tagged MAX4 (axin residues 1-229) as a negative control, 400 ng (unless otherwise stated in figure legends) w.t GSK3β or the empty vector pMT23, 50 or 500 ng of GSK-3 constructs (S9A, K216R) or the empty vector pCDNA3, 100 ng of GFP-tagged FRAT or FRATΔC or GFP as control, 8 ng w.t or H874Y AR or AR mutants (S81A, S94A and S650A). For RNAi experiments, cells were first transfected with 1 μg β-catenin RNAi vector (pTERβi or the control RNAi vectors, control 1 and 2), and after 24 hours transfected again with the reporter vectors as well as another 200 ng of the RNAi vectors. For GSK-3 inhibitor experiments, cells were transfected with the reporter plasmids only (pOT or pMMTV-luciferase) together with pMT23 as the empty vector. In all transfections, after incubating with transfection reagents (3 hours for Lipofectamine Plus, 5 hours for Lipofectin and Fugene6), cells were grown in their normal growth medium for 40-42 hours, or in their stripped medium for 18 hours until addition of R1881 or ethanol for further 24 hours. Cells were harvested up to 48 hours after transfection. For protein analysis, cells were extracted using the appropriate lysis buffer (for cell extraction and IP techniques, see sections 2.9 and 2.10, respectively). For Luciferase assays cells were first rinsed in Dulbecco's Phosphate Buffered Saline (PBS, Sigma) and then lysed using the Reporter Lysis Buffer (Promega) at 0.3 ml/well. After 15 minutes incubation at room temperature, plates were incubated at -80°C for a further 15 minutes. Thawed cells were collected into tubes and centrifuged for 5 minutes at 15,000 g. Cell supernatants were then used for transcription assays.

2.8 Transcription assays

Luciferase and β-galactosidase assays were performed using the LucLite Plus (PerkinElmer Life Sciences) and Galacto-light Plus (Applied Biosystems) kits, respectively, according to manufacturer's instructions. All reactions were carried out at room temperature and in white opaque 96-well plates (PerkinElmer Life Sciences) covered with foil to protect from light. The LucLite Plus assay is a mixture of substances that modify the enzymatic reaction of the luciferase reporter enzyme in cell lysates to produce a highly sensitive, stable (half-life of several hours), glow-type signal. A 20 μl aliquot of each cell lysate was incubated with 20 μl thawed

reconstituted substrate (lypophilized substrate reconstituted with substrate buffer solution) in two duplicate wells for 15 minutes. The β-galactosidase assay is designed for rapid and sensitive detection of β-galactosidase reporter enzyme in cell lysates. 5 µl aliquot of each cell lysate supernatant per well was incubated with 50 µl Reaction Buffer (100 mM sodium phosphate pH 8, 1 mM MgCl2) (diluted 1:100 with Galacton substrate) in two duplicate wells for 1 hour. This was followed by addition of 75 µl/well of Accelerator(-II) (containing Sapphire-II enhancer) for a further 15 minutes incubation, for termination of enzyme activity and in order to trigger light emission. Plates were read on a NXT TopCount Luminometer (Packard Bioscience) and the average was calculated between duplicate samples. Counts are presented as the ratio between Luciferase and β-galactosidase activities. Each transfection was carried out in triplicate and repeated at least twice (-/+ standard-deviation is measured from triplicate values).

2.9 Cell extraction

Reagents were from Sigma unless otherwise stated. Cell lysates were obtained using various lysis methods depending on the desired cellular fraction of the cell. Cells were grown to 50-70% confluence in 100 mm dishes or in 6-well plates in their growing medium or stripped medium with R1881.

2.9.1 Whole cell extracts

Cells were rinsed in cold Tris-Buffered Saline (TBS; 50 mM Tris pH 7.5, 150 mM NaCl), lysed in cold improved RIPA (0.5% deoxycholate, 1% Triton X-100, 20 mM Tris pH 8.0, 0.1% SDS, 100 mM NaCl, 50 mM NaF) or Nonidet P-40 (1% NP-40 (Anachem), 20 mM Tris pH 8.0, 150 mM NaCl, 50 mM NaF) lysis buffer, containing 1 mM EDTA pH 8, 10 µg/ml aprotinin (Roche Diagnostics), 10 µg/ml leupeptin and protease inhibitor cocktail (Roche Diagnostics) at 1:100 dilution. Lysates were collected into tubes by squirting off the plates, incubated on ice for 10 minutes and then centrifuged for 12 min at 15,000 g.

2.9.2 Non-nuclear and nuclear extracts

Cells were lysed using the commercial extraction kit NE-PER, according to the manufacturer's instructions (Perbio Science UK Ltd). This kit supplies a complete set of reagents (components of reagents in this kit are protected information) that enables the separation of non-nuclear and nuclear fractions from cultured cells. Each

reagent was mixed with protease inhibitor cocktail (Roche Diagnostics) at 1:100 dilution and all steps were carried out at 4°C, using ice-cold reagents. Lysis buffer volumes and mixing times were doubled in order to improve extraction. Cells were first collected into tubes using cold PBS and centrifuged at 500 g for 3 minutes. After supernatant was discarded, pellet was dried properly using a pipette and Cytoplasmic Extraction Reagent I (CERI) was added (reagent volumes were adjusted according to cell pellet size). Pellets were resuspended by vortexing and after 10 minutes incubation on ice Cytoplasmic Extraction Reagent II (CERII) was added. Following further vortexing and incubation on ice, lysates were centrifuged for 5 minutes at 16,000 g and supernatants (the *non-nuclear extracts*) were immediately transferred into pre-chilled tubes. Insoluble pellets, which contained the nuclei, were resuspended with the Nuclear Extraction Reagent (NER), and after 4 cycles of vortexing and 10 minutes incubation on ice, centrifuged for 10 minutes at 16,000 g. Supernatants (the *nuclear extracts*) were immediately transferred into pre-chilled tubes.

2.9.3 Cytosolic extracts

Cells were lysed in hypotonic lysis buffer (0.02% 1M MgCl2, 1% 1M Tris pH 7.5) as described in Orford *et al.* (Orford *et al.*, 1997). Lysis was followed by homogenisation, centrifugation at 500 g for 5 minutes to remove nuclei, and then a second centrifugation at 20,000 g for 30 minutes to produce the cytosolic extract.

An aliquot of lysate, obtained using whichever method, was heated for 3 minutes at 95°C with an equal volume of Laemmli sample buffer in order to denature the proteins.

2.10 Immunoprecipitation (IP)

All steps were carried out at 4°C, using ice-cold reagents. IP samples were prepared using two methods- 1. Lysis using NP-40 lysis buffer for NP-40-soluble cell extracts and centrifugation at 15,000 g for 12 minutes (Figures 32 and 34). 2. As described in Truica *et al.* (Truica *et al.*, 2000), cells were lysed in 0.5% NP-40 lysis buffer and then passed several times through a 30.5-gauge needle, in order to disrupt the nuclei. Cell lysate was incubated on ice for 5 minutes and centrifuged at 12,000 g for 10 minutes to remove insoluble material (Figures 11 and 33).

Extracts, obtained by either method, were incubated on ice with antibody for 1 hour in order to form antibody/protein complexes. To precipitate these complexes, 30 minutes incubation was carried out with beads on a wheel in the cold room. For IP antibodies, the beads used were either Protein A Sepharose (Amersham Pharmacia) at 40 µl/sample (for rabbit antibody) or protein A/G-agarose (Cambridge Biosciences) at 20 µl/sample (for mouse antibody). Following this incubation, beads were washed 4 times with NP-40 lysis buffer and once with TBS, with a one minute 500 g centrifugation after each wash to pellet the beads. Following washes, beads were resuspended in 10 µl of Laemmli sample buffer and heated at 95°C for 3 minutes. Samples were separated from the beads by making three holes at the bottom of each tube, and centrifuged for 1 minute at 2000 g in order to collect the IP samples in fresh tubes underneath.

2.11 SDS polyacrylamide gel electrophoresis and Western Blotting

Reagents were from Sigma unless otherwise stated. Proteins were separated by SDS-polyacrylamide gels that were made using different concentrations of acrylamide depending on the size of the protein (9% gels for detection of proteins smaller than 50 KDa, and 6% gels for detection of proteins larger than 50 KDa) and according to Sambrook and Russell (2001) (Sambrook, 2001). Gels were run at 10 mA/gel in running buffer (250 mM glycine, 25 mM Tris, 0.1% SDS), after which they were incubated in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, 0.0375% SDS) for two minutes and transferred to nitrocellulose membrane (0.2 µm pore size, Schleicher and Schuell) using a Trans-Blot semi-dry transfer apparatus (Bio-Rad Laboratories). After transfer for 30 minutes, membranes were washed once in transfer buffer and once in TBS-T (10 mM Tris pH 7.5, 0.1M NaCl, 0.1% Tween-20), and then blocked in blocking buffer (3% bovine albumin, fraction V (BSA), 1% Ovalbumin, in TBS-T) for 1 hour at room temperature. Membranes were then probed with different primary antibodies (for antibodies, see section 2.12) in blocking buffer for 2 hours at room temperature or over-night at 4°C. This incubation was followed by six 10-minutes washes with TBS-T, 45 minutes incubation with secondary antibodies and a further six 10-minutes washes in TBS-T. Levels of proteins were determined by chemiluminescence using Enhanced Chemiluminescence (ECL, Amersham Biosciences) reagents, followed by exposure to film (Amersham

Biosciences) using a film processor (Konica medical film processor SRX-101A (Konica)). Each experiment was repeated at least twice and results presented are representative and reproducible.

2.12 Antibodies

Western Blots were probed using various antibodies (all antibodies were used at 1:1000 dilution unless otherwise stated). For β-catenin levels in cell extracts, the monoclonal antibody anti-β-catenin (clone 14, BD Biosciences) was used at dilution 1:2000. For Tcf-4, the monoclonal antibody against Tcf-4 (Upstate) was used. Levels of GSK-3 were detected using the monoclonal antibodies anti-GSK3β (BD transduction laboratories) or anti-GSK-3 (α+β) (Upstate). Phosphorylated GSK-3 (α +β) were detected using the phospho-specific polyclonal antibodies p-GSK-3 S21/S9 (Cell Signaling) or Y279/Y216 (Biosource). For myc-epitope tagged proteins, a monoclonal antibody (clone 9E10, Sigma Aldrich) was used. AR was detected using polyclonal anti-AR (P111A, Affinity Bioreagents, (ABR)). To detect AR in β-catenin IP samples (Figure 11) the monoclonal anti-AR (AR441, DAKO) was used. To detect phosphorylated AR, a phospho-specific polyclonal antibody against p-AR (S81) (Upstate) was used at 1:400. Blots from whole cell extracts or non-nuclear extracts were also probed using monoclonal anti-γ-tubulin (clone GTU-88, Sigma Aldrich), monoclonal anti-HSP60 (LK-1, Stressgen biotechnologies) or monoclonal anti-β-actin (AC-15, Abcam) to verify equal loading levels. Monoclonal antibody anti-PARP (poly ADP-ribose polymerase) was used as a control for nuclear extracts (F-2, Santa Cruz Biotechnology). A polyclonal anti-Pan-Cadherin (Sigma) was used to estimate the level of membrane protein contamination of cytosolic extracts. The following antibodies were used for immunoprecipitations: P110 polyclonal anti-AR (Affinity Bioreagents) at 1:50, polyclonal anti-β-catenin (Kypta *et al.*, 1996) at 1:500 dilution, 5 μg polyclonal anti-GFP serum (Kypta *et al.*, 1996) as a control (Figures 11 and 32). 2 μg 9E10 and 2 μg anti-GFP mAb (Roche) as control were used in Figures 32 and 34. 1 μg of normal polyclonal IgG (Oncogene research products) as control was used in Figure 33. Secondary antibodies were peroxidase-conjugated (HRP) (Jackson Laboratories) anti-mouse and anti-rabbit, used at 1:5000 and 1:10000, respectively.

3. Results

3.1 Chapter 1- Wnt and AR signalling pathways in PCa cells- Protein studies

3.1.1 Protein expression levels of Wnt signalling pathway components

β-catenin, a key component of the Wnt signalling pathway has been shown to regulate AR activity (Truica *et al.*, 2000; Mulholland *et al.*, 2002; Pawlowski *et al.*, 2002; Yang *et al.*, 2002). For this reason, β-catenin was the first protein to be characterised in PCa cells. A comparison of β-catenin protein expression levels among several PCa cell lines as well as control non-PCa cell line is presented in Figure 8a. Whole cell extracts from LNCaP, LNCaP-r, LAPC-4, 22Rv1, PC3, DU145 and HCT116 cells were probed with antibody against β-catenin. Lysates were also probed with antibody against HSP60, as an internal loading control. The colon cancer cell line, HCT116, which has stabilised β-catenin as a result of an activating mutation in the β-catenin gene, was used as a positive control. Results indicate that all PCa cell lines expressed broadly similar total levels of β-catenin. Compared with the loading control, PC3 and DU145 cells expressed slightly higher levels of total β-catenin.

Nuclear β-catenin levels in LNCaP, LNCaP-r, LAPC-4, 22Rv1, PC3, DU145 and HCT116 cells are shown in Figure 8b. Nuclear extracts were probed with antibodies against β-catenin and PARP (as an internal loading control for a nuclear protein). Most PCa cells contained a high level of nuclear β-catenin with the exception of PC3 and DU145 cells. LAPC-4 cells contained less nuclear β-catenin than LNCaP, LNCaP-r and 22Rv1 cells. The control cell line, HCT116, contained nuclear β-catenin, as expected.

GSK3β, as one of the key regulators of β-catenin in the Wnt signalling pathway, was also examined among several PCa cell lines. Whole cell and nuclear extracts from LNCaP, LNCaP-r, LAPC-4, 22Rv1, PC3, DU145 and HCT116 cells were probed with antibody against GSK3β and the appropriate loading controls (Figures 8c and 8d, respectively). LNCaP and LNCaP-r cells expressed higher levels of GSK3β compared with the other PCa cell lines. PC3 cells expressed high levels of GSK3β in whole cell extracts but relatively low levels of GSK3β in the nucleus. DU145 cells expressed very little GSK3β in the nucleus.

β-catenin activates transcription by binding to the Tcf/lef family of transcription factors (reviewed in Akiyama, 2000; Polakis, 2000). Therefore, the level of Tcf-4 in nuclear extracts from HCT116 and several PCa cells lines was examined. It has been reported that the levels of Tcf-1, LEF-1 and Tcf-3 are extreminley low in LNCaP cells (Chesire *et al.*, 2002). Results shown in Figure 8e indicate that LNCaP cells had relatively little nuclear Tcf-4 protein. In contrast to the relative levels of β-catenin, Tcf-4 levels were higher in PC3 than in LNCaP cells. HCT116 cells expressed high levels of nuclear Tcf-4.

Figure 8

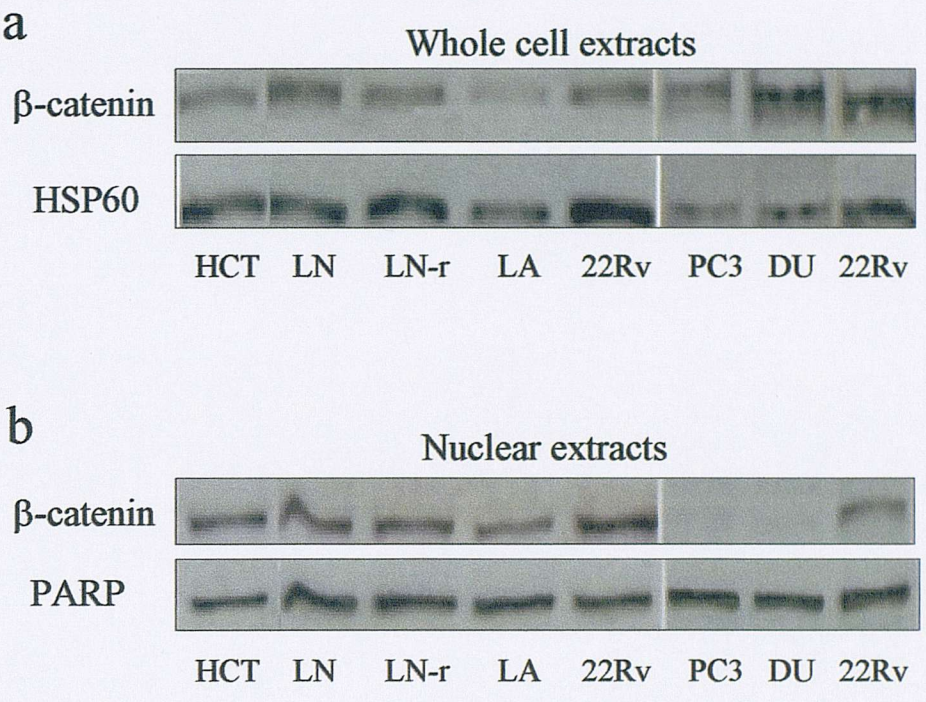


Figure 8 (a-b). A comparison of β-catenin protein levels among PCa cell lines and the colon cancer cell line, HCT116. (a) Whole cell extracts from HCT116 (HCT), LNCaP (LN), LNCaP-r (LN-r), LAPC-4 (LA), 22Rv1 (22Rv), PC3 and DU145 (DU) were probed with antibodies against β-catenin and HSP60. (b) Nuclear extracts from HCT116 (HCT), LNCaP (LN), LNCaP-r (LN-r), LAPC-4 (LA), 22Rv1 (22Rv), PC3 and DU145 (DU) cells were probed with antibodies against β-catenin and PARP.

Figure 8 continued-

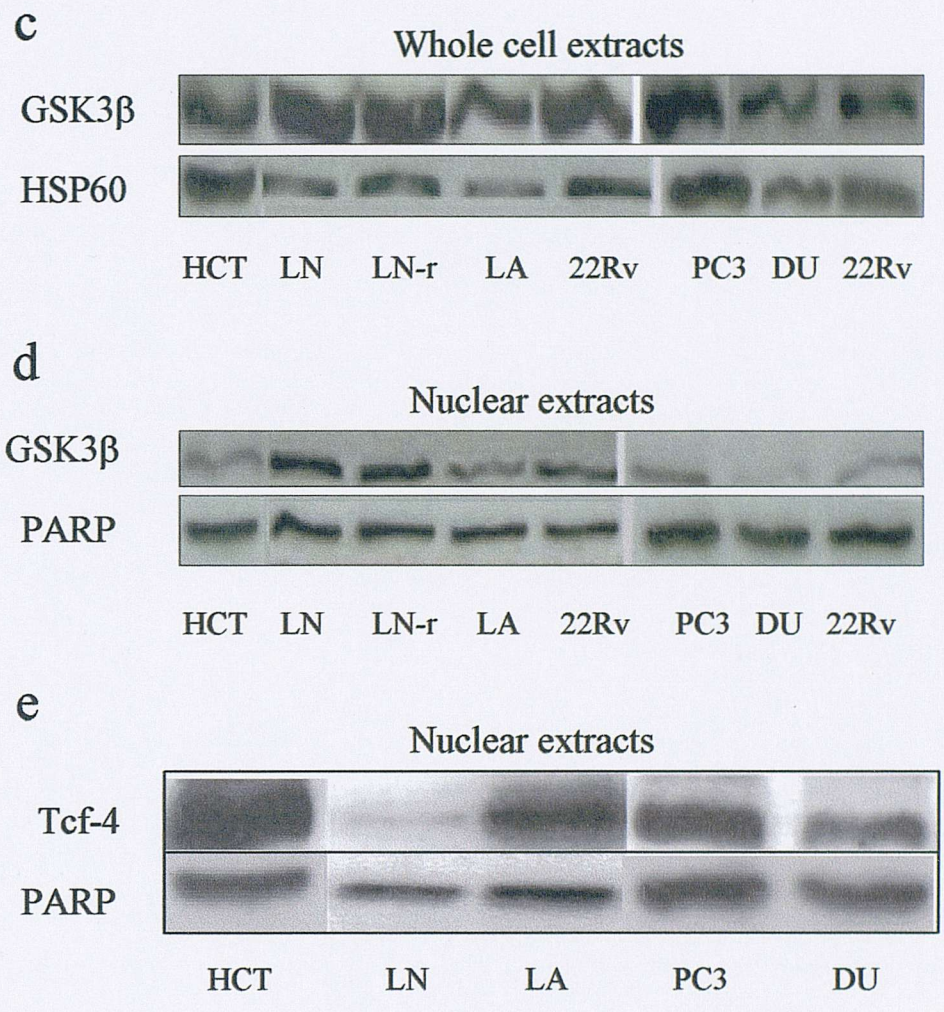


Figure 8 (c-e). A comparison of GSK3β and Tcf-4 protein levels among PCa cell lines and the colon cancer cell line, HCT116. Whole cell extracts (c) or nuclear extracts (d) from HCT116 (HCT), LNCaP (LN), LNCaP-r (LN-r), LAPC-4 (LA), 22Rv1 (22Rv), PC3 and DU145 (DU) were probed with antibodies against GSK3β and HSP60 (c) or GSK3β and PARP (d). (e) Nuclear extracts from HCT116 (HCT), LNCaP (LN), LAPC-4 (LA), PC3 and DU145 (DU) cells were probed with antibodies against Tcf-4 and PARP.

3.1.2 AR protein expression levels

Since β-catenin has been shown to regulate AR, it was important to also compare the levels of AR in the different cell lines. Whole cell and nuclear extracts were probed with antibody against AR (Figure 9a and 9b, respectively). As expected, the PCa lines PC3 and DU145 and the non-PCa line HCT116 did not express AR. 22Rv1 cells expressed two additional AR protein products (migrating with a relative mass of 75-80 KDa). These have been shown to be C-terminal truncated mutants of AR that lack the LBD domain (Tepper *et al.*, 2002). LAPC-4, LNCaP and LNCaP-r cells, expressed similar levels of AR protein. All the cell lines that expressed AR protein, also contained nuclear AR protein. This was expected, as the cells were grown in media containing serum, which contains ligands for AR. These results confirm the known pattern of AR protein expression in PCa cells.

3.1.3 Stabilisation of β-catenin in PCa cells

As part of this initial study of Wnt signalling in PCa cell lines, the ability of the cells to respond to a Wnt stimulus was investigated. β-catenin stabilisation is an early response to a Wnt stimulus and can indicate whether the cells can mount a Wnt signalling response. Wnt signalling activation can be achieved in two ways: 1. by culturing cells with conditioned medium containing Wnt3A, obtained from transfected L cells (LW) 2. by treating cells with LiCl, a GSK-3 inhibitor. In order to be able to detect the effects of these stimuli on β-catenin, it was necessary to isolate cytosolic extracts thereby avoiding the detection of β-catenin from the membrane pool, which is not significantly affected by activation of the Wnt pathway. Therefore, cells were lysed in hypotonic lysis buffer and lysates were probed with an antibody against β-catenin, as well as an antibody against γ- tubulin as an internal loading control. As a control, the effect of Wnt3A on the level of β-catenin was examined in cytosolic extracts from control L cells. Figure 10a shows that cytosolic β-catenin levels were elevated in the Wnt3A-treated L cells. In PC3 cells, β-catenin was stabilised by LiCl and, to a smaller extent, by LW. In contrast, β-catenin levels in LNCaP cells were not detectably affected by either treatment (Figure 10b).

Figure 9

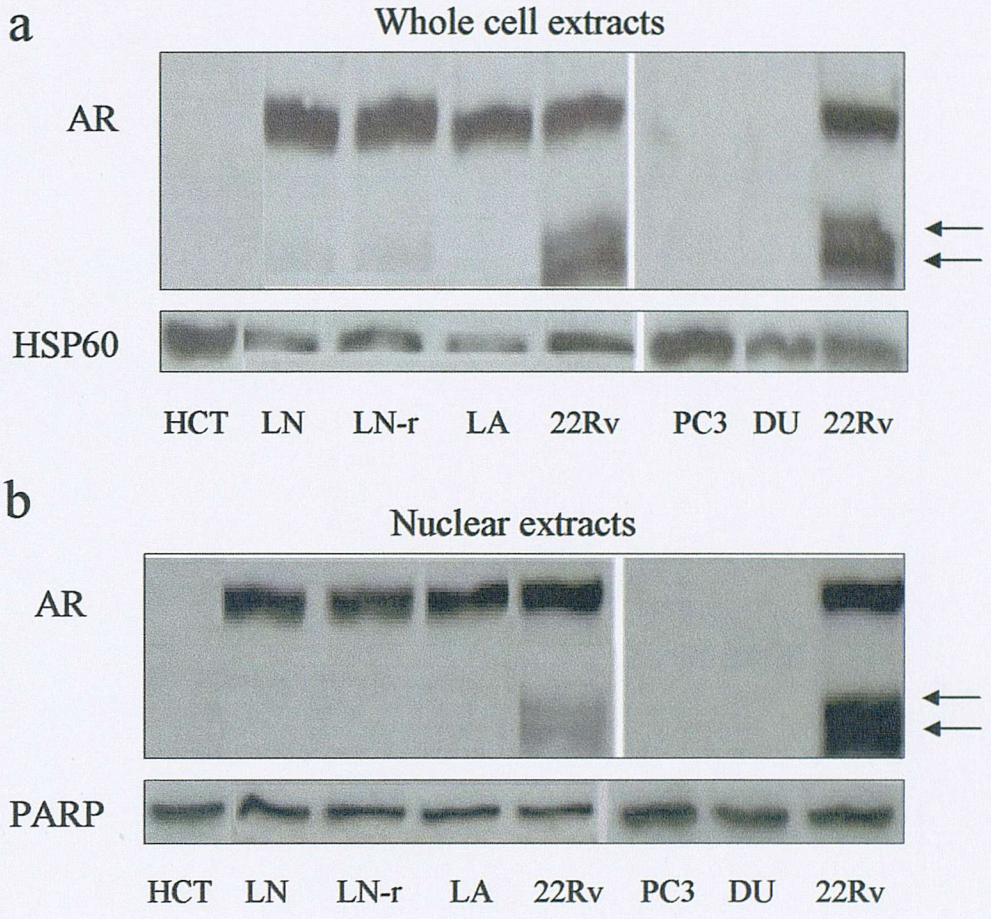


Figure 9. A comparison of AR protein levels among PCa cell line and the colon cancer cell line, HCT116. (a) Whole cell extracts from HCT116 (HCT), LNCaP (LN), LNCaP-r (LN-r), LAPC-4 (LA), 22Rv1 (22Rv), PC3 and DU145 (DU) were probed with antibodies against AR and HSP60. 22Rv1 cells also express C-terminal truncated AR species, migrating with a relative mass of 75-80 KDa (see arrows). (b) Nuclear extracts from HCT116 (HCT), LNCaP (LN), LNCaP-r (LN-r), LAPC-4 (LA), 22Rv1 (22Rv), PC3 and DU145 (DU) were probed with antibodies against AR and PARP as an internal control for a nuclear protein. The expression pattern of AR in nuclear extracts was similar to that in whole cell extracts.

Figure 10

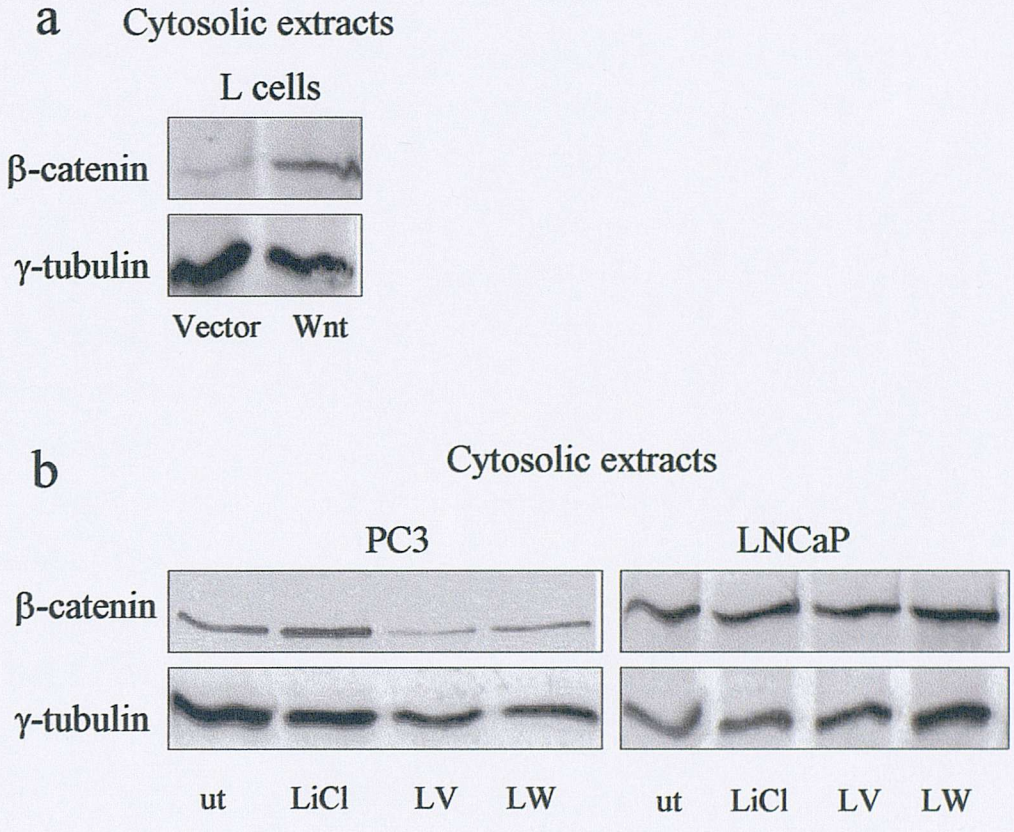


Figure 10. LiCl treatment and Wnt stimulation stabilise β-catenin in PC3 cells but have no effect in LNCaP cells. (a) β-catenin levels (and γ- tubulin as an internal loading control) were compared in cytosolic extracts from L cells treated with either vector or Wnt3A. (b) Cells were treated with (LiCl) or without (ut) 20 mM LiCl, or supplemented with conditioned medium obtained from L cells expressing vector (LV) or Wnt3A (LW) for 24 hours. Cytosolic extracts were probed with antibodies against β-catenin and γ- tubulin as an internal loading control.

3.1.4 AR and β-catenin protein complex in LNCaP cells

It has been reported that β-catenin promotes AR-mediated transcription by binding to AR (Truica *et al.*, 2000; Pawlowski *et al.*, 2002; Yang *et al.*, 2002; Mulholland *et al.*, 2003). In order to confirm the published work, LNCaP cells, which express AR and high levels of β-catenin, were used to try to detect the complex between AR and β-catenin. LNCaP cells were grown in hormone-depleted medium for 24 hours prior to the experiment, and then treated with 10 nM R1881 or the carrier ethanol for 45 minutes. Whole cell extracts were immunoprecipitated with an antibody against AR (or with anti-GFP, as a control), and probed with antibodies against β-catenin and AR. Figure 11 presents the result achieved after optimising IP conditions using several techniques; the detection of this complex required very specific conditions (for details on IP technique, see section 2.10-2 in Materials and Methods). Results indicate that β-catenin and AR can interact in LNCaP cells but the low level of complex detected suggests that it is either unstable or not very abundant. This complex is slightly more abundant in the presence of hormone, as previously reported (Truica *et al.*, 2000; Yang *et al.*, 2002) (Verras *et al.*, 2004). Probing IP samples with antibody against AR indicated that equal amounts of AR were immunoprecipitated and that control IP's did not contain AR.

Figure 11

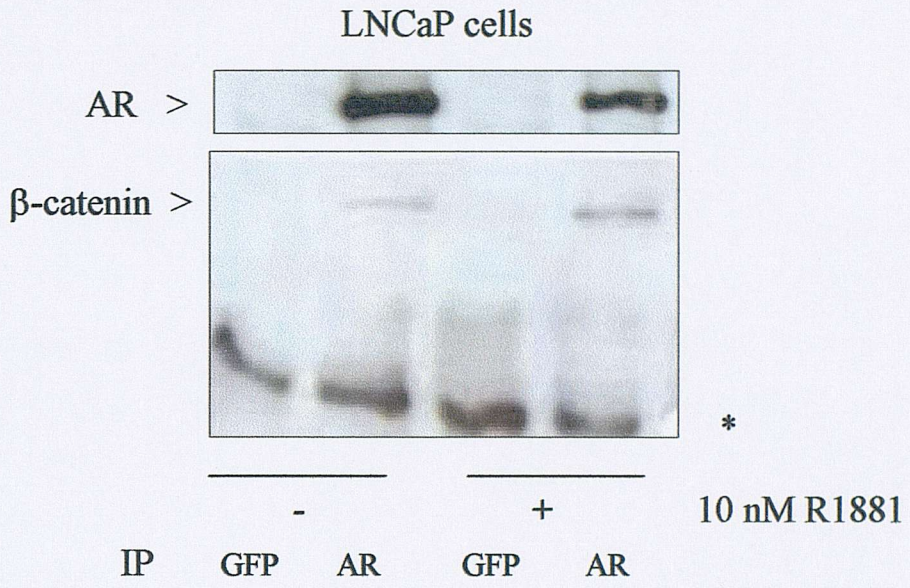


Figure 11. Detection of AR/β-catenin complexes in LNCaP cells is increased in the presence of R1881. Whole cell extracts from LNCaP cells treated with 10 nM R1881 (+) or the carrier ethanol (-) for 45 minutes, were immunoprecipitated with antibodies against AR or GFP and probed with antibodies against β-catenin and AR (indicated by arrow heads). IgG (indicated by *) appears at the bottom of the gel.

3.1.5 AR and β-catenin levels in nuclear and non-nuclear extracts in the presence and absence of androgens

AR transcriptional activity is strongly regulated by androgens. The next goal was to examine how β-catenin behaves in the presence and absence of androgens, and whether the cellular localisation of the AR/β-catenin complex is regulated by hormone treatment. LNCaP, LAPC-4 and CWR-R1 cells were starved for 18 hr and then treated with 10 nM R1881 or the carrier ethanol for 40 minutes. Samples of extracts (nuclear or non-nuclear) were probed with antibodies against β-catenin and AR as well as for HSP60 (for non-nuclear protein) or PARP (for nuclear protein), respectively, as loading controls. In the non-nuclear extracts (Figure 12a), β-catenin levels in the three cell lines tested were not significantly affected by androgen treatment. In contrast, AR levels in the non-nuclear extracts of all three lines were reduced in the presence of androgens. In Figure 12b, nuclear extracts were examined. There was no significant change in nuclear β-catenin in any of the cell lines after androgen treatment (after normalisation to PARP levels). In contrast, AR levels increased in the nucleus of LNCaP and CWR-R1 cells treated with androgens. Results in LAPC-4 cells were less conclusive. Previous studies using overexpressed β-catenin and AR showed that β-catenin is predominantly cytoplasmic in the absence of androgens and, upon androgen treatment, translocates to the nucleus with agonist-bound AR to activate AR-mediated transcription (Mulholland *et al.*, 2002; Pawlowski *et al.*, 2002; Yang *et al.*, 2002). My results indicate that AR translocates to the nucleus upon androgen treatment, however, the effects on endogenous β-catenin translocation were minimal. Similarly, Chesire *et al.* (2002) were unable to detect β-catenin ligand-mediated nuclear localisation in PCa cells (Chesire *et al.*, 2002).

Figure 12

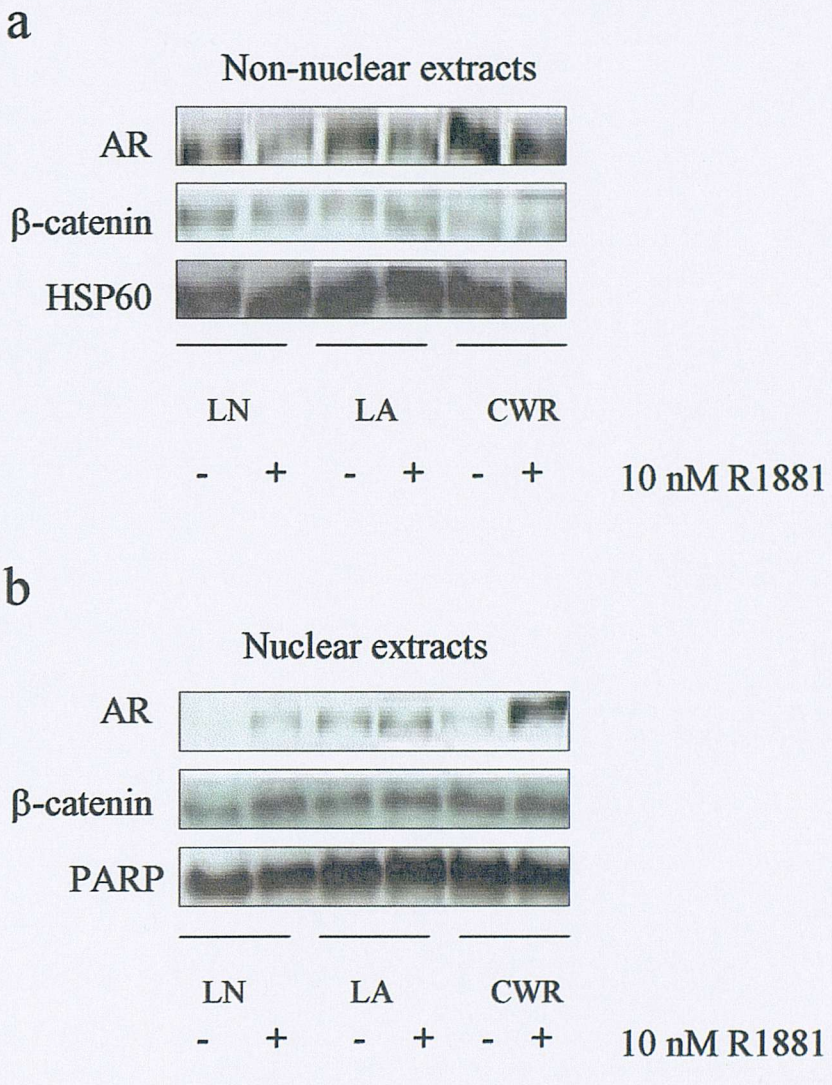


Figure 12. AR and β-catenin localisation to the nucleus in PCa cells. LNCaP (LN), LAPC-4 (LA) and CWR-R1 (CWR) cells were treated with 10 nM R1881 (+) or the carrier ethanol (-) for 40 minutes. Non-nuclear (a) and nuclear (b) extracts were probed with antibodies against β-catenin and AR as well as for HSP60 (a) or PARP (b), as loading controls.

3.2 Chapter 2- Wnt and AR signalling pathways in PCa cells- transcription studies

3.2.1 β-catenin/Tcf signalling responses to expression of Wnt components in PCa cells

In order to examine the transcriptional response to Wnts, LAPC-4, LNCaP and CWR-R1 cells were transfected with the DNA plasmids of two canonical Wnts, Wnt1 and Wnt3A (or their respective empty vectors as controls), as well as the reporter plasmids (pOT-luciferase (pOT-Luc), a reporter of β-catenin/Tcf-dependent transcription, and RSV-β-Gal as a control for transfection efficiency). In Figure 13, results are presented as fold-change comparing cells transfected with either Wnt to control cells. β-catenin/Tcf-dependent transcription significantly increased in CWR-R1 cells, in response to expression of both Wnt1 and Wnt3A compare to their respective empty vectors ($p<0.00005$). pOT-Luc activity was reduced, albeit to a much lesser extent compare to the increase in CWR-R1 cells, in LNCaP cells expressing both Wnts ($p=0.002$ for Wnt1, $p=0.0006$ for Wnt3A) and in LAPC-4 cells expressing Wnt3A ($p=0.02$) compare to their respective empty vectors.

Previous results showed that although nuclear levels of β-catenin are high in LNCaP cells, levels of Tcf-4 protein are very low. In order to examine the possibility that low levels of Tcf-4 prevent an increase in β-catenin/Tcf-dependent transcriptional activity in response to Wnt, Tcf-4 was next expressed in LNCaP cells. Cells were transfected with either β-catenin (or its empty vector pMT23), Tcf-4 or both. According to Figure 14, results indeed indicate that expression of Tcf-4 alone can activate β-catenin/Tcf-dependent transcription in LNCaP cells ($p<0.00005$). Therefore, the low level of Tcf-4 expression may explain why the Wnt signalling pathway cannot be switched on by LiCl or Wnts in LNCaP cells (see Figure 10).

Figure 13

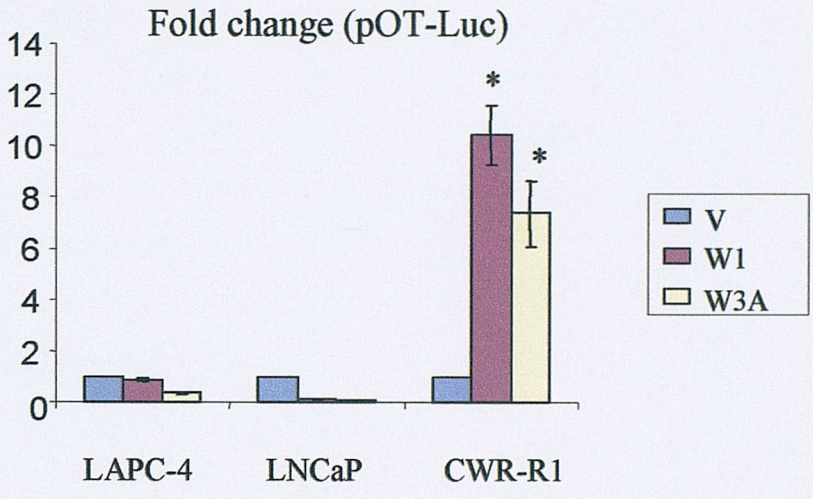


Figure 14

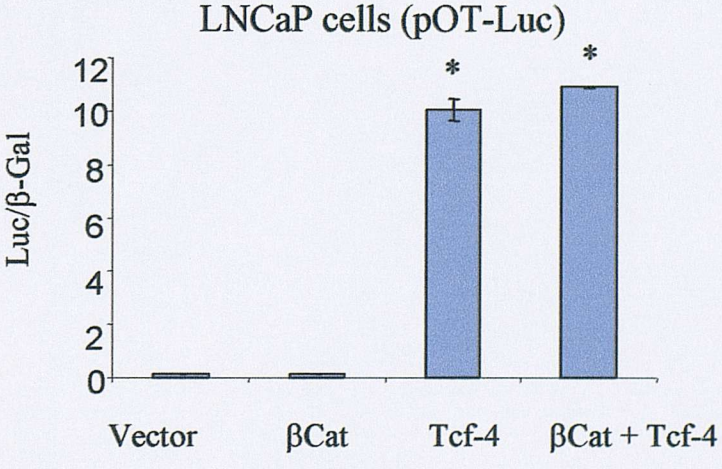


Figure 13 β-catenin/Tcf-4 signalling response to Wnt expression in PCa cells. LAPC-4, LNCaP and CWR-R1 cells were transfected with the reporter vectors pOT-Luc and RSV-β-Gal plus Wnt1 (W1) or plus Wnt3A (W3A), or plus their respective empty vectors pMT23 and pGK (V). Cells were assayed for luciferase (Luc) and β-galactosidase (β-Gal) activities, and results are presented as the activity relative to each cell line transfected with empty vector. (*) pOT-Luc activity significantly increased in CWR-R1 cells expressing either Wnt compare to cells expressing their respective empty vector ($p<0.00005$ for Wnt1 and Wnt3A). Experiment was done at least three times in triplicates.

Figure 14. Tcf-4 restores β-catenin/Tcf-4-dependent transcription in LNCaP cells. Cells were transfected with the reporter vectors pOT-Luc and RSV-β-Gal plus β-catenin (βCat) or plus Tcf-4 or plus both. pMT23, the empty vector was transfected as control (Vector). Cells were assayed for luciferase (Luc) and β-galactosidase (β-Gal) activities, and results are presented as the ratio between luciferase and β-Gal activities. (*) pOT-Luc activity significantly increased in cells expressing Tcf-4 compare to cells expressing vector ($p<0.00005$ for Tcf-4 alone and βCat+Tcf-4). Experiment was done twice in triplicates.

β-catenin/Tcf-dependent transcription was also not activated by Wnt expression in LAPC-4 cells, even though these cells express Tcf-4. It is possible that LAPC-4 cells do not express receptors for Wnt1 or Wnt3A. In order to address this question, the Wnt signalling receptor, LRP5, was used. A constitutively-active form of LRP5, C2, and an inactive truncated form of LRP5, C6, were transfected in LAPC-4 cells, in the presence and absence of 10 nM R1881. The results in Figure 15a show that the Wnt signalling pathway can be activated when C2 is expressed.

In order to follow this further downstream, the effect of β-catenin overexpression on β-catenin/Tcf-dependent transcription was examined. As shown in Figure 15b, β-catenin/Tcf-dependent transcription can also be activated in LAPC-4 cells when they overexpress β-catenin. Since LAPC-4 cells already have nuclear β-catenin (see Figure 8b), this might suggest that the endogenous pool of nuclear β-catenin is not able to interact with the endogenous Tcf-4.

Figure 15

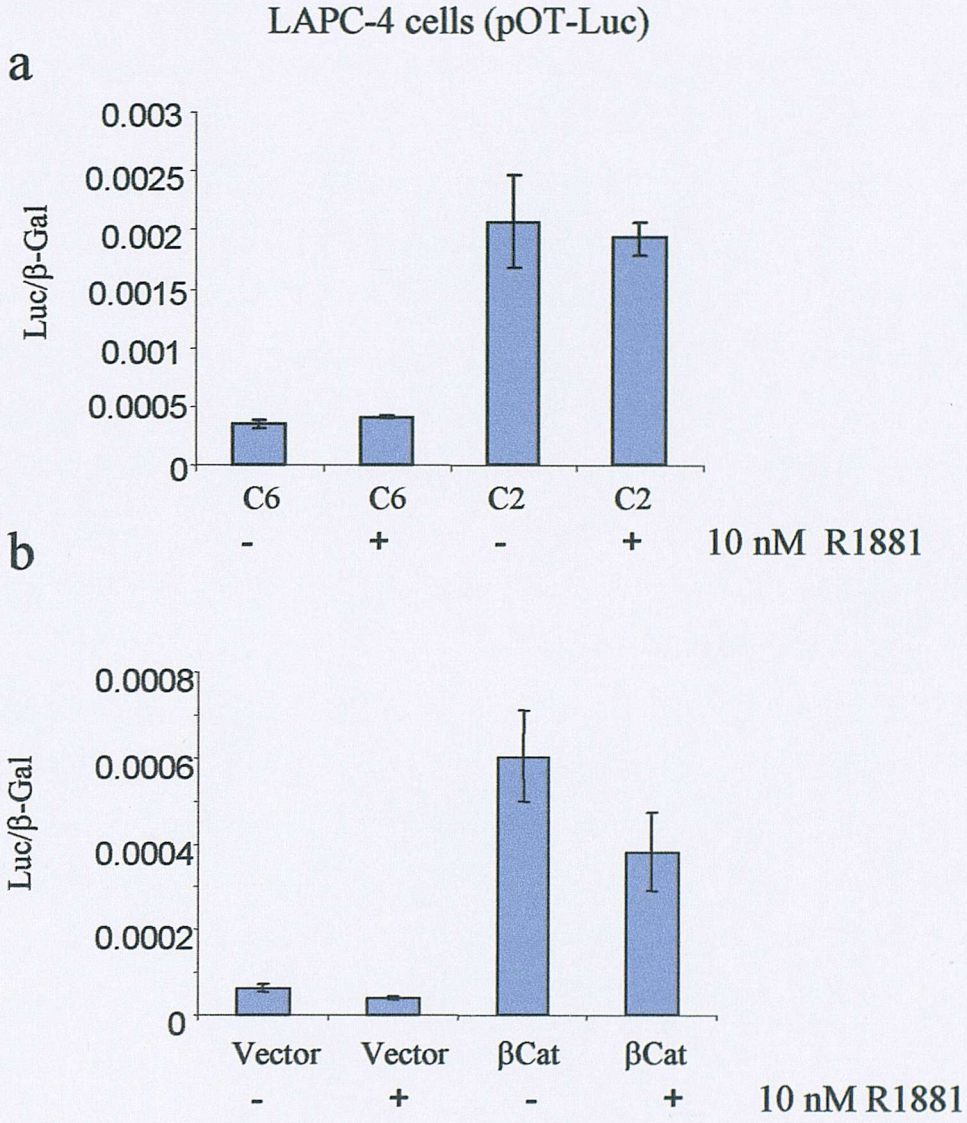
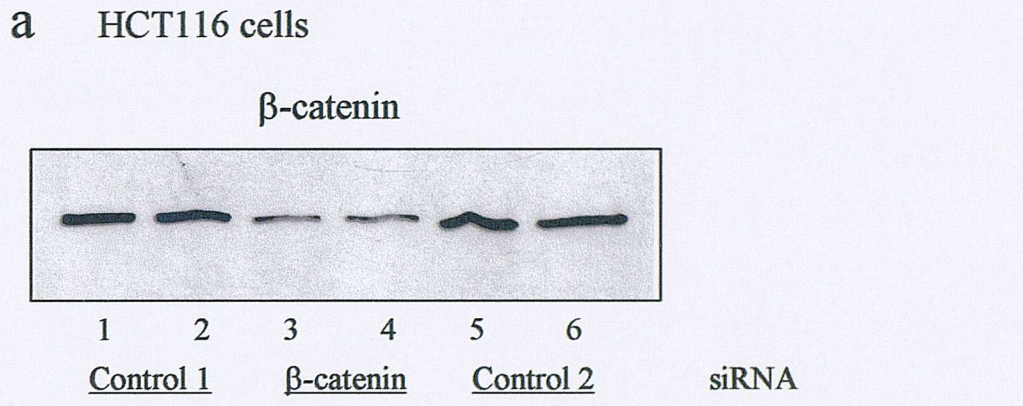


Figure 15. (a) Activation of β-catenin/Tcf-4-dependent transcription by expression of a constitutively-active Wnt receptor. LAPC-4 cells were transfected with the reporter vectors pOT-Luc and RSV-β-Gal plus the constitutively-active plasmid of LRP5, C2, or truncated LRP5, C6. **(b) Activation of β-catenin/Tcf-4-dependent transcription by overexpression of β-catenin.** LAPC-4 cells were transfected with the reporter vectors pOT-Luc and RSV-β-Gal plus β-catenin (βCat) or plus the empty vector pMT23 as control (Vector). Cells were treated with (+) or without (-) 10 nM R1881 for 24 hours and then lysed and assayed for luciferase (Luc) and β-galactosidase (β-Gal) activities. Results are presented as the ratio between luciferase and β-Gal activities.

As shown earlier, β-catenin levels are already high in PCa cells (Figures 8a and 8b). In order to determine whether endogenous β-catenin contributes to basal pOT activity, β-catenin levels were reduced using a β-catenin RNAi plasmid. As described by Van de Wetering *et al.* (van de Wetering *et al.*, 2003), β-catenin RNAi plasmid (pTERβi) can reduce β-catenin protein levels and downregulate β-catenin/Tcf-dependent transcription in colon cancer cells. Owing to the low transfection efficiency of PCa cells, it was not possible to examine the effects of pTERβi on endogenous β-catenin protein levels by western blotting in these cells. In order to confirm that the pTERβi plasmid reduces β-catenin protein levels, the colon cancer cell line HCT116, which is easier to transfect and has a high endogenous level of β-catenin, was used. HCT116 cells were transfected with an AR plasmid and either control siRNA plasmids (control 1 or 2), in the absence or presence of 1 nM R1881, or pTERβi plasmids. Whole cell extracts were probed with antibody against β-catenin. Expression of pTERβi led to a significant reduction in the level of β-catenin protein (Figure 16a). Additionally, the effects on β-catenin were determined by measuring β-catenin/Tcf-dependent transcriptional activity. Figure 16b shows the effect of pTERβi plasmid expression on β-catenin/Tcf-dependent transcription in PCa cells as well as in HCT116 cells (as a control for cells with high basal β-catenin/Tcf activity). As expected, depletion of β-catenin levels reduced β-catenin/Tcf-dependent transcriptional activity in HCT116 cells. pTERβi also repressed basal β-catenin/Tcf-dependent transcription in all PCa cell lines that were examined, with the greatest inhibition occurring in CWR-R1 cells. This may reflect the higher basal β-catenin/Tcf activity in these cells.

Figure 16



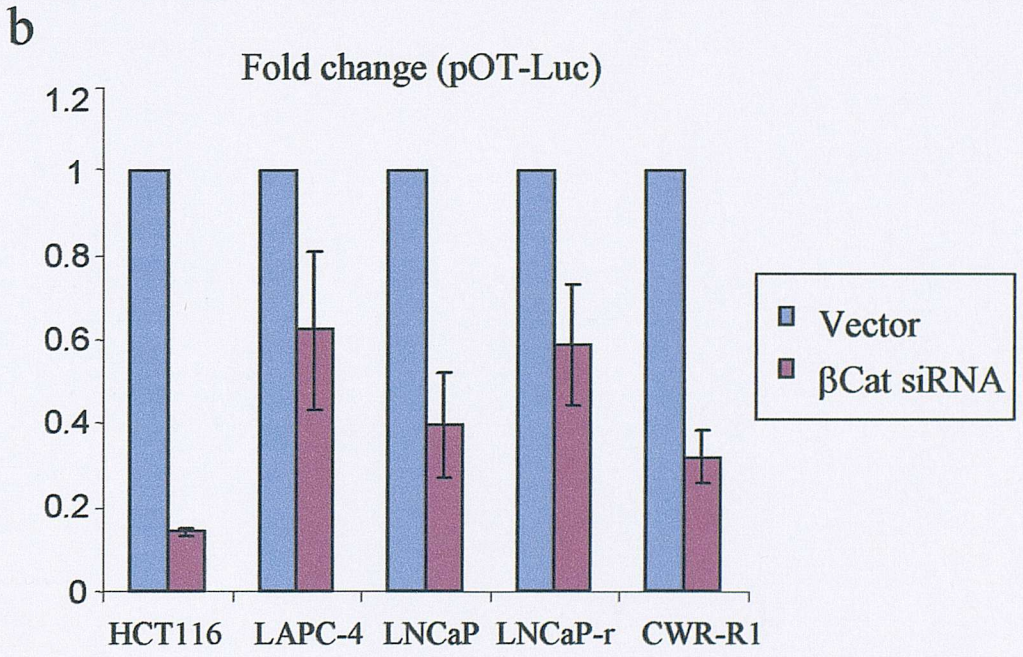


Figure 16. Depletion of endogenous β-catenin inhibits β-catenin/Tcf-4-dependent transcription in HCT116 and PCa cells. (a) HCT116 cells were transfected with pSG5 AR and either control 1 (lanes 1 and 2), β-catenin (lanes 3 and 4) or control 2 (lanes 5 and 6) siRNA expression plasmids. Cells were treated with (lanes 2,4 and 6) or without (lanes 1,3 and 5) 1 nM R1881 and whole cell extracts were probed with antibody against β-catenin. (b) The indicated cell lines were transfected with the reporter vectors pOT-Luc and pDM-β-Gal plus either control 1 (Vector) or β-catenin siRNA (βCat siRNA) expression plasmids. Cells were assayed for luciferase (Luc) and β-galactosidase (β-Gal) activities and results are presented as the activity relative to each cell line transfected with empty vector.

Investigating the effect of various Wnt components on the β-catenin/Tcf-dependent transcriptional activity in the presence of androgens, we noticed that androgens have a repressive effect on the OT promotor. For example, as shown in Figure 15b, β-catenin/Tcf activity in LAPC-4 cells was activated by the expression of β-catenin in the presence and absence of hormone. However, β-catenin/Tcf activity was also slightly (but reproducibly) repressed in the presence of hormone compared to when it was absent. Therefore, the effects of androgen treatment on β-catenin/Tcf signalling were examined in more detail. In order to test the effects that androgen treatment and AR have on Wnt signalling, the AR-negative cell line, PC3, was transfected with control or AR plasmid and treated with or without 10 nM R1881. The results in Figure 17 indicate that AR inhibited β-catenin/Tcf activity in PC3 cells, suggesting that the β-catenin/Tcf basal activity in these cells is higher in the absence of AR. This result is in agreement with reports showing that AR has an inhibitory effect on β-catenin/Tcf activity due to a competition between AR and Tcf over binding to β-catenin (Pawlowski *et al.*, 2002; Mulholland *et al.*, 2003; Song *et al.*, 2003).

Figure 17

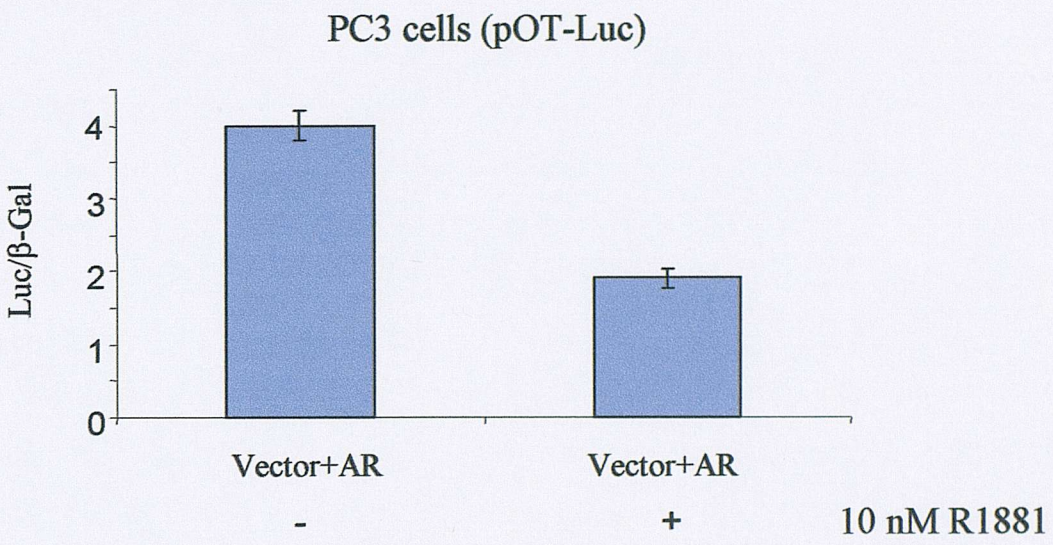


Figure 17. Ligand-dependent AR activity repression of β-catenin/Tcf-4-dependent transcription in PCa cells. PC3 cells were transfected with the reporter vectors pOT-Luc and RSV-β-Gal plus the empty vector pMT23 (Vector) and pSG5 AR. Cells were treated with (+) or without (-) 10 nM R1881 for 24 hours and then lysed and assayed for luciferase (Luc) and β-galactosidase (β-Gal) activities. Results are presented as the ratio between luciferase and β-Gal activities.

3.2.2 The AR transcriptional response to regulation of Wnt signalling in PCa cells

In order to determine how the AR responds to Wnt overexpression, cells were transfected with Wnt1 or Wnt3A plasmids (or empty vector as control) and the reporter plasmids MMTV-Luciferase (MMTV-Luc) (MMTV has ARE's, and is used as a measure of AR activity) and RSV-β-Gal. Wnt expression did not affect AR-dependent transcription greatly in PCa cells (Figure 18). Since Wnt expression did not activate β-catenin/Tcf signalling in LNCaP and LAPC-4 cells, it perhaps was not surprising that AR activity was not activated in these cells either. However, Wnt1 and Wnt3A also did not affect AR activity in CWR-R1 cells, even though they activated β-catenin/Tcf dependent transcription (see Figure 13). One possibility is that the level of endogenous β-catenin in these cells is already regulating AR activity. Therefore, the effect of β-catenin overexpression on AR activity, which has been reported to be positive (Truica *et al.*, 2000; Chesire *et al.*, 2002; Yang *et al.*, 2002), was examined. LAPC-4 and CWR-R1 cells were transfected with β-catenin (or the control vector), and the effect on AR signalling was tested in the presence of 10 nM R1881. Results in Figure 19 (LAPC-4 (a), CWR-R1 (b)) show that in both cell lines, AR activity was not significantly affected by the expression of β-catenin. Clearly the effects of β-catenin overexpression on Tcf and AR activities differ.

Since PCa cells already have high levels of β-catenin, the effects of β-catenin depletion on AR transcriptional activity were next tested. HCT116, CWR-R1, LNCaP cells were transfected with either control siRNA (control 1) or pTERβi plasmids and grown in androgen-depleted medium in the presence of androgens (Figure 20b). 22Rv1 cells were transfected with two different siRNA controls (control 1 and 2) and grown in the presence or absence of androgens (Figure 20c). In order to test the effect of β-catenin depletion on AR activity in the control cell line, HCT116 cells, AR was ectopically expressed in these cells. As might be expected from published β-catenin overexpression studies, depletion of β-catenin in HCT116 cells significantly reduced AR activity ($p=0.01$) (Figure 20b). In order to determine if the effects of β-catenin siRNA resulted from a reduction in the level of expression of cotransfected AR, western blots were also conducted as described in section 3.2.1. As shown in Figure 16a, expression of β-catenin siRNA led to a reduction in β-catenin protein levels, however according to Figure 20a, AR protein levels were unaffected. Surprisingly, depletion of β-catenin significantly increased endogenous

AR transcriptional activity in CWR-R1 ($p=0.004$), LNCaP ($p=0.003$) (Figure 20b) or 22Rv1 cells ($p<0.00005$) (Figure 20c). These results indicate that endogenous β-catenin in PCa cells is somehow regulating AR activity, but not in the manner predicted by studies using overexpressed β-catenin in PCa cell lines (Truica *et al.*, 2000; Mulholland *et al.*, 2002; Pawlowski *et al.*, 2002).

Figure 18

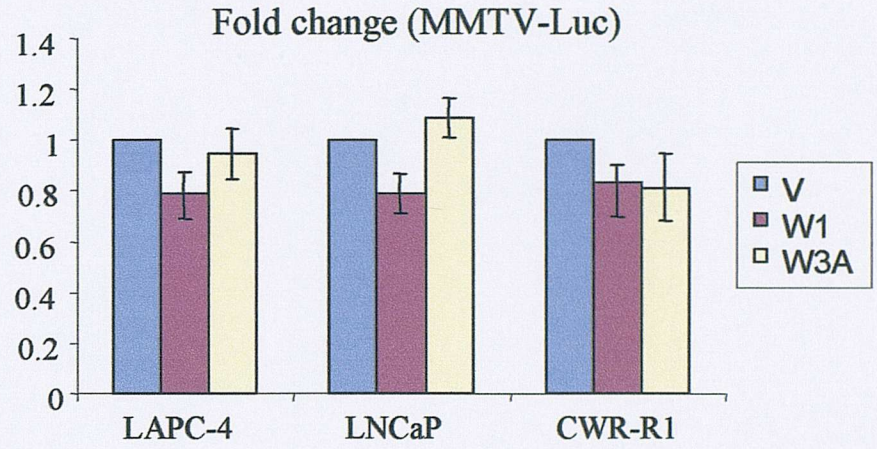


Figure 19

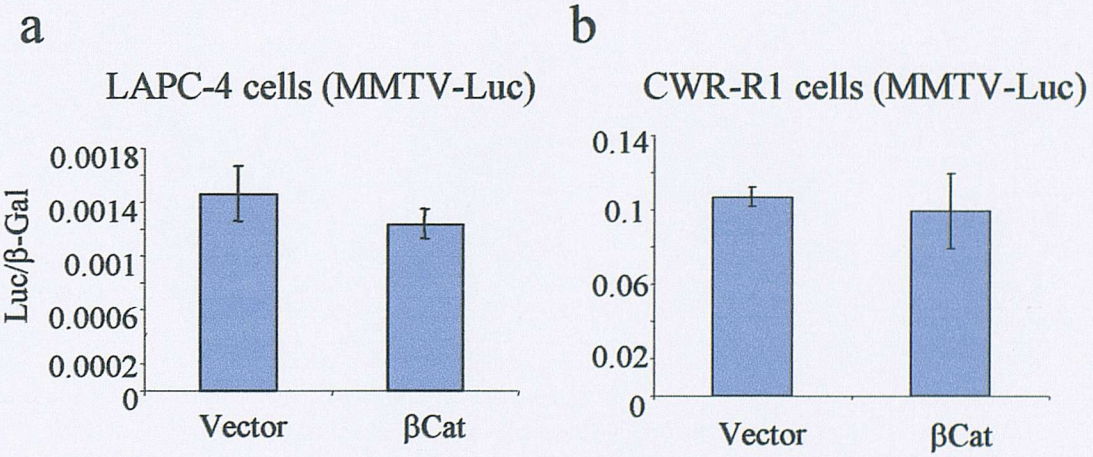


Figure 18. Wnt expression does not affect AR-dependent transcription in PCa cells. LAPC-4, LNCaP and CWR-R1 cells were transfected with the reporter vectors MMTV-Luc and RSV-β-Gal plus Wnt (W1) or Wnt3A (W3A), or their respective empty vectors pMT23 and pGK (V) Cells were treated with 10 nM R1881 for 24 hours and then lysed and assayed for luciferase (Luc) and β-galactosidase (β-Gal) activities. Results are presented as the activity relative to each cell line transfected with empty vector.

Figure 19. β-catenin overexpression does not affect AR-dependent transcription in LAPC-4 (a) and CWR-R1 (b) cells. Cells were transfected with the reporter vectors MMTV-Luc and RSV-β-Gal plus β-catenin (βCat) or the empty vector pMT23 as control (Vector). Cells were treated with 10 nM R1881 for 24 hours and then lysed and assayed for luciferase (Luc) and β-galactosidase (β-Gal) activities. Results are presented as the ratio between luciferase and β-Gal activities.

Figure 20

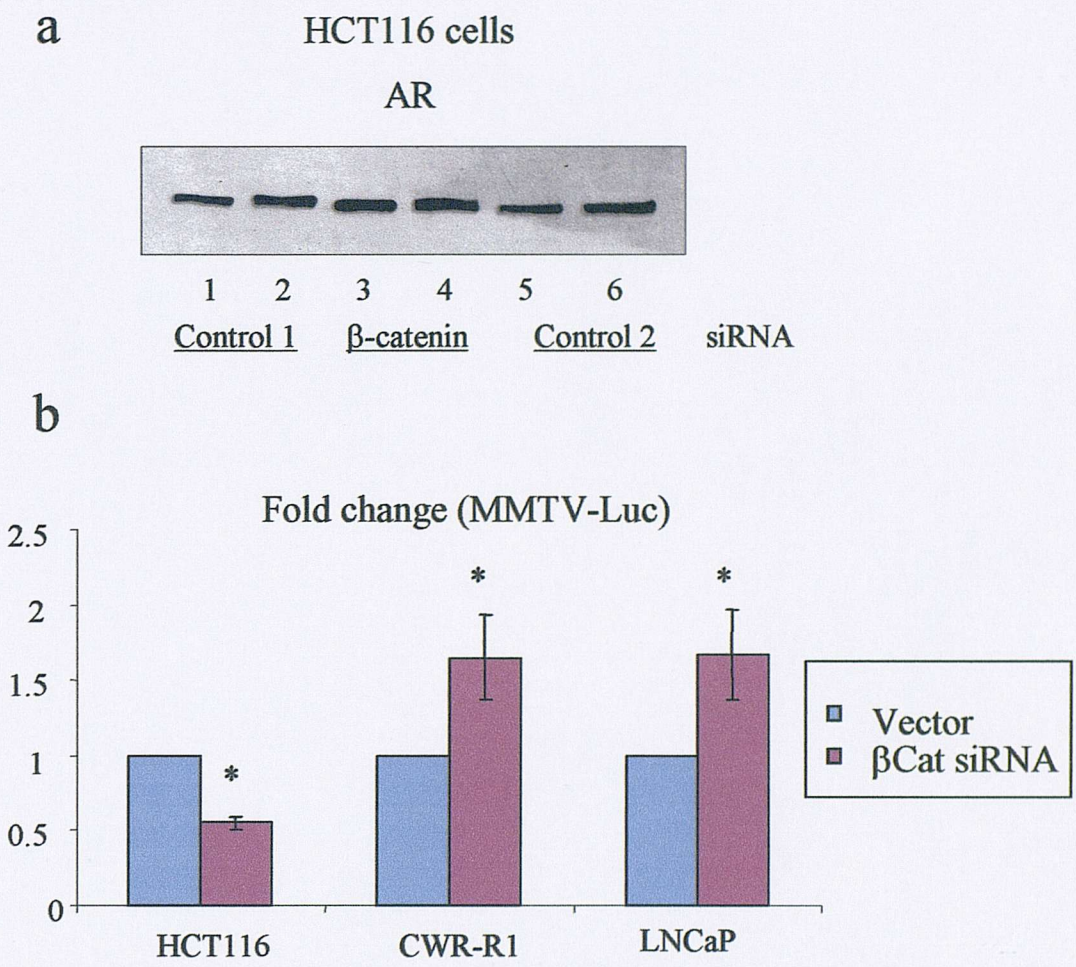


Figure 20. Depletion of endogenous β-catenin leads to an increase in AR activity in PCa cells. (a) HCT116 cells were transfected with pSG5 AR and either control 1 (lanes 1 and 2), β-catenin (lanes 3 and 4) or control 2 (lanes 5 and 6) siRNA expression plasmids. Cells were treated with (lanes 2,4 and 6) or without (lanes 1,3 and 5) 1 nM R1881 and whole cell extracts were probed with antibody against β-catenin (Figure 16a) and then reprobed with antibody against AR. (b) HCT116, CWR-R1 and LNCaP cells were transfected with the reporter vectors MMTV-Luc and pDM-β-Gal, pSG5 AR (HCT116 cells only) and either control 1 (Vector) or β-catenin siRNA (βCat siRNA) expression plasmids. Cells were treated with 10 nM (CWR-R1 cells) or 1 nM (HCT116 and LNCaP cells) R1881 for 24 hours. Results are presented as the activity relative to each cell line transfected with vector. (*) AR activity was significantly increased in βCat siRNA-transfected cells compare to vector-transfected cells in CWR-R1 ($p=0.004$) and LNCaP cells ($p=0.003$), and significantly reduced in HCT116 cells ($p=0.01$). Experiment was done at least three times in triplicates.

Figure 20 continued-

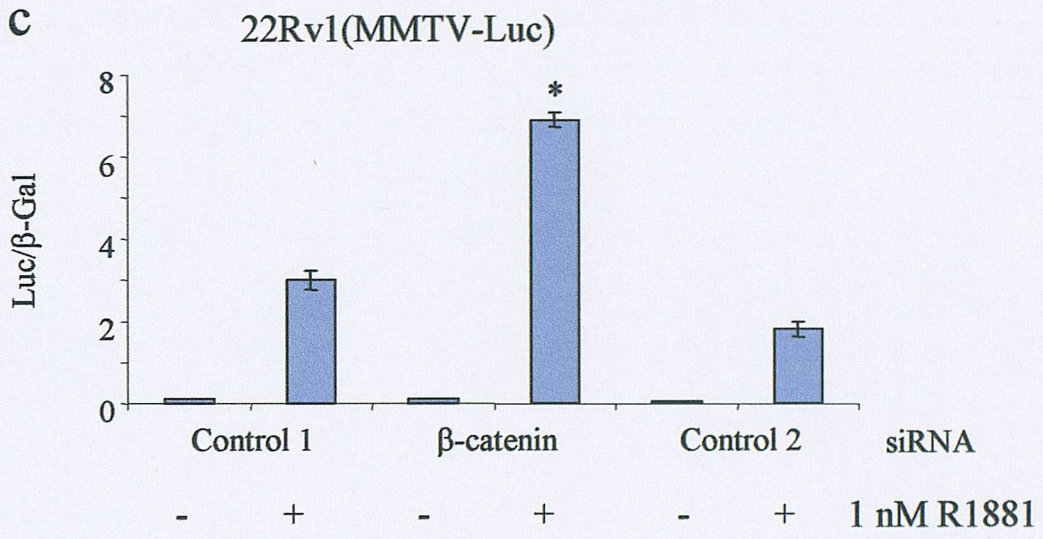


Figure 20 (c). Depletion of endogenous β-catenin leads to an increase in AR activity in PCa cells. 22Rv1 cells were transfected with the reporter vectors MMTV-Luc and pDM-β-Gal and either control 1, β-catenin, or control 2 siRNA expression vectors. Cells were grown in hormone-depleted medium in the presence (+) or absence (-) of 1 nM R1881 for 24 hours. Cells were assayed for luciferase (Luc) and β-galactosidase (β-Gal) activities, and results are presented as the ratio between luciferase and β-Gal activities. (*) AR activity was significantly increased in β-catenin siRNA-transfected cells compare to control 1 and control 2-transfected cells ($p < 0.00005$). Experiment was done twice in triplicates.

3.3 Chapter 3- Regulation of AR transcriptional activity by GSK3β and GSK3β-binding proteins

3.3.1 Regulation of AR transcriptional activity by Axin deletion mutants

The results in chapter 1 and 2 suggested that CWR-R1 cells could be most suitable for further studies on the crosstalk between Wnt and AR signalling in PCa lines. This is due to several reasons; first and importantly, because CWR-R1 cells respond to Wnts (see Figure 13). Second, CWR-R1 cells express AR, and have a very good hormone response at the transcriptional level and partially at the level of androgen-regulated growth (Wainstein *et al.*, 1994; Tan *et al.*, 1997; Gregory *et al.*, 2001b), which is typical of the majority of tumours *in vivo*. Third, these cells are relatively easy to transfect. During this work I also used 22Rv1 cells, which share the advantages of CWR-R1 cells and, in addition, grow under less stringent culture conditions.

The RNAi experiments suggested that endogenous β-catenin does not activate AR in CWR-R1 cells. However, depletion of β-catenin may have other effects in addition to its effects on transcription, for example, on cell-cell adhesion. Therefore, Axin was used to inhibit β-catenin/Tcf signalling. Axin is a scaffold protein that promotes phosphorylation and degradation of β-catenin in the cytosol and its expression in several cell lines leads to downregulation and inactivation of β-catenin (Kishida *et al.*, 1998; Ross *et al.*, 2000; Kishida *et al.*, 2001; Reya *et al.*, 2003). CWR-R1 cells were transfected with several GFP-tagged Axin plasmids (presented in Figure 21) as well as the reporter plasmids MMTV-Luc and RSV-β-Gal. The full-length Axin fused to GFP (GAX), a form of GAX with a point mutation (L521P) in the GSK3β-binding domain (GAXP), and GFP alone were used. Figure 22a shows that GAX significantly reduced AR activity ($p<0.00005$), but GAXP did not ($p=0.5$). GAXP has a mutation in a conserved proline residue in the GSK3β-binding domain of Axin, which prevents binding to GSK3β and also reduces binding to β-catenin (Smalley *et al.*, 1999). This indicates that Axin also has the ability to inhibit AR-mediated transcription but requires an intact GSK3β-binding domain. Next, in order to locate the exact domain required for Axin to inhibit AR activity, different deletion mutants of Axin that lack certain protein-binding domains were used: GAXΔAPC (GAX1), which cannot bind APC, GAX1ΔGSK3, which cannot bind APC and

GSK3β, or GAX1Δβ-catenin, which cannot bind APC and β-catenin. As shown in Figure 22b, the interaction of Axin with APC was not required for AR inhibition since GAX1 inhibited AR as well as GAX ($p<0.00005$). However, deletion of the GSK3β-binding site in Axin (GAX1ΔGSK3), reduced the ability of Axin to repress AR activity. GAX1ΔGSK3 still weakly binds to GSK3β (Hinoi *et al.*, 2000), which might explain why it still repressed AR activity compared to GFP ($p<0.00005$). Importantly, the interaction of Axin with β-catenin was not required for the inhibition of AR, as GAX1Δβ-catenin still inhibited AR ($p<0.00005$).

In order further to delineate the region of Axin required for inhibition of AR, four additional Axin constructs were used (also shown in Figure 21). GAX7, which is a GFP-tagged construct of Axin that can only bind β-catenin and GSK3β, its mutated derivative GAX7P, which has a point mutation in the GSK3β-binding domain, or GFP alone as a control. A FLAG-tagged Axin construct, AX2, that can bind GSK3β only and its mutated derivative AX2P, were also used. The control for AX2 and AX2P constructs was a c-myc-tagged construct (MAX4), containing residues 1-229 of Axin, which do not contain a binding site for any known proteins. According to Figure 23a, GAX7 repressed AR activity ($p<0.00005$) while the mutated form, GAX7P, repressed less ($p=0.0005$). Similarly, Figure 23b shows that AX2 repressed AR activity ($p<0.00005$) and AX2P repressed less ($p=0.02$). Moreover, co-expression of w.t GSK3β with AX2 rescued the inhibitory effect of AX2. The effect of w.t GSK3β and AX2 co-expression did not have a significant effect on AR activity compare to the control plasmid expression ($p=0.09$). These results (Figures 23a and 23b) support what was suggested from the results in Figure 22, namely, that GSK3β binding is required for repression of AR by Axin, and also show that the GSK3β-binding domain of Axin is sufficient for the inhibition of AR activity. Moreover, the fact that the β-catenin-binding domain of Axin is not required for the inhibition of AR activity suggests that the effects of Axin are independent of β-catenin. In view of these results, GSK3β and its effect on the AR signalling pathway became the focus of subsequent studies.

Figure 21

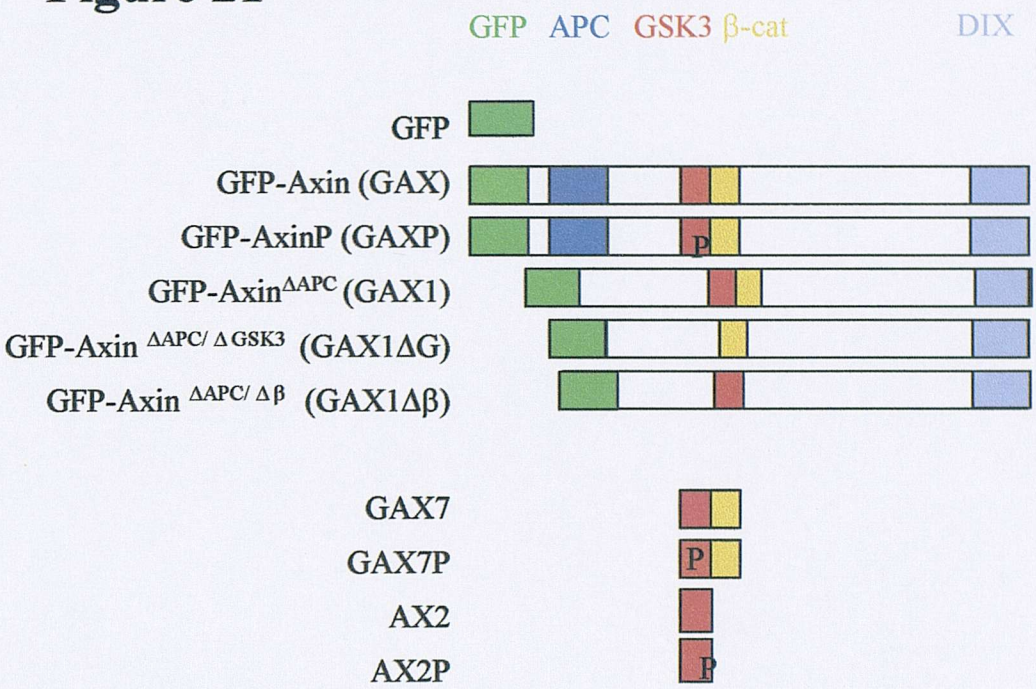


Figure 21. Cartoon of Axin constructs used in transient transfections. GFP-tagged Axin (GAX) was used as the full length Axin to create the following Axin deletion mutants; GFP-AxinΔAPC (GAX1) that cannot bind to APC, GFP-AxinΔAPCΔGSK3 (GAX1ΔG) that cannot bind to APC and GSK3β, GFP-AxinΔAPCΔβ (GAX1Δβ) that cannot bind to APC and β-catenin. GAX7 is a construct that can only bind to GSK3β and β-catenin. AX2 is a FLAG-tagged construct of Axin that can only bind to GSK3β. P denotes the L521P mutation that disrupts binding of Axin to GSK3β.

Figure 22

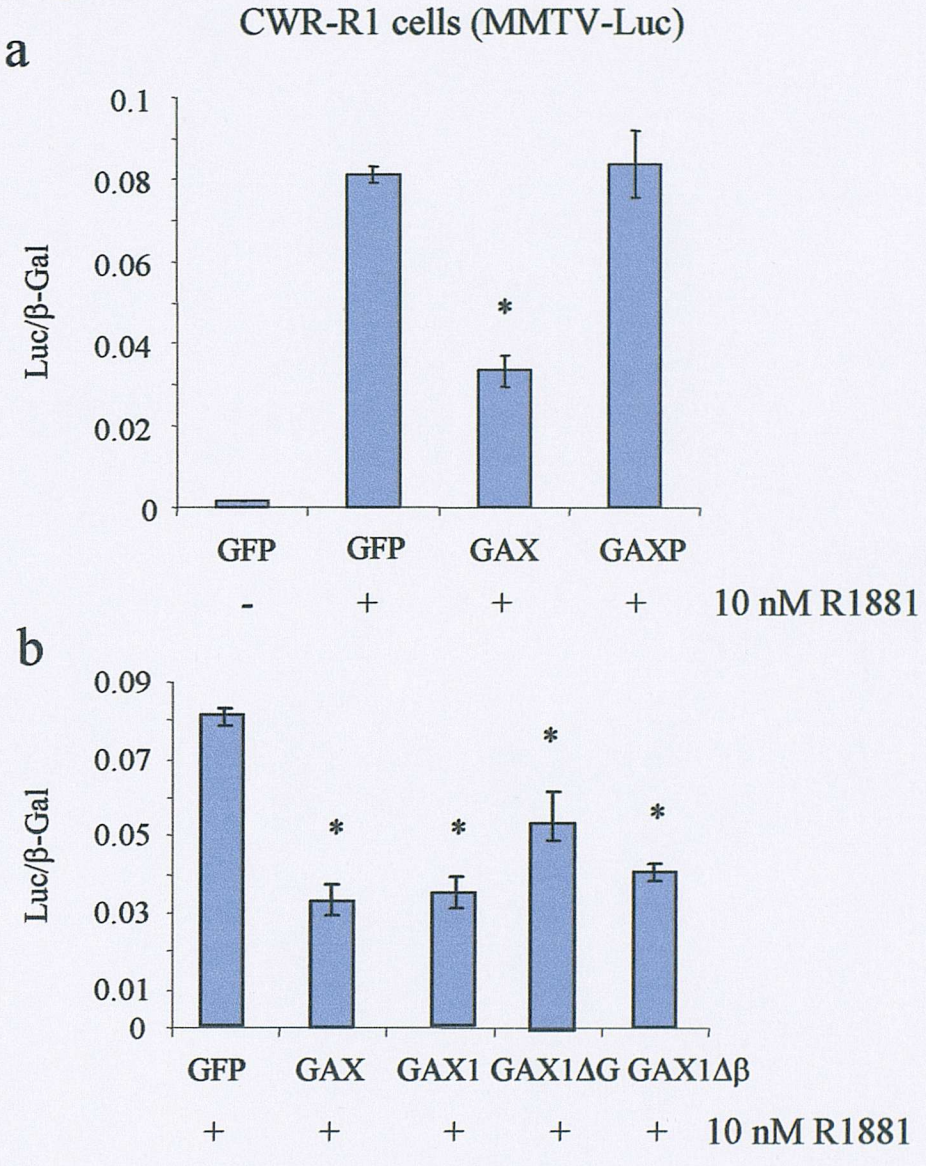


Figure 22. (a) AR-dependent transcription is inhibited by Axin overexpession. CWR-R1 cells were transfected with the reporter vectors MMTV-Luc and RSV-β-Gal plus either GFP, GAX or GAXP. Cells were treated with (+) or without (-) 10 nM R1881 for 24 hours. **(b) GSK3β interaction with Axin is necessary for the inhibition of AR activity.** CWR-R1 cells were transfected with the reporter vectors MMTV-Luc and RSV-β-Gal plus either GFP, GAX, GAX1, GAX1ΔG or GAX1Δβ. Cells were treated with 10 nM R1881 for 24 hours. Cells were assayed for luciferase (Luc) and β-galactosidase (β-Gal) activities, and results are presented as the ratio between luciferase and β-Gal activities. (*) AR activity was significantly reduced in cells expressing GFP-tagged Axin mutants compare to cells expressing GFP alone in the presence of R1881 ($p<0.00005$). Experiment was done at least three times in triplicates.

Figure 23

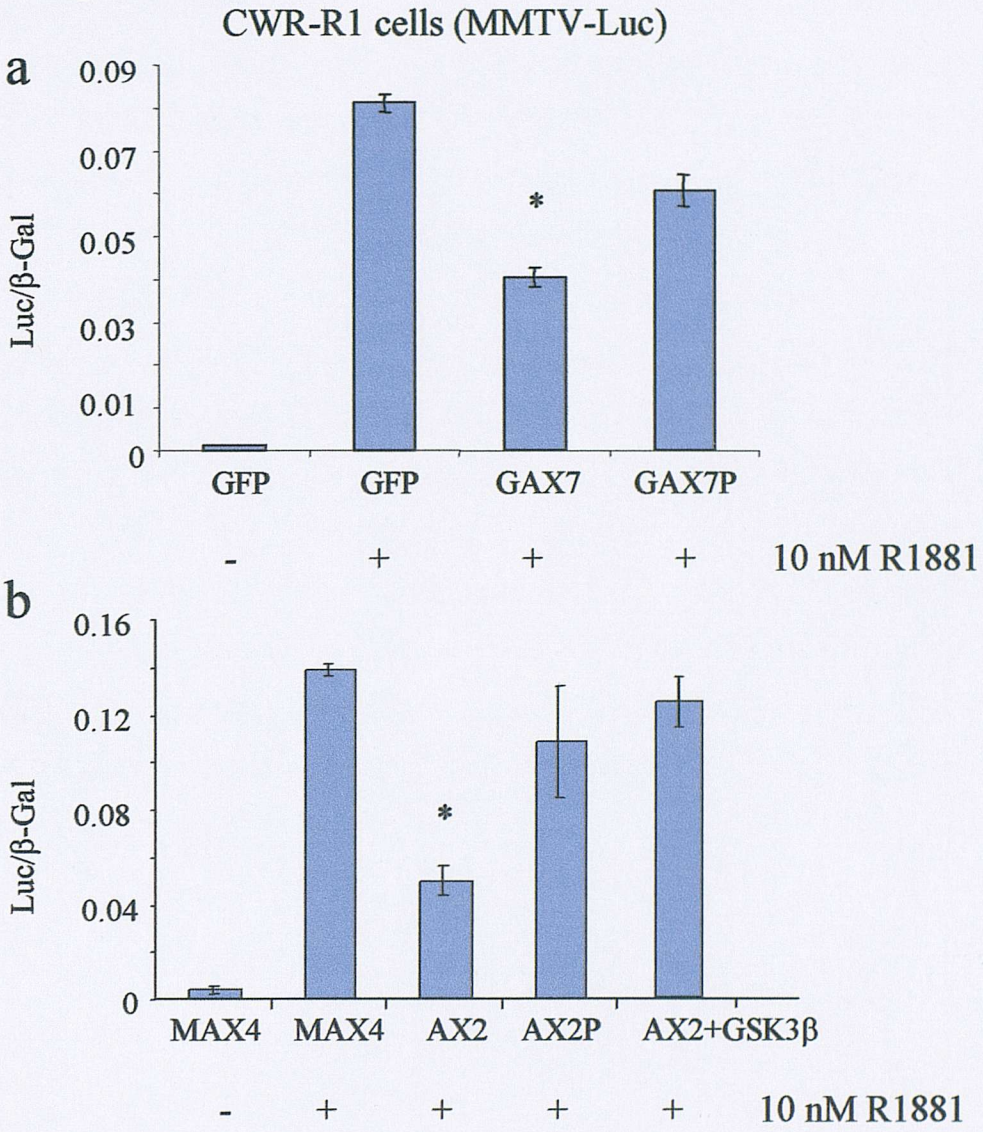


Figure 23. GSK3β interaction with Axin is sufficient for the inhibition of AR activity. (a) CWR-R1 cells were transfected with the reporter vectors MMTV-Luc and RSV-β-Gal plus either GFP, GAX7 or GAX7P. (b) CWR-R1 cells were transfected with the reporter vectors MMTV-Luc and RSV-β-Gal plus either a c-myc-tagged Axin construct containing the N-terminus only (MAX4) as control, AX2, AX2 with w.t GSK3β or AX2P. Cells were treated with (+) or without (-) 10 nM R1881 for 24 hours and assayed for luciferase (Luc) and β-galactosidase (β-Gal) activities. Results are presented as the ratio luciferase and β-Gal activities. (*) AR activity was significantly reduced in cells expressing Axin mutants compare to cells expressing control plasmid (a-GFP, b-MAX4) in the presence of R1881 ($p<0.00005$ for GAX7 and AX2). Experiment was done at least three times in triplicates.

3.3.2 Regulation of AR transcriptional activity by FRAT

A second approach was used to inhibit GSK3β. This involved the GSK3β-binding protein, FRAT, which binds to GSK3β and sequesters it from its interactions with other proteins in the cell (Li *et al.*, 1999; Franca-Koh *et al.*, 2002; Fraser *et al.*, 2002). FRAT was first tested for its effects on β-catenin/Tcf-dependent transcription in HEK 293 cells. GFP-tagged FRAT, or a mutated form that cannot bind to GSK3β, GFP-tagged FRATΔC (or the empty vector, encoding GFP) were transfected into HEK 293 cells, together with the reporter plasmids pOT-Luc and and RSV-β-Gal. As shown in Figure 24a, FRAT expression activated the Wnt pathway, but FRATΔC did not. Next, the effect of FRAT expression on AR activity was examined in CWR-R1 cells. CWR-R1 cells were transfected with FRAT, FRATΔC or GFP and MMTV-Luc and treated with 10 nM R1881 (+) or carrier (-) for 24 hours. As presented in Figure 24b, in the presence of hormone, FRAT repressed AR activity and, FRATΔC did not. These results contribute to the idea that GSK3β normally binds and activates AR. Thus, by sequestering GSK3β (by expression of FRAT or Axin), AR-mediated transcription is repressed.

Figure 24

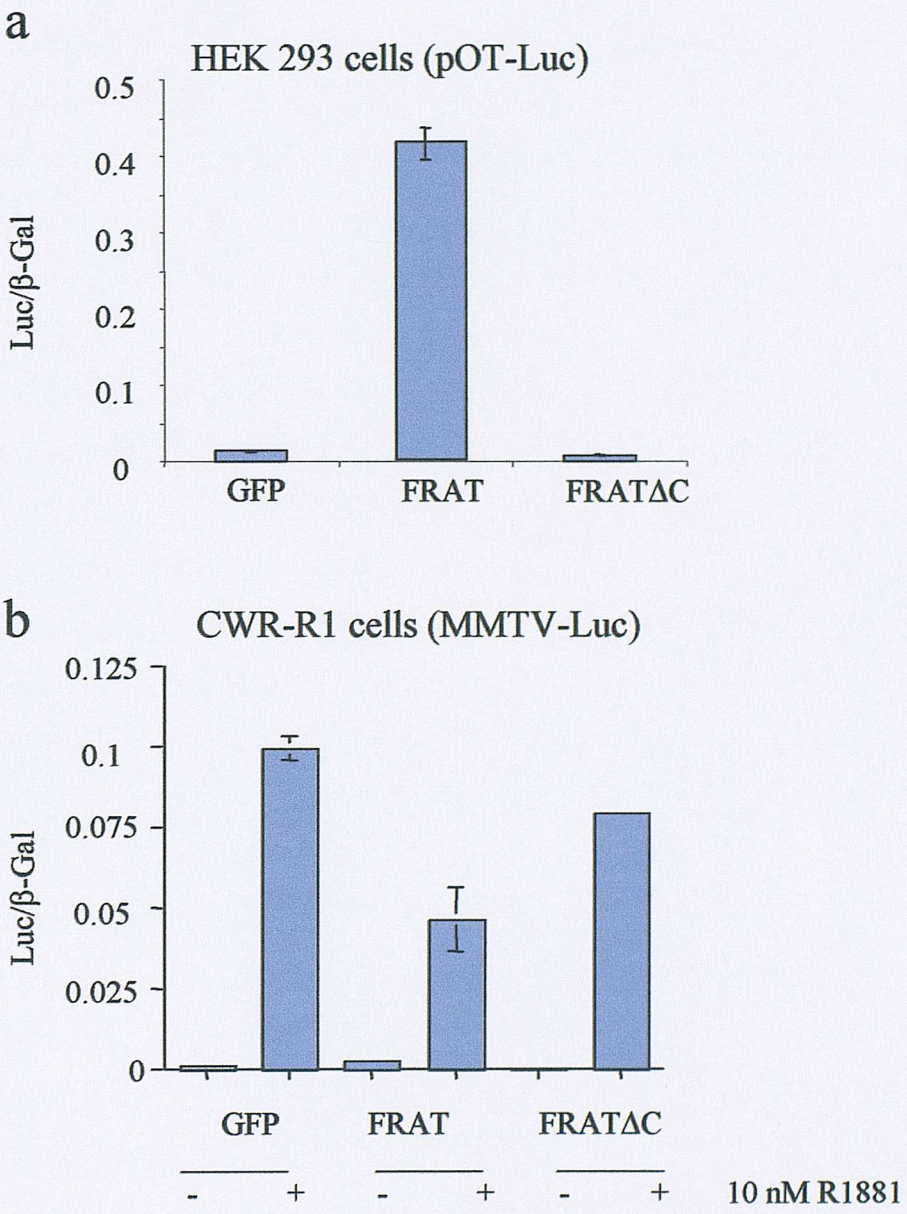


Figure 24. (a) FRAT activates β-catenin/Tcf-4-dependent transcription in HEK 293 cells. Cells were transfected with the reporter vectors pOT-Luc and RSV-β-Gal plus either GFP as control, GFP-FRAT or GFP-FRATΔC. **(b) FRAT represses AR-dependent transcription in CWR-R1 cells.** Cells were transfected with the reporter vectors MMTV-Luc and RSV-β-Gal plus either GFP, GFP-FRAT or GFP-FRATΔC and treated with (+) or without (-) 10 nM R1881 for 24 hours. Cells were assayed for luciferase (Luc) and β-galactosidase (β-Gal) activities, and results are presented as the ratio between luciferase and β-Gal activities.

3.3.3 The effects of GSK3β expression on AR activity in PCa cells

The effects of GSK3β-binding proteins on AR signalling suggested an important role for GSK3β in the regulation of AR activity. Therefore, the effects of GSK3β overexpression on AR-mediated transcription were next assessed. W.t GSK3β, a constitutively-active form of GSK3β that has a mutation at S9 (S9A), the inhibitory phosphorylation site, and a catalytically-inactive form of GSK3β (K216R) were expressed in PCa cell lines together with the reporter plasmids MMTV-Luc and RSV-β-Gal.

AR transcriptional activity was not significantly affected by expression of any of these constructs in 22Rv1 cells ($p=0.5$ for w.t GSK3β, $p=0.3$ for S9A, $p=0.2$ for K216R) (Figure 25a). The lack of effect of GSK3β on AR activity might be because endogenous GSK3β is already active in 22Rv1 cells. Therefore, the effect of GSK3β overexpression was examined in LNCaP cells, in which GSK3β is known to be inactive as a result of phosphorylation at S9 (Salas *et al.*, 2004). When expressed at high levels, w.t GSK3β significantly increased AR activity in LNCaP cells ($p=0.02$) (Figure 25b). Constitutively-active GSK3β significantly increased AR transcriptional activity both at low and high levels of expression ($p=0.0006$ and $p=0.0004$, respectively). Catalytically-inactive GSK3β did not appear to affect AR activity ($p=0.3$). These results indicate that overexpression of GSK3β can activate AR in PCa cell line with low endogenous GSK3β activity. It is not clear why the catalytically-inactive form of GSK3β did not inhibit AR activity, but it may be that the level of expression achieved was too low.

Lastly, the effect of GSK3β overexpression in PC3 cells was tested. PC3 cells were transfected with small amount of pSG5 AR and either w.t GSK3β or empty vector and were treated with 0.15 nM R1881 (close to the Kd of AR for R1881) (Gregory *et al.*, 2001b), 10 nM R1881 or a carrier, 24 hours prior harvesting the cells. As shown in Figure 25c, AR transcriptional activity significantly increased when GSK3β was overexpressed at both hormone doses ($p<0.0005$ at the 0.15 nM dose, and $p=0.02$ at the10 nM dose).

Taken together, results in this chapter support the hypothesis that GSK3β increases AR transcriptional activity in PCa cells.

Figure 25

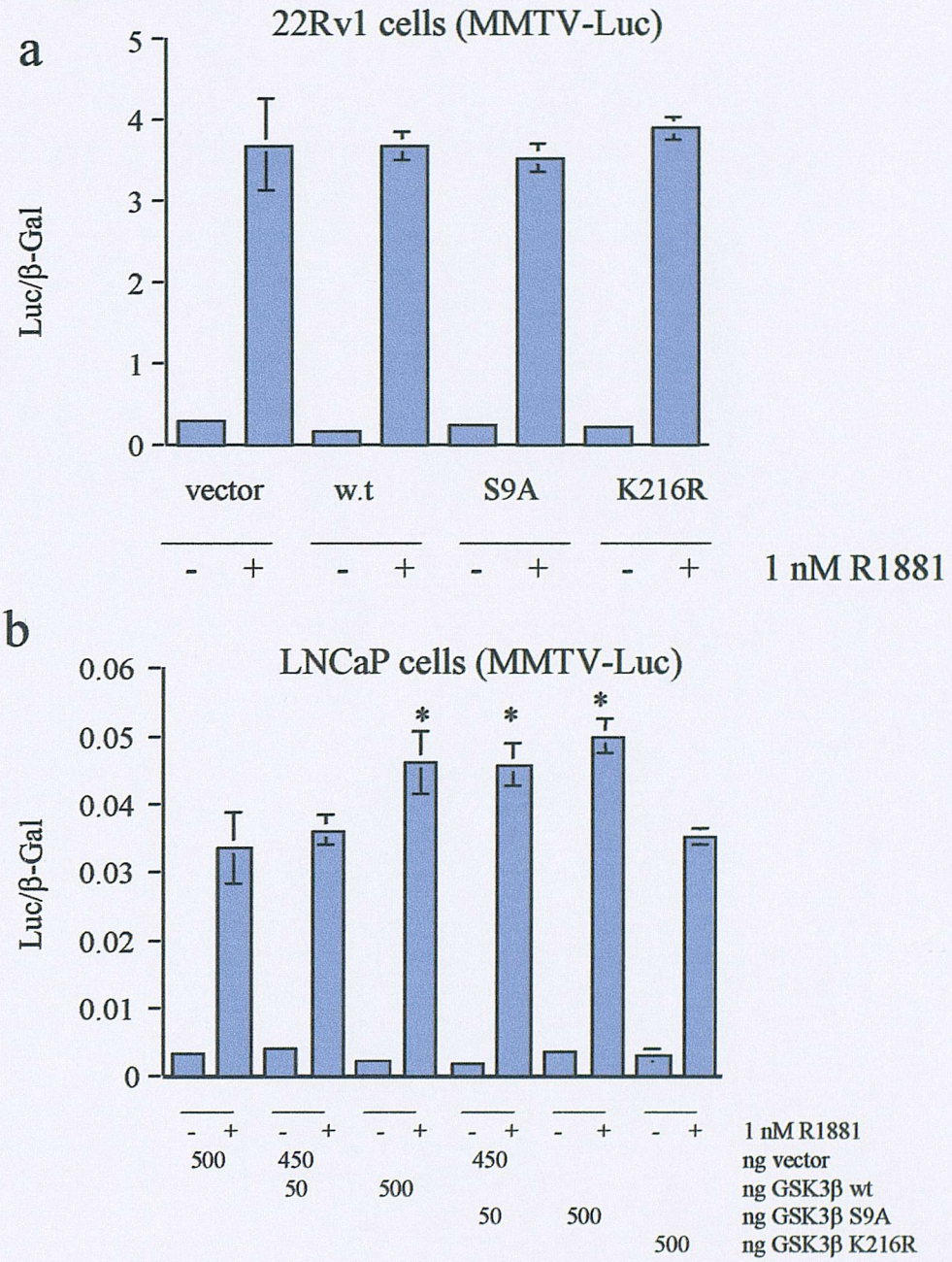


Figure 25 (a-b). GSK3β overexpression increases AR-dependent transcription in LNCaP cells. (a) 22Rv1 cells were transfected with the reporter vectors MMTV-Luc and RSV-β-Gal plus either the empty vector pCDNA3 (vector), w.t GSK3β, constitutively-active GSK3β, S9A, or the inactive form of GSK3β, K216R. Cells were treated with (+) or without (-) 1 nM R1881 for 24 hours. (b) LNCaP cells were transfected with the reporter vectors MMTV-Luc and RSV-β-Gal and the indicated amounts of empty vector pCDNA3 (vector), w.t GSK3β, GSK3β S9A, or GSK3β K216R. Cells were treated with (+) or without (-) 1 nM R1881 for 24 hours. Cells were assayed for luciferase (Luc) and β-galactosidase (β-Gal) activities, and results are presented as the ratio between luciferase and β-Gal activities. (*) AR activity significantly increased in cells expressing GSK3β constructs compare to cells expressing empty vector in the presence of R1881 at the higher dose of w.t GSK3β ($p=0.02$) as well as at the lower ($p=0.0006$) and higher dose ($p=0.0004$) of S9A GSK3β. Experiment was done three times in triplicates.

Figure 25 continued-

c

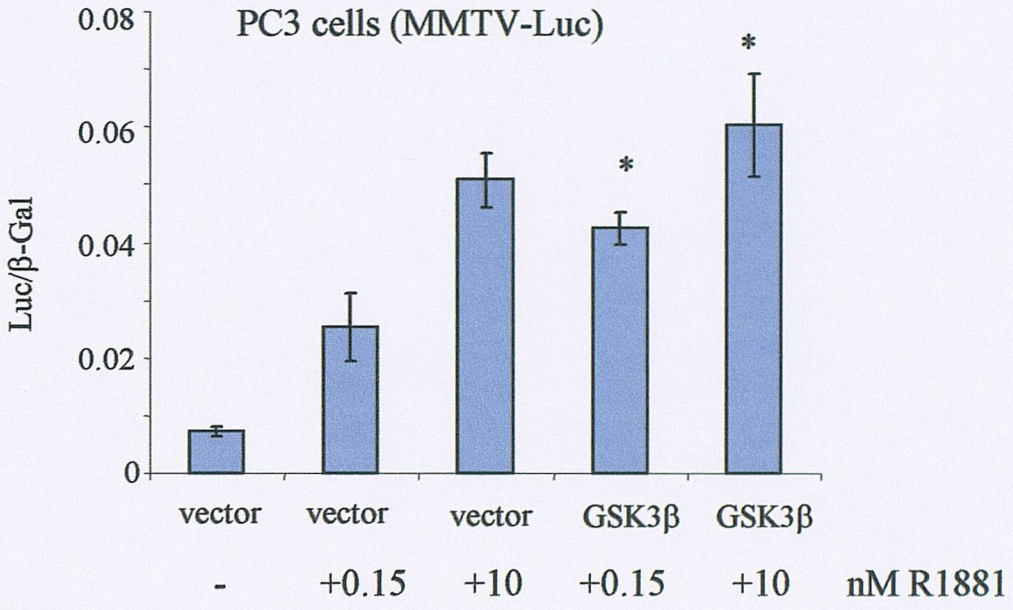


Figure 25 (c). GSK3β overexpression increases AR-dependent transcription in PC3 cells. (c) PC3 cells expressing AR were transfected with the reporter vectors MMTV-Luc and RSV-β-Gal and either w.t GSK3β or the empty vector pMT23 (vector), and treated with either 0.15, 10 nM R1881 or the carrier ethanol (-). Cells were assayed for luciferase (Luc) and β-galactosidase (β-Gal) activities, and results are presented as the ratio between luciferase and β-Gal activities. (*) AR activity significantly increased in cells expressing GSK3β constructs compare to cells expressing empty vector in the presence of R1881 at the 0.15 nM dose ($p<0.0005$) and at the 10 nM dose ($p=0.02$). Experiment was done twice in triplicates.

3.4 Chapter 4- Inhibition of AR activity and PCa cell growth by GSK-3 inhibitors

3.4.1 The effects of GSK-3 inhibitors on GSK-3 and β-catenin stabilisation

Another approach to analyse the effects of inhibition of GSK3β in PCa cells is to use chemical inhibitors of GSK-3. LiCl, which has been shown by several groups to stabilise β-catenin by inhibiting GSK-3, was first used (Stambolic *et al.*, 1996; Garcia-Perez *et al.*, 1999; Jones and Veale, 2003; Orme *et al.*, 2003). However, since LiCl has other effects on cells, two commercially available inhibitors, SB415286 and SB216763, were also tested. Initially, in order to confirm that the inhibitors were working properly, their effects on β-catenin protein levels in 22Rv1 cells were tested. Cells were treated with LiCl, SB216763 or SB415286 for 24 hr and lysed in hypotonic lysis buffer in order to prepare cytosolic extracts. Cytosolic extracts were probed with anti-β-catenin and anti-β-actin (as a loading control) antibodies. Results (Figure 26a) show that LiCl and SB415286 stabilised β-catenin. SB216763 did not appear to change β-catenin protein levels. Therefore, it was unclear if SB216763 affected GSK-3 in this cell line.

GSK-3 is known to have two phosphorylation sites: S9 or S21 are inhibitory phosphorylation sites for GSK3β and GSK3α, respectively; phosphorylation on Y216 and Y279 leads to activation of GSK3β and GSK3α, respectively. Therefore, inhibition of GSK-3 protein activity can be measured by changes in its phosphorylation state. 22Rv1 cells that had been treated for 24 hours with each of the three inhibitors were lysed in RIPA lysis buffer and then probed for total GSK-3 (α+β), GSK-3 phosphorylated on S9/S21 and for GSK-3 phosphorylated on Y216/Y279. Figure 26b shows that total GSK-3 levels were not greatly affected by the inhibition. LiCl treatment led to a small increase in S21 phosphorylation on GSK3α, but not in S9 phosphorylation on GSK3β. Treatment with SB415286 and SB216763 reduced, rather than increased, serine phosphorylation on GSK-3 (S9 and S21). In addition, both SB216763 and SB415286 led to a reduction in Y279 phosphorylation on GSK3α. Y216 phosphorylation on GSK3β was slightly reduced by all three inhibitors. These results suggest that the mechanism of inhibition of GSK-3 by LiCl, SB216763 and SB415286 differs: LiCl increases S21 phosphorylation, whereas SB216763 and SB415286 reduce GSK-3 tyrosine

phosphorylation. In general, the effects of these inhibitors in 22Rv1 cells appear to be stronger on GSK3α compared to GSK3β.

Next, the effects of the inhibitors on β-catenin/Tcf-dependent transcription were tested in CWR-R1 cells. Cells were treated with 20 mM LiCl, 5 or 20 μM of SB216763 and SB415286, respectively, 24 hr after transfection (for 24 hr). Results in Figure 26c indicate that all three inhibitors activated β-catenin/Tcf-dependent signalling as expected. Taken together, the results from Figure 26 suggested that all three GSK-3 inhibitors inhibit GSK-3, albeit in different ways, and therefore could be further used to investigate the effect of GSK3β inhibition on AR signalling in PCa cells.

3.4.2 Regulation of AR transcriptional activity by GSK-3 inhibitors

In order to examine the effects of these inhibitors on AR activity, CWR-R1 cells were transfected with the reporter plasmid MMTV-Luc and treated with the inhibitors and 10 nM R1881 for 24 hours. Both SB415286 and SB216763 repressed AR activity, while LiCl had no effect (Figure 27a). SB415286 and SB216763 also repressed AR-mediated transcription in assays using a second reporter plasmid (Figure 27b). Here CWR-R1 cells were transfected with the reporter Probasin-Luciferase (PB-Luc), which measures transcription of probasin, an AR target gene. These results show that the inhibition of AR activity by GSK-3 inhibitors is not restricted to the MMTV promotor.

Figure 26

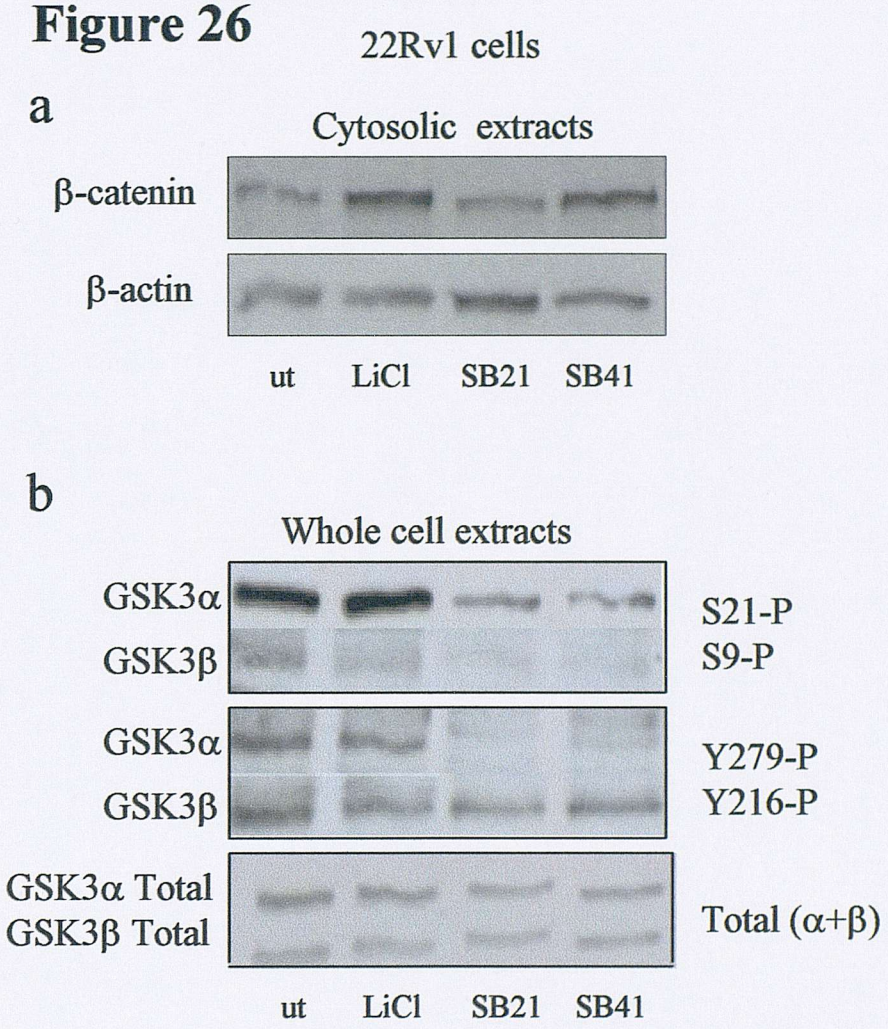


Figure 26 (a-b). (a) Inhibition of GSK-3 leads to β-catenin stabilisation in 22Rv1 cells. Cells were treated with either the carrier ethanol (ut), 20 mM LiCl, 5 μM SB216763 (SB21) or 20 μM SB415286 (SB41) for 24 hours. Cytosolic extracts were probed with antibodies against β-catenin and β-actin as an internal loading control. **(b) Changes in serine and tyrosine phosphorylation of GSK-3 in 22Rv1 cells.** Cells were treated with either the carrier ethanol (ut), 20 mM LiCl, 5 μM SB216763 (SB21) or 20 μM SB415286 (SB41) for 24 hours. Whole cell extracts were probed with the phospho-specific antibodies against S21/S9 (upper panel) and Y279/Y216 (middle panel) in GSK3α/GSK3β, respectively. Extracts were also probed for total GSK-3 (α+β) (lower panel).

Figure 26 continued-

c

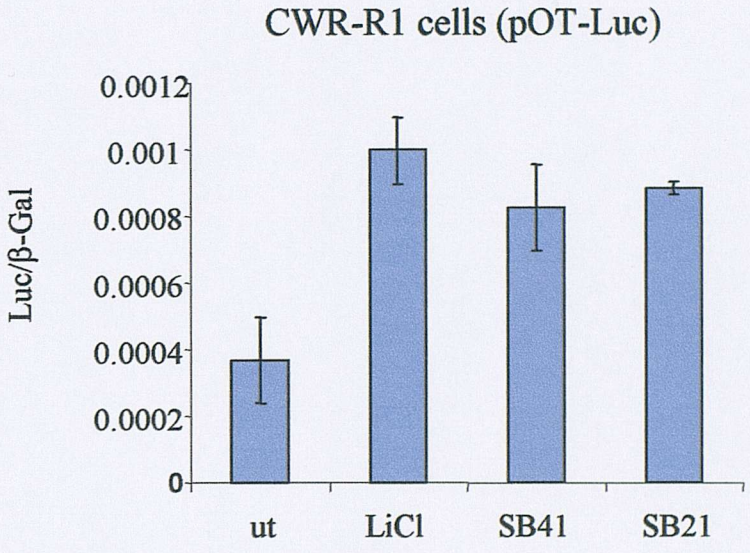


Figure 26 (c). Activation of β-catenin/Tcf-4-dependent transcription by GSK-3 inhibitors in CWR-R1 cells. Cells were transfected with the reporter vectors pOT-Luc and RSV-β-Gal and treated with either the carrier ethanol (ut), 20 mM LiCl, 5 μM SB216763 (SB21) or 20 μM SB415286 (SB41) for 24 hours. Cells were assayed for luciferase (Luc) and β-galactosidase (β-Gal) activities and results are presented as the ratio between luciferase and β-Gal activities.

Figure 27

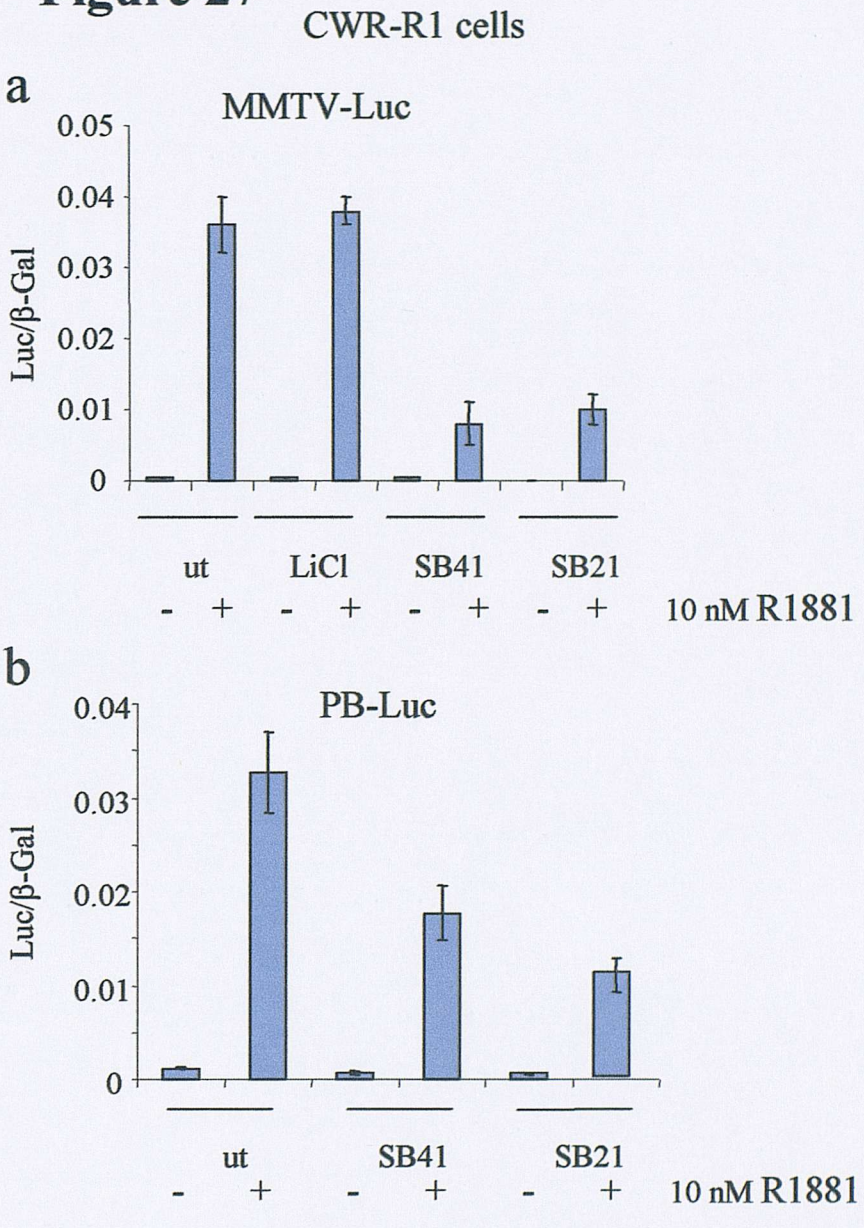


Figure 27. Inhibition of AR-dependent transcription by GSK-3 inhibitors in CWR-R1 cells. Cells were transfected with the reporter vectors MMTV-Luc (a) or PB-Luc (b) and RSV-β-Gal and treated with either the carrier ethanol (ut), 20 mM LiCl (a only), 5 μM SB216763 (SB21) or 20 μM SB415286 (SB41) for 24 hours. Cells were treated with (+) or without (-) 10 nM R1881 half an hour after addition of inhibitors and assayed for luciferase (Luc) and β-galactosidase (β-Gal) activities. Results are presented as the ratio between luciferase and β-Gal activities.

3.4.3 PCa cell growth regulation by GSK-3 inhibitors

The effects of GSK-3 inhibitors were next examined with respect to PCa cell growth. First, in order to find the optimal dose required for a significant effect on cell growth, CWR-R1 cells were treated with increasing amounts of SB216763 and SB415286 and counted after 72 hours. The inhibitory effects of SB216763 on cell growth were maximal at 3 μM (Figure 28a), whereas the inhibitory effects of SB415286 increased with dose up to the maximal dose tested (50 μM) (Figure 28b). The effects of SB415286 at higher doses appeared to result from general toxicity of this inhibitor. We chose to use it at 20 μM which is close to the Ki for this inhibitor in other assays (Coghlan *et al.*, 2000; Cross *et al.*, 2001).

Next, the effects of the inhibitors at an optimal dose were tested over time. CWR-R1 cells were treated with 5 μM SB216763 and 20 μM SB415286 and counted over a period of 6 days. As shown in Figure 29 both SB216763 and SB415286 repressed CWR-R1 cell growth.

In order to determine whether inhibition of GSK-3 specifically inhibited growth of PCa cells that express AR, I examined the effects of SB216763 (5 μM) on the growth of several PCa cell lines (Figure 30a). Some of the lines express AR (CWR-R1, 22Rv1 and LNCaP cells) and some do not (PC3, DU145 cells). SB216763 similarly repressed the growth of CWR-R1 and 22Rv1 cells. In contrast, SB216763 did not significantly affect the growth of PC3 and DU145 cells. The inhibitory effect on LNCaP cell growth was only weak, consistent with the low endogenous GSK-3 activity in this cell line. These results suggest that inhibition of GSK-3 reduces the growth rate of AR-positive PCa cells and supports the idea that AR is required for the growth inhibitory response.

The effect of GSK-3 inhibition on AD PCa cell growth was then tested. This experiment was done in the same way as described in Figure 30a, but conducted in 22Rv1 cells that were grown in hormone-depleted medium in the absence or presence of R1181. 22Rv1 cells are able to grow to same the extent in hormone-depleted medium but their growth can be stimulated with the addition of androgens (Sramkoski *et al.*, 1999). According to Figure 30b, R1881 stimulated 22Rv1 cell growth and this was blocked by treatment with SB216763. In addition, SB216763 inhibited hormone-independent growth of 22Rv1 cells to a certain extent (compare lanes 1 and 4), suggesting that there is also an effect on AI cell growth.

Figure 28

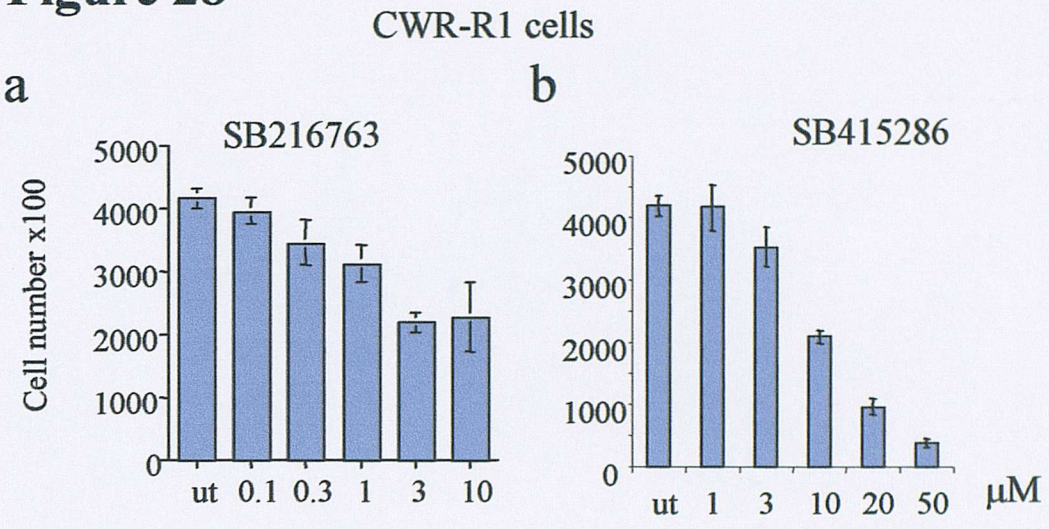


Figure 29

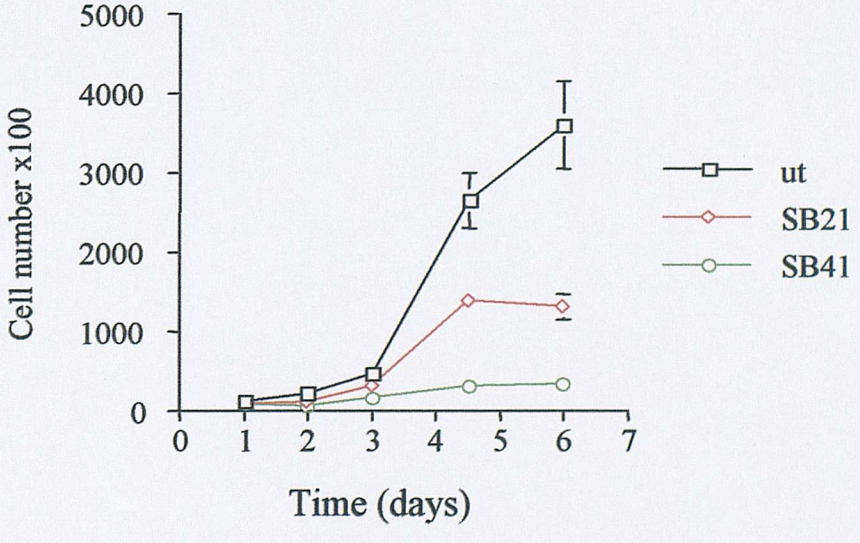


Figure 28. Chemical inhibition of GSK-3 inhibits CWR-R1 cell growth in a dose-dependent manner. Cells were grown for 72 hours in complete growth medium in the presence of the indicated concentrations of SB216763 (a) and SB415286 (b), and the number of cells were counted.

Figure 29. chemical inhibitors of GSK-3 inhibit CWR-R1 cell growth over a period of 6 days. Cells were grown in complete growth medium in the presence of either the carrier ethanol (ut), 5 μM SB216763 (SB21) or 20 μM SB415286 (SB41) for up to 6 days and the number of cells was counted.

Figure 30

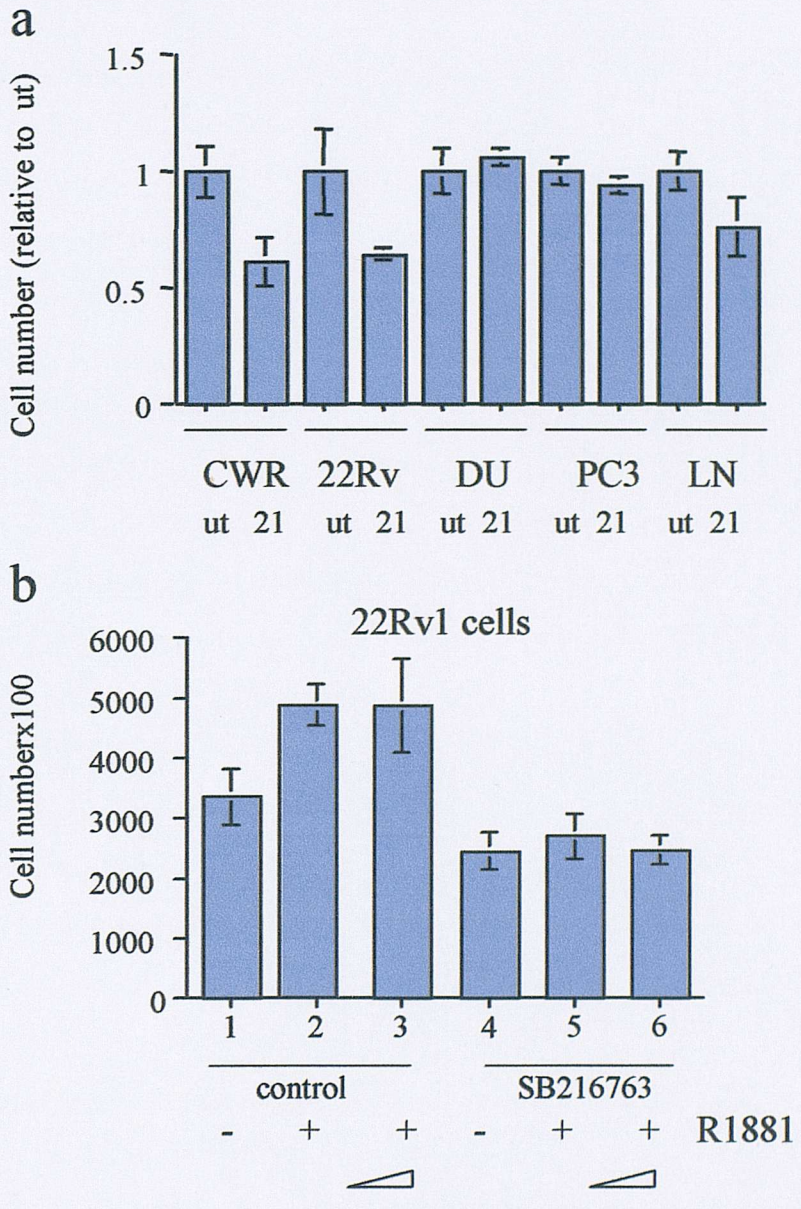


Figure 30. (a) Inhibition of GSK-3 reduces AR-positive PCa cell growth. CWR-R1 (CWR), 22Rv1 (22Rv), DU145 (DU), PC3 and LNCaP (LN) cells were treated either with a carrier (ut) or 5 μM SB216763 (21). The number of cells was counted after 72 hours (or after 5 days for LNCaP cells). **(b) Inhibition of GSK-3 reduces AD and AI cell growth in 22Rv1 cells.** Cells were grown in hormone-depleted medium in the absence of hormone (lanes 1 and 4), in the presence of 10^{-12} M R1881 (lanes 2 and 5) or 10^{-9} M R1881 (lanes 3 and 6) and either with carrier (control, lanes 1-3) or 5 μM SB216763 (lanes 4-6). The number of cells was counted after 72 hours.

3.5 Chapter 5- Investigation of the mechanism by which GSK3β regulates AR activity

3.5.1 Regulation of AR protein level by GSK3β

As a first step in determining the mechanism of GSK3β regulation of AR transcriptional activity, the effects of GSK3β inhibition on AR protein levels were examined. Whole cell extracts from CWR-R1 cells that had been treated with GSK-3 inhibitors for 24 hours were probed with antibodies against AR and γ-tubulin as a loading control (Figure 31a). Interestingly, compared with untreated cells, AR protein levels were reduced after treatment with both inhibitors. SB415286 appeared to reduce AR protein levels more than SB216763, although reprobing with anti-tubulin revealed that part of this reduction resulted from a reduction in the number of cells. However, after taking into account the loading controls, both inhibitors specifically reduced AR protein levels in CWR-R1 cells, suggesting that GSK3β plays a role in AR protein stability.

Next, in order to try to determine in which cellular compartment GSK3β regulation of AR protein occurs, different cellular fractions were isolated. CWR-R1 cells grown in hormone-depleted medium were treated with GSK-3 inhibitors in the absence or presence of 10 nM R1881 for 24 hours. Non-nuclear extracts were probed with antibodies against AR and γ-tubulin as a loading control (Figure 31b). Addition of R1881 to control cells induced a small increase in non-nuclear AR in CWR-R1 cells (lane 2) (in contrast to the 40 minute treatment in Figure 12). An increase was also seen using SB216763 (lane 4) and to a lesser extent using SB415286 (lane 6). The main difference was that SB216763 and SB415286 reduced the level of non-nuclear AR in cells that were not treated with androgen (lanes 3 and 5), although SB415286 also reduced AR in androgen-treated cells (lane 6). Nuclear extracts from the same cells were loaded on gels and probed with antibodies against AR and PARP as a loading control. Taking into account levels of the control protein, results presented in Figure 31c show that the GSK-3 inhibitors did not have a significant effect on nuclear AR protein levels, compared to untreated cells, in the presence of androgens. However, there appeared to be a small decrease in AR levels in the absence of hormone (compare loading controls in lanes 1, 3 and 5). These results suggest that GSK3β might play a role in AR protein stability mainly when it is in the unliganded state.

Figure 31

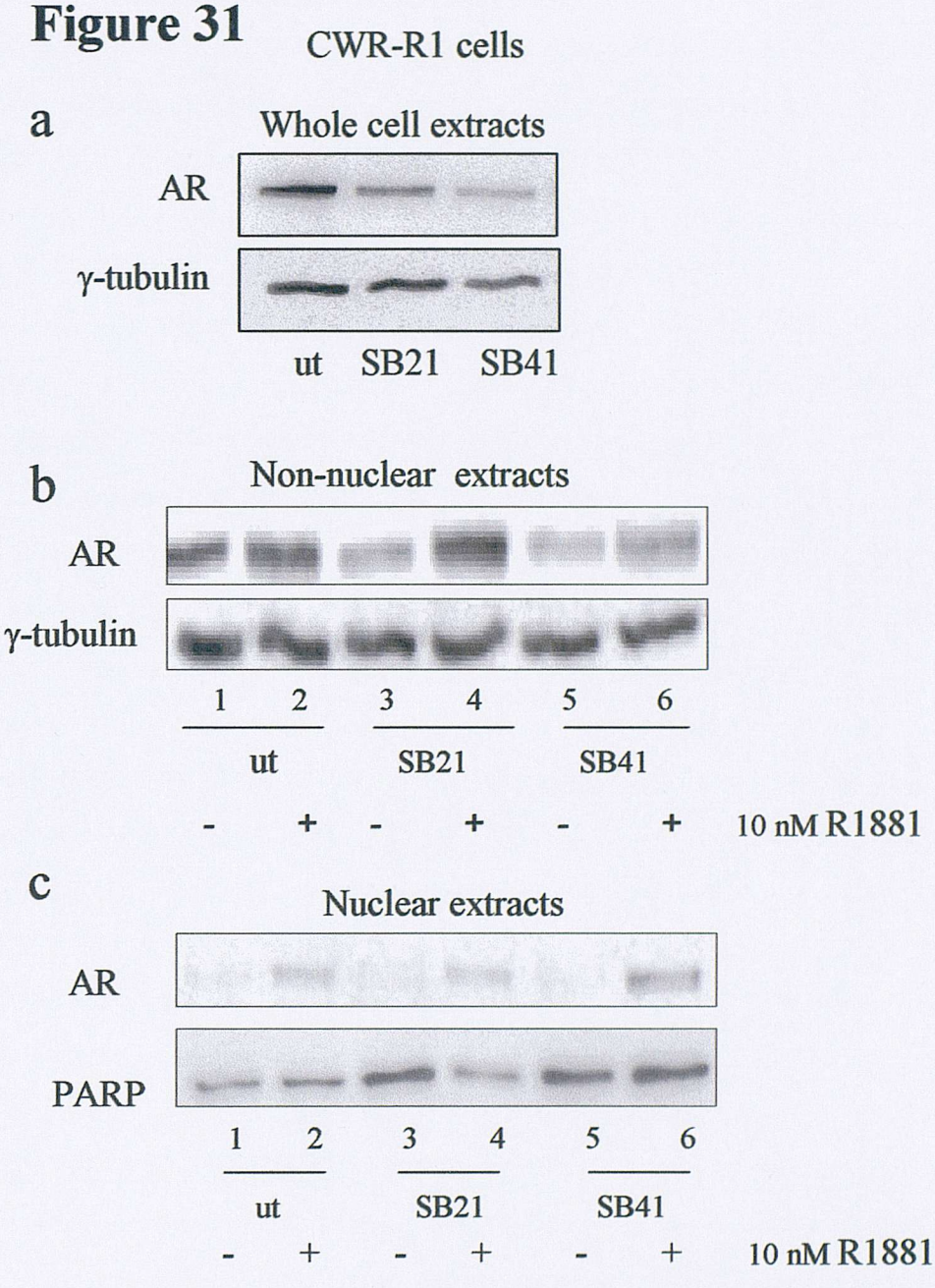


Figure 31. Inhibition of GSK-3 leads to a reduction in AR protein levels. (a) CWR-R1 cells were grown in their complete growth medium and treated with either the carrier ethanol (ut), 5 μM SB216763 (SB21) or 20 μM SB415286 (SB41) for 24 hours. Whole cell extracts were probed with antibodies against AR and γ-tubulin. (b-c) CWR-R1 cells were grown in hormone-depleted medium in the presence (+, lanes 2, 4 and 6) or absence (-, lanes 1, 3 and 5) of 10 nM R1881, and either the carrier ethanol (ut, lanes 1-2), 5 μM SB216763 (SB21, lanes 3-4) or 20 μM SB415286 (SB41, lanes 5-6) for 24 hours. Non-nuclear extracts were probed with antibodies against AR and γ-tubulin (b). Nuclear extracts were probed with antibodies against AR and PARP (c).

3.5.2 Association between AR and GSK3β

The possibility of an *in vivo* complex between AR and GSK3β was next studied. COS7 cells were transfected with AR and a myc-tagged GSK3β. 24 hr after transfection, cells were lysed in NP-40 lysis buffer and immunoprecipitated with different antibodies. Samples that were immunoprecipitated with anti-AR were probed with 9E10 (an anti-myc tag antibody in order to detect GSK3β). Samples that were immunoprecipitated with 9E10 were probed with anti-AR. Extracts were also immunoprecipitated with monoclonal and polyclonal anti-GFP as control antibodies (Figure 32). GSK3β was detected in anti-AR immunoprecipitates and not in control immunoprecipitates (Figure 32a), and AR was detected in anti-GSK3β immunoprecipitates and not in control immunoprecipitates (Figure 32b). Total cell extracts were probed with antibody against 9E10 (Figure 32a) or AR (Figure 32b) to confirm the expression and migration of the respective proteins. These results show that the two proteins have the ability to form a complex, and, together with the previous results, further support the hypothesis that GSK3β increases the AR-mediated transcriptional activity by binding to AR.

The next goal was to try and detect an endogenous complex between AR and GSK3β. 22Rv1 cells treated with or without 1 nM R1881 for 1 hour were lysed in NP-40 lysis buffer and immunoprecipitated with anti-AR or control (normal IgG) antibodies and probed with antibodies against AR and GSK3β (Figure 33) (for details on IP technique, see section 2.10-2 in Materials and Methods). GSK3β was detectable in anti-AR immunoprecipitates in the presence and absence of androgens (lower panel, lanes 2 and 4). However, a small amount of GSK3β was also detected in normal IgG immunoprecipitates (lanes 1 and 3) indicating that non-specific binding was also occurring. Reprobing of the immunoprecipitates with an antibody against AR confirmed that similar amounts of AR were immunoprecipitated (upper panel, lanes 2 and 4). Total cell extracts that were probed with antibodies against AR and GSK3β indicated that cells expressed slightly higher amounts of AR protein in the presence of androgens (upper panel, lane 6) and equal amounts of GSK3β protein (lower panel, lanes 5 and 6). These results suggest that an endogenous complex between AR and GSK3β may be present, but that this complex is present at very low levels or is unstable.

Figure 32 COS7 cells

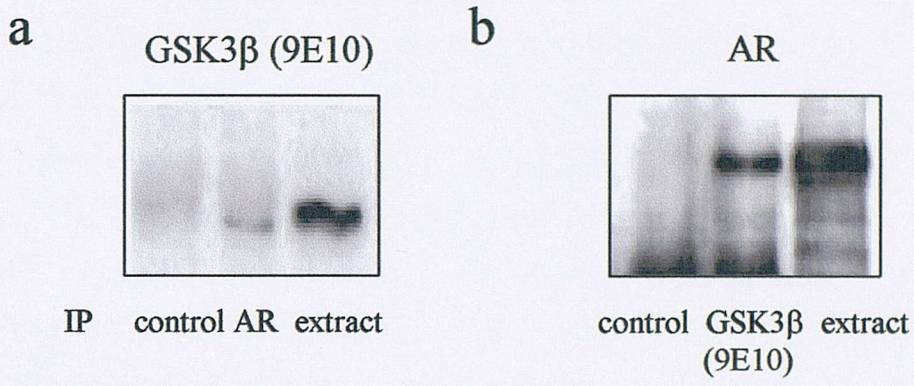


Figure 33 22Rv1 cells

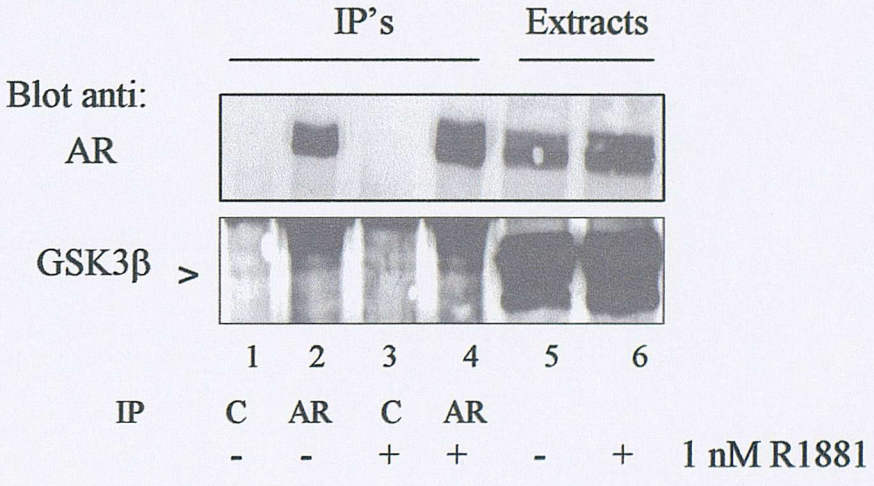


Figure 32. AR and GSK3β form a complex *in vivo* in COS7 cells. Cells were transfected with AR and myc epitope-tagged GSK3β. Whole cell extracts were immunoprecipitated with either a polyclonal antibody against AR and probed with antibody against myc (9E10) for detection of GSK3β (a), or with a monoclonal antibody against myc (9E10) and probed with an antibody against AR (b). Cells were also immunoprecipitated with polyclonal and monoclonal antibody against GFP (a, b respectively) as control. Total cell extracts were probed with antibody against myc (a, 9E10) and AR (b).

Figure 33. Detection of endogenous complex between AR and GSK3β in 22Rv1 cells. 22Rv1 cells treated with (+) (lanes 3 and 4) or without (-) (lanes 1 and 2) 1 nM R1881 for 1 hour, were immunoprecipitated with antibodies against AR (lanes 2 and 4) or control (anti-normal IgG) (lanes 1 and 3) and probed with antibodies against AR and GSK3β. R1881-treated total cell extracts (lane 6) and untreated extracts (lane 5) were probed with antibodies against AR and GSK3β. The arrow head indicate the position of GSK3β in IP samples and cell extracts.

3.5.3 Axin disrupts the complex between AR and GSK3β

To determine a possible mechanism for the inhibition of AR activity by Axin, AX2 or AX2P (or empty vector as a control) were expressed in COS7 cells together with AR and myc-tagged GSK3β (Figure 34). AR was detected in GSK3β immunoprecipitates from COS7 cells expressing empty vector (lane 6) or AX2P (lane 4). Interestingly, the amount of complex between AR and GSK3β detected in cells expressing AX2P (lane 4) was higher than the amount detected in cells expressing the empty vector (lane 6). The reason for this is not known. Importantly, AX2 expression prevented detection of a complex between AR and GSK3β. Total cell extracts were also probed with antibodies against AR and 9E10 and this confirmed that the cells expressed similar amounts of AR and myc-tagged GSK3β (lanes 1, 2 and 3). These results suggest that AX2 inhibits AR transcriptional activity by preventing interaction between GSK3β and AR and further support a model that the association between AR and GSK3β is required for the enhancement in AR transcriptional activity.

Figure 34

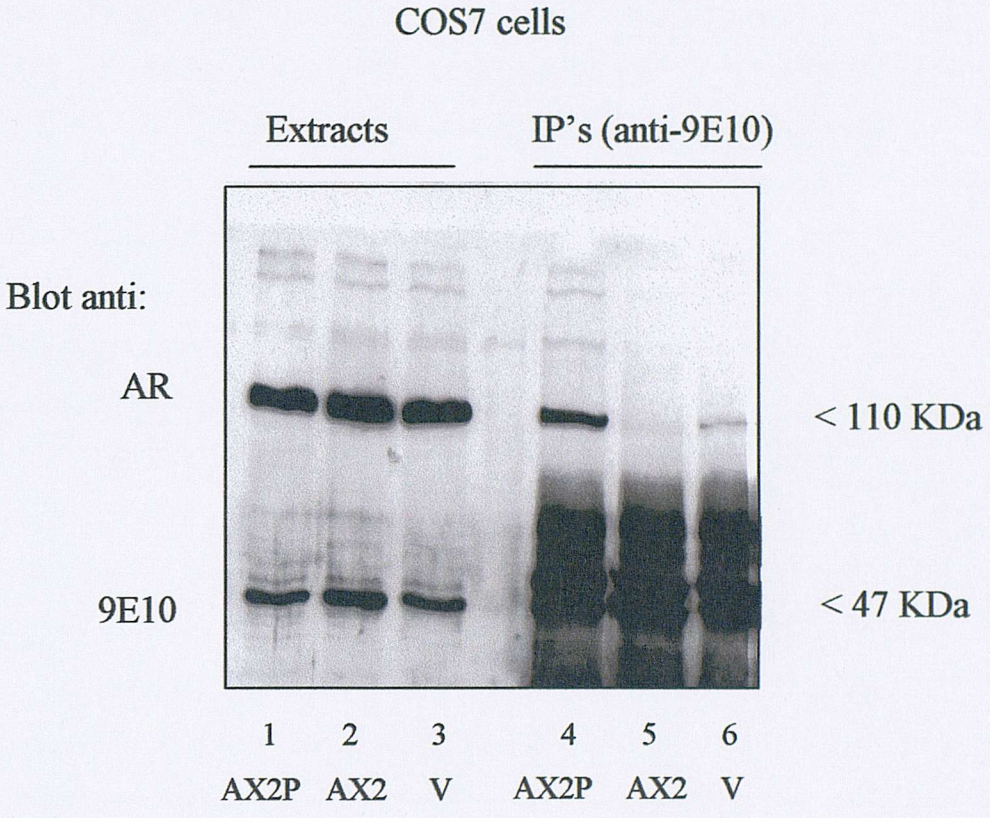


Figure 34. Association between AR and GSK3β is disrupted by AX2 overexpression. Whole cell extracts from COS7 cells transfected with AR and myc epitope-tagged GSK3β, plus either AX2P (lane 4), AX2 (lane 5), or an empty vector (lane 6) were imuunoprecipitated with an antibody against myc (9E10), and probed with antibodies against AR and 9E10. Total cell extracts from cells transfected with either AX2P (lane 1), AX2 (lane 2) or the empty vector (lane 3) were probed with antibodies against AR and 9E10. The upper arrow head indicates the position of AR (at 110 KDa) and the lower arrow head indicates the position of GSK3β (at 47 KDa) in the IP samples and cell extracts. The bands migrating above GSK3β are IgG recognised by the secondary antibody.

3.5.4 Investigation of GSK3β regulation of AR transcriptionl activity through phosphorylation

GSK-3 is known to phosphorylate and regulate the activities of several transcription factors (Woodgett, 1990). GSK-3 phosphorylates serine and threonine residues and one of its consensus sites for phosphorylation (S/T P) can be found in three major AR phosphorylation sites (S81, S94 and S650) (Jenster *et al.*, 1994; Zhou *et al.*, 1995; Yeh *et al.*, 1999; Wen *et al.*, 2000; Lin *et al.*, 2001; Gioeli *et al.*, 2002). Some of these proline-directed phosphorylation sites have been previously reported to be involved either in AR transcriptional activity (S650) and/or are phosphorylated after addition of hormone (S81 and S650) (Gioeli *et al.*, 2002). Therefore, the possibility that GSK3β regulates AR through phosphorylation on these three candidate sites was examined. Since most of the previous experiments were done in CWR-R1 cells, initially an AR plasmid with the same mutation as AR in CWR-R1 cells (H874Y) was used. The H874Y AR plasmid was mutated to alanines at serines 81, 94 and 650. The mutants were first expressed in COS7 cells together with w.t GSK3β and their expression levels compared. Transfected cells were grown in hormone-depleted medium for 16 hours and then treated with 10 nM R1881 or its carrier ethanol for 6 hours and whole cell extracts were probed with antibodies against total AR (Figure 35a, lower panel). H874Y AR and all three mutants were expressed equally and were all stabilised by R1881 treatment (lanes 2, 4, 6 and 8). In order to examine phosphorylation at S81, a commercial antibody that recognises phosphorylated S81 was used (Figure 35a, upper panel). This revealed R1881-dependent phosphorylation of AR at S81 (lane 2). The S650A mutant was also phosphorylated on S81 (lane 6), as expected, whereas the S81A mutant was not (lane 4). However, this result was not reproducible in several other attempts. The reason for that is not known but possibly this is due to an antibody batch problem. The phosphorylation status of the AR mutants will have to be examined using other techniques.

Next, the effect of the serine mutations on AR transcriptional activity was tested. PC3 cells were transfected with H874Y AR and H874Y phosphorylation site mutants of AR, together with the reporter plasmids MMTV-Luc and pDM-β-Gal and treated with 0.15 nM R1881 or the carrier ethanol for 24 hours (Figure 35b). Results show that AR transcriptional activity of H874Y AR and all three mutants increased in response to androgens, indicating that mutation at these three phosphorylation sites

did not compromise hormone-dependent transcriptional activation. Similar results were reported by other groups who used AR mutants to examine the effect of serine phosphorylation on AR (Jenster *et al.*, 1994; Zhou *et al.*, 1995; Yeh *et al.*, 1999; Wen *et al.*, 2000; Lin *et al.*, 2001; Gioeli *et al.*, 2002).

In order to test the effect of GSK3β on AR activity, PC3 cells were transfected with the AR phosphorylation site mutants together with w.t GSK3β (or empty vector) and treated with 0.15 nM R1881 (or the carrier ethanol) for 24 hours (Figure 36a). The results indicate that the AR transcriptional activity of H874Y AR, S81A and S650A was higher in cells expressing GSK3β compared to cells expressing an empty vector (compare lanes 2 and 3, 5 and 6, 10 and 11). However, AR activity of S94A was not greatly affected by the expression of GSK3β (compare lanes 8 and 9). Figure 36b is a combination of results from three independent experiments carried out in triplicates in PC3 cells in the same way as in Figure 36a. The results are presented as the fold activation of AR comparing cells expressing GSK3β and cells expressing an empty vector in the presence of androgens and relative to H874Y AR with the activity of H874Y in the presence of GSK3β set at 1. The effect of GSK3β expression on S81A and S650A was similar to the effect on H874Y AR transcriptional activity. In contrast, GSK3β-dependent AR activity of S94A was significantly reduced ($p=0.00002$) compared to H874Y AR, suggesting S94 is involved in the regulation of AR transcriptional activity by GSK3β. These results indicate that GSK3β activation of the mutated form of AR found in CWR-R1 and 22Rv1 cells (AR H874Y), requires S94.

Figure 35

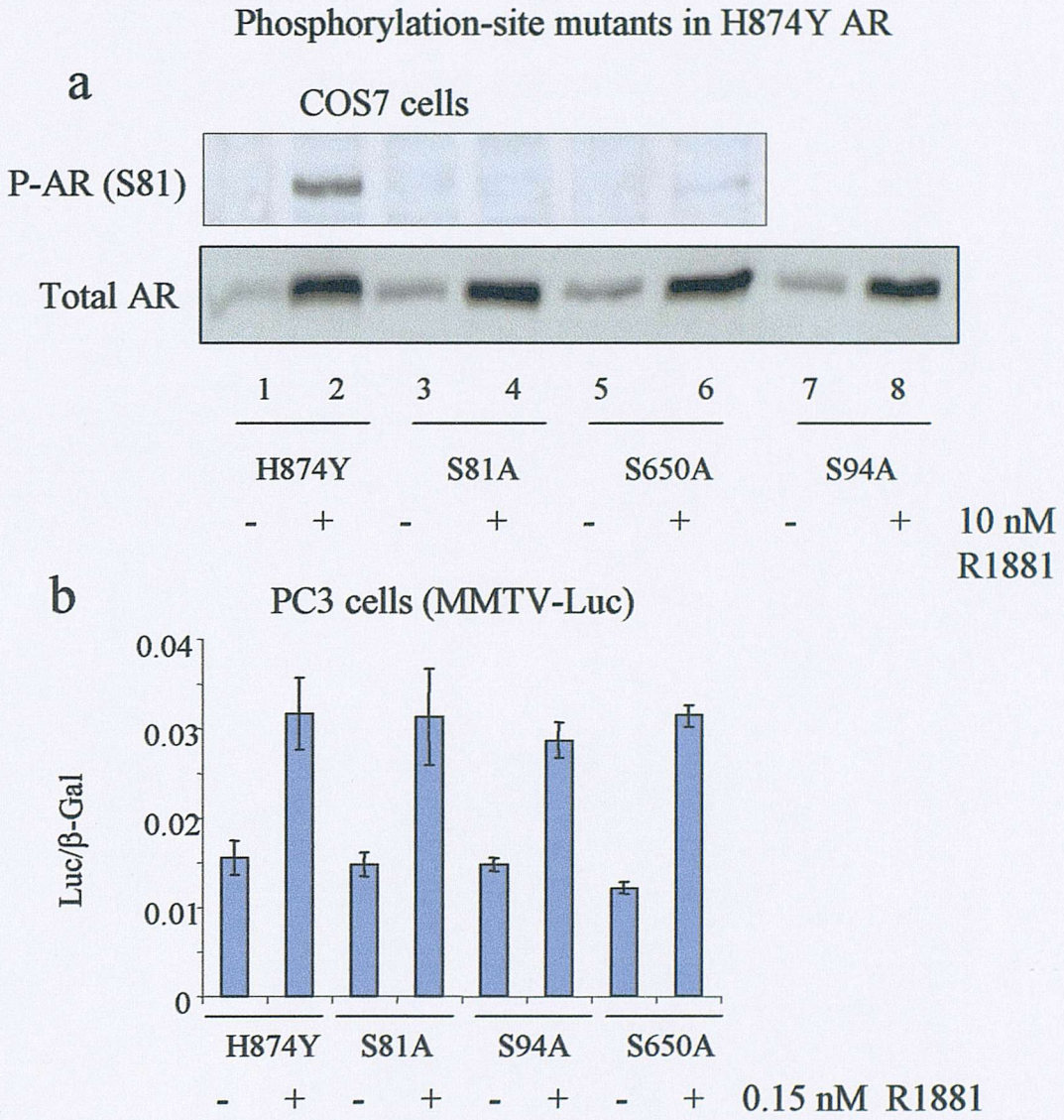


Figure 35. (a) Phosphorylation site mutations in H874Y AR do not affect AR hormone-dependent protein stabilisation. COS7 cells were transfected with the AR mutants S81A (lanes 3 and 4), S650A (lanes 5 and 6) and S94A (lanes 7 and 8) as well as the non-mutated H874Y AR (lanes 1 and 2), and treated with (+, lanes 2, 4, 6 and 8) or without (-, lanes 1, 3, 5, and 7) 10 nM R1881. Whole cell extracts were probed with the phospho-specific antibody against S81 in AR (upper panel), and with the antibody against total AR (lower panel). **(b) Phosphorylation site mutations in AR do not affect ligand-dependent AR activity in PC3 cells**. Cells were transfected with the reporter vectors MMTV-Luc and pDM-β-Gal plus either the non-mutated H874Y AR or the AR phosphorylation site mutants; S81A, S94A or S650A. Cells were treated with 0.15 nM R1881 and assayed for luciferase (Luc) and β-galactosidase (β-Gal) activities. Results are presented as the ratio between luciferase and β-Gal activities.

Figure 36 Phosphorylation-site mutants in H874Y AR

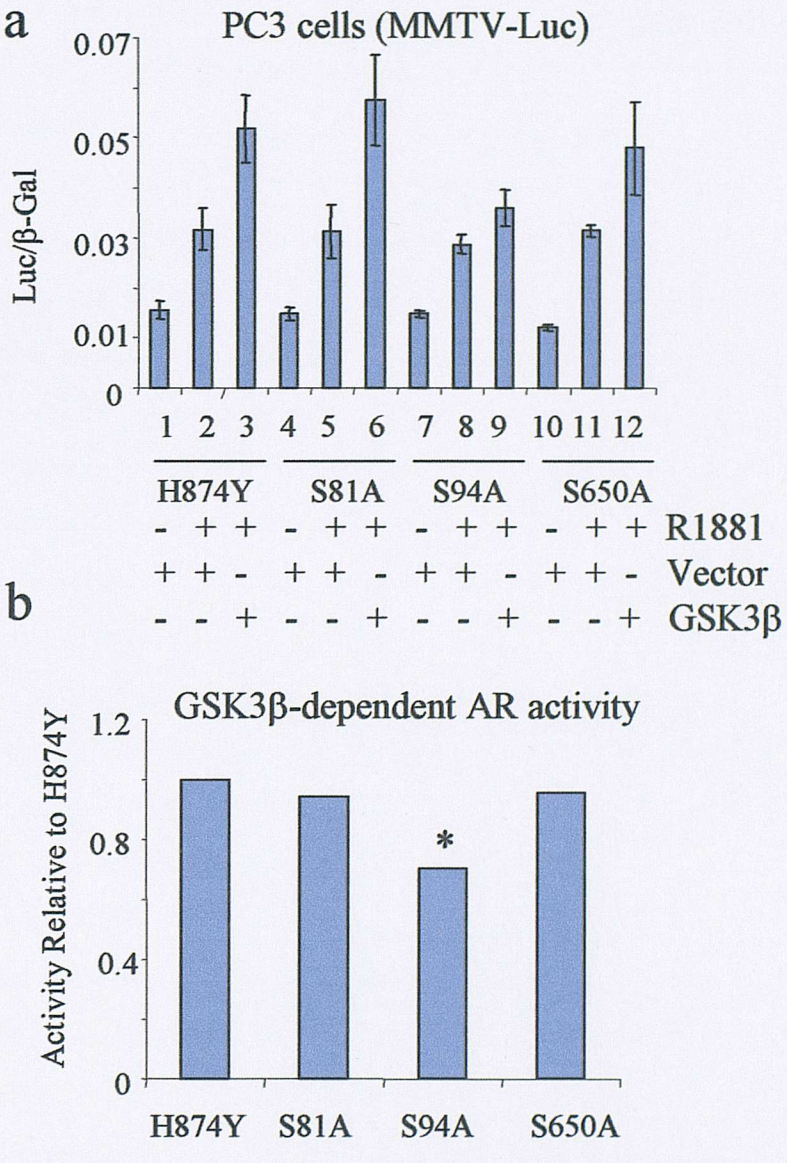


Figure 36. GSK3β activation of AR requires S94 in PC3 cells expressing H874Y AR. (a) Cells were transfected with the reporter vectors MMTV-Luc and pDM-β-Gal plus either the non-mutated H874Y AR (lanes 1-3) or the AR phosphorylation site mutants S81A (lanes 4-6), S94A (lanes 7-9) or S650A (lanes 10-12), and either w.t GSK3β (lanes 3,6,9 and 12) or the empty vector pMT23 (lanes 1,2,4,5,7,8,10 and 11). Cells were treated with (+) or without (-) 0.15 nM R1881 for 24 hours and assayed for luciferase (Luc) and β-galactosidase (β-Gal) activities. Results are presented as the ratio between luciferase and β-Gal activities. (b) An average was taken from the results of three experiments that were carried out in PC3 cells in the same way as in Figure 36a. Results are presented as the fold activation of AR comparing cells expressing GSK3β and cells expressing an empty vector in the presence of androgens and relative to H874Y AR (the activity of H874Y in the presence of GSK3β is set at 1). (*) GSK3β-dependent AR activity of S94 was significantly reduced (p=0.00002) compared to H874Y AR.

To investigate further the role of S94, the S94A mutation was also created in w.t AR. In parallel, a S650A mutation was created in w.t AR. The protein expression levels of w.t AR as well as the w.t-based mutants S94A and S650A are shown in Figure 37a and indicate that they were all expressed in COS7 cells to a similar level, and all stabilised by R1881 (lanes 2, 4 and 6). S94A and S650A migrated more slowly than w.t AR, particularly in the absence of hormone (lanes 3 and 5).

GSK3β-dependent AR transcriptional activity was next measured in PC3 cells transfected with w.t AR and the w.t-based mutants S94A and S650A (Figures 37b and 37c, in the same way as described in Figures 36a and 36b). Both S94A and S650A (w.t-based) mutants maintained their hormone response and, in some experiments S94A responded better than w.t AR (Figure 37b, compare lanes 1 and 2, 5 and 6), although this was not consistently observed. The activity of w.t AR and the w.t-based S650A was higher in cells co-transfected with GSK3β compared to cells expressing empty vector (Figure 37b, compare lanes 2 and 4,10 and 12). In contrast, GSK3β expression did not lead to an increase in the transcriptional activity of w.t-based S94A (Figure 37b, compare lanes 6 and 8). This result was highly reproducible. Figure 37c shows the results from six independent experiments and indicates that the w.t-based S94A AR mutant has lost its ability to respond to GSK3β ($p=0.00007$).

In conclusion, the results in this chapter suggest that the positive regulation of GSK3β on AR transcriptional activity involves association between AR and GSK3β, and this might lead to an increase in unliganded AR protein. Disruption of this interaction occurs when Axin binds to GSK3β and interferes with the complex between AR and GSK3β. Finally, mutation analysis indicates that S94 in AR is required for GSK3β to activate AR signaling and is therefore a strong candidate for being a GSK3β-dependent phosphorylation site in AR.

Figure 37 Phosphorylation-site mutants in w.t AR

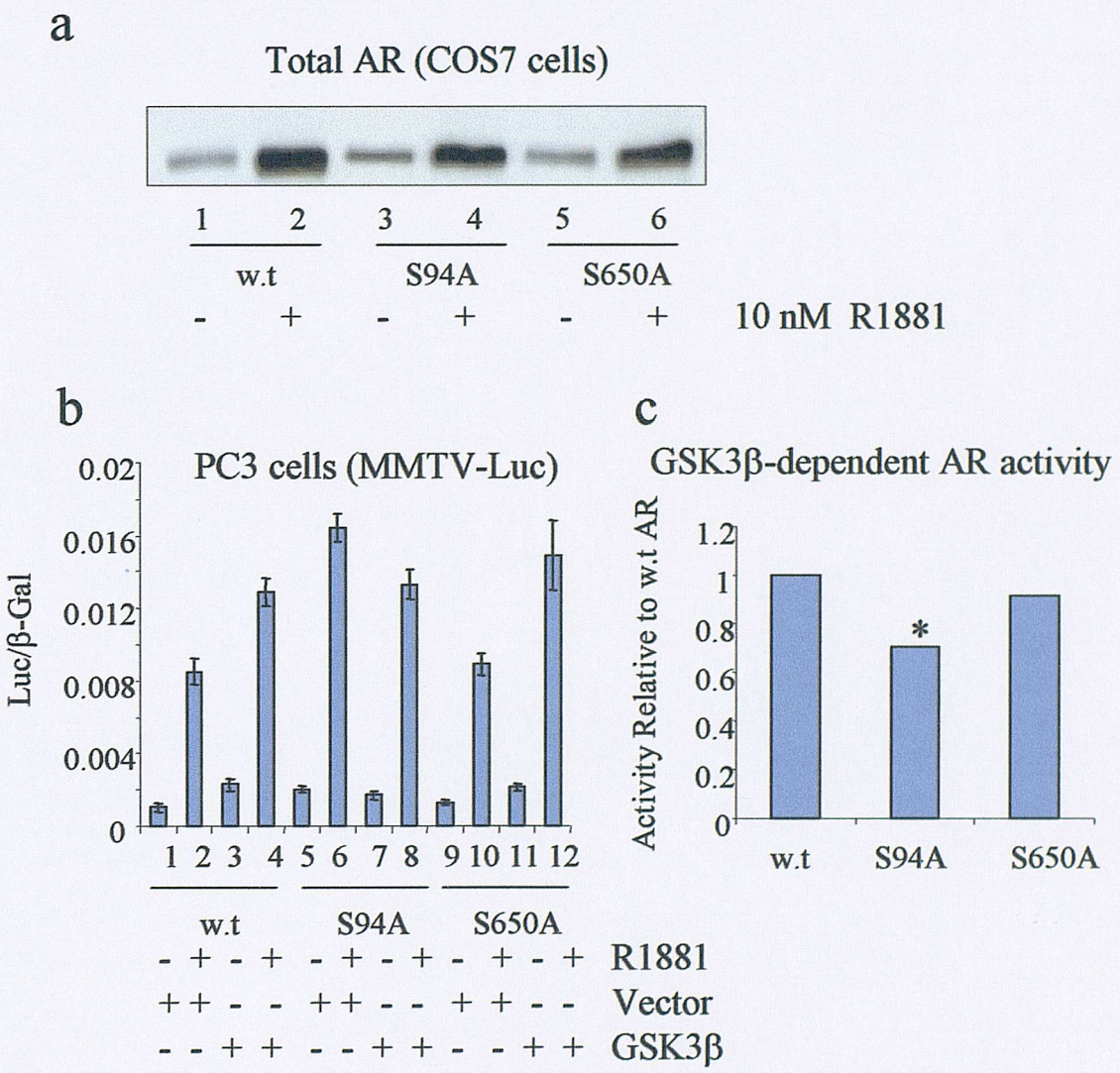


Figure 37. (a) Phosphorylation site mutations in w.t AR do not affect AR hormone-dependent protein stabilisation. COS7 cells were transfected with either w.t AR (lanes 1 and 2) or the w.t-based AR mutants S94A (lanes 3 and 4) or S650A (lanes 5 and 6) and treated with (+) or without (-) 10 nM R1881. Whole cell extracts were probed with an antibody against total AR. **(b-c) GSK3β activation of AR requires S94 in PC3 cells expressing w.t AR.** (b) Cells were transfected with the reporter vectors MMTV-Luc and pDM-β-Gal plus either w.t AR (lanes 1-4) or the AR phosphorylation site mutants S94A (lanes 5-8) or S650A (lanes 9-12), and either w.t GSK3β (lanes 3,4,7,8,11 and 12) or the empty vector pMT23 (lanes 1,2, 5,6,9 and 10). Cells were treated with (+) or without (-) 0.15 nM R1881 for 24 hours and assayed for luciferase (Luc) and β-galactosidase (β-Gal) activities. Results are presented as the ratio between luciferase and β-Gal activities. (c) An average was taken from the results of six independent experiments that were carried out in triplicates in PC3 cells in the same way as in Figure 37b. Results are presented as the fold activation of AR comparing cells expressing GSK3β and cells expressing an empty vector in the presence of androgens and relative to w.t AR (the activity of w.t AR in the presence of GSK3β is set at 1). (*) GSK3β-dependent AR activity of S94A was significantly reduced (p=0.00007) compared to w.t AR.

4. Discussion

Several reports have suggested that β-catenin is a transcriptional coactivator of AR (Truica *et al.*, 2000; Chesire *et al.*, 2002; Mulholland *et al.*, 2002; Yang *et al.*, 2002). In this study, the Wnt signalling pathway in PCa cell lines was characterised and the role of endogenous β-catenin in the regulation of AR endogenous activity was investigated. The results suggested that endogenous β-catenin is not a transcriptional coactivator for endogenous AR, however, GSK3β, independently of the Wnt signalling pathway, plays an important role in AR activity regulation.

One common feature of the PCa cell lines studied is that they have high levels of endogenous β-catenin in the cytoplasm and/or nucleus, suggesting that the Wnt signalling pathway is already active. Overexpression of Wnt1 or Wnt3A, which increase endogenous β-catenin levels in many other cell lines, such as HEK 293 (reviewed in Willert and Nusse, 1998; Polakis, 2000), had no significant effect on AR activity in PCa cell lines. Possibly, the lack of response in the PCa cell lines being examined is due to cell-specific effects. However, it is also possible that the stabilised pool of β-catenin in PCa cells is already regulating AR activity. In addition, the 1.5-2 fold activation of AR that has been reported to occur upon β-catenin overexpression (Truica *et al.*, 2000; Chesire *et al.*, 2002; Mulholland *et al.*, 2002; Yang *et al.*, 2002) was not reproducible in our laboratory. This discrepancy is possibly due to the fact that the expression vector that we used for β-catenin (driven by the adenovirus early promoter) is unlikely to express β-catenin to such high levels as those used by others (mainly driven by the CMV promoter). Since the high levels of β-catenin achieved by overexpression are unlikely to be physiological and they could have indirect effects via other signalling pathways (Damalas *et al.*, 1999; Oren *et al.*, 2002), it might be more relevant to examine the effects of the loss of endogenous β-catenin in PCa cell lines. This was achieved using two approaches – RNAi (to deplete β-catenin mRNA levels) and Axin (to induce degradation of β-catenin protein). The results suggested that endogenous β-catenin does not activate AR in PCa cells, and that its role is more complex than suggested by previous work. The results of these experiments also suggested that Axin has a unique β-catenin-independent function in regulating AR activity. Deletion analysis indicated that the ability of Axin to repress AR activity requires the GSK3β-binding domain. Indeed,

expression of this domain was sufficient to repress AR activity. This suggested a link between GSK3β and AR and led to further investigation into the role of GSK3β in the regulation of AR activity. Inhibition of GSK3β, either by expression of Axin or FRAT or by treating cells with chemical inhibitors of GSK-3, repressed AR-mediated transcription. Overexpression of GSK3β activated AR-mediated transcription, albeit only in cell lines with low endogenous GSK3β activity. Moreover, IP studies using transfected COS7 cells and the PCa cell line 22Rv1 indicated that AR and GSK3β can form a complex *in vivo*. Moreover, the COS7 cell studies showed that Axin, by binding to GSK3β, prevents AR from interacting with GSK3β. The role of GSK3β in the regulation of AR signalling was also tested in PCa cell growth assays using GSK-3 chemical inhibitors. The results of these studies confirmed that GSK3β activity was required for maximal proliferation in CWR-R1 and 22Rv1 cells. In addition, treatment of CWR-R1 cells with GSK-3 chemical inhibitors suggested a role for GSK3β in AR protein stability, particularly at the level of unliganded AR. Lastly, mutation analysis indicated that S94 in AR is required for GSK3β to activate AR signaling. Therefore, it is possible that the effect of GSK3β on AR signalling is mediated through changes in phosphorylation of AR on S94.

4.1 Characterisation of the Wnt and AR signalling pathways in PCa cell lines

Western blotting experiments revealed that the levels of β-catenin and Tcf-4, which are required for activation of β-catenin/Tcf-4-dependent transcription, differed among the PCa cell lines. In whole cell extracts, β-catenin was similarly expressed in all PCa cell lines. However, the level of nuclear β-catenin was lower in PC3 and DU145 cells than in the other PCa cell lines examined, including LNCaP cells (Figures 8a and 8b). This is probably why it was possible to detect an increase in the β-catenin cytosolic protein levels after treating with Wnt3A-conditioned medium and LiCl in PC3 cells, but not in LNCaP cells (Figure 10b). In a similar study conducted by Verras *et al.* (2004), treatment with W3A-conditioned medium obtained from L cells led to an increase in β-catenin in whole cell extracts from DU145 cells (Verras *et al.*, 2004). However, in contrast with our results, this group also showed that β-catenin cytosolic protein levels increased in LNCaP cells treated with W3A-

conditioned medium. The reason for this discrepancy in our results is not known but possibly it results from differences in our cell extraction methods.

Tcf-4 protein levels in the nucleus, on the other hand, were higher in PC3 and LAPC-4 cells than in LNCaP cells (Figure 8e). Therefore, it was not surprising to find that the β-catenin/Tcf-4-dependent transcription in LNCaP cells was extremely low and was not increased by Wnt expression (Figure 13). The limiting amount of Tcf-4 and other Tcf/Lef family members has been previously reported in LNCaP cells (Chesire *et al.*, 2002), and here was demonstrated by expression of Tcf-4, which alone, restored β-catenin/Tcf-4-dependent transcription (Figure 14).

GSK3β protein expression levels were higher (especially in nuclear extracts) in LNCaP and LNCaP-r cells compared to the other PCa cell lines tested (Figure 8c and 8d). In a recent study reported by Salas *et al.* (2004), protein expression levels of phosphorylated and total GSK3β in whole cell extracts from LNCaP, PC3 and DU145 cells were compared (Salas *et al.*, 2004). Their results indicated that, although total GSK3β protein levels were similar among these cell lines, S9 phosphorylation on GSK3β was higher in LNCaP cells, indicative of low endogenous activity of GSK3β. Results in our study, obtained using whole cell and nuclear extracts, revealed that GSK3β protein levels varied among the PCa cell lines to a greater extent in the nucleus, with LNCaP and LNCaP-r cells expressing high levels of GSK3β. Together with the results of Salas *et al.* (2004), this indicates that GSK3β protein level does not necessarily reflect its level of activity.

Surprisingly, LAPC-4 cells expressed high levels of nuclear Tcf-4 protein and some nuclear β-catenin, but the Wnt signalling pathway could not be activated by expression of Wnt1 or Wnt3A (Figures 8b, 8e and 13). However, expression of a constitutively-active form of the Wnt receptor LRP5 (C2) could activate β-catenin/Tcf-dependent transcription (Figure 15a). Moreover, β-catenin/Tcf-dependent transcription was also activated by overexpression of β-catenin (Figure 15b). This suggests that LAPC-4 cells might not have the ability to respond to Wnt1 and Wnt3A, for example, because they do not express the relevant Wnt receptors. However, downstream of the receptor, the Wnt signalling pathway is working normally. Another possibility is that the endogenous pool of β-catenin is not able to interact with the endogenous Tcf-4. This could be explained by the presence of Tcf-4 corepressors in these cells that prevent interaction between β-catenin and Tcf-4. For

example, the Groucho proteins can bind to Tcf/Lef and are able to inhibit gene activation even in the presence of β-catenin (Polakis, 2000; Seidensticker and Behrens, 2000). In addition, CBPs, which are usually coactivators, can also act as corepressors by acetylating Tcf and thereby reducing the interaction between Tcf and β-catenin (Akiyama, 2000; Polakis, 2000; Seidensticker and Behrens, 2000).

The results of RNAi experiments indicated that the basal level of β-catenin/Tcf-4-dependent transcription could be repressed when the level of β-catenin is reduced in the PCa cell lines. Interestingly, among the PCa cell lines tested, basal β-catenin/Tcf-4 activity was highest in CWR-R1 cells, and RNAi-mediated inhibition of β-catenin/Tcf-dependent transcription was most effective in these cells (Figure 16b). CWR-R1 cells also had the strongest β-catenin/Tcf transcriptional response to expression of Wnt1 and Wnt3A (Figure 13). Taken together, these results suggest that factors that normally repress the Wnt pathway in PCa cell lines are less active in CWR-R1 cells. Thus, CWR-R1 cells appear to be primed to respond to Wnt signals. This might be related to the unique conditions under which they are grown, with low levels of serum, but high levels EGF (Gregory *et al.*, 2001b) (for CWR-R1 growth condition also see section 2.6.1 in Materials and Methods).

All the AR-positive PCa cell lines express similar amounts of AR (Figure 9). Western blotting revealed that, in addition to the expected full-length AR protein, 22Rv1 cells contained two additional faster-migrating proteins, as has been previously reported (Tepper *et al.*, 2002). These bands represent C-terminally-truncated AR species that lack the LBD as a result of a mutation that occurred during progression to the AI stage of the cancer. This mutation is characterised by an in-frame tandem duplication of exon 3 that encodes the second zinc finger of the AR DBD. According to Tepper *et al.* (2002), full-length AR from 22Rv1 cells is approximately 5 KDa larger than expected and is accompanied by the C-terminally-truncated AR species (Tepper *et al.*, 2002). Results in Figure 9 clearly showed the truncated AR species, however, the slightly larger full-length AR was less apparent.

Previous reports have shown that AR has a repressive effect on β-catenin/Tcf-4 signalling (Chesire and Isaacs, 2002; Pawlowski *et al.*, 2002; Mulholland *et al.*, 2003; Song *et al.*, 2003). This effect was also observed here in LAPC-4 cells when they were treated with R1881 (Figure 15b). In addition, ectopic expression of AR in the AR-negative PC3 cells reduced basal β-catenin/Tcf-4 signalling activity in the

presence of androgens (Figure 17). These results indicate that AR signalling has a strong impact on β-catenin/Tcf-4 signalling in PCa cells. According to the published reports, the repression of β-catenin/Tcf-4 signalling is facilitated by a competition between AR and Tcf-4 over binding to β-catenin. Results obtained by Mullholand *et al.* (2003) demonstrated several points to support this model using the colon cancer cell line SW480. Their results showed that AR-mediated repression affected β-catenin/Tcf-4-dependent transcription, cell cycle progression and cell growth (Mulholland *et al.*, 2003). In addition, Chesire *et al.* (2002) suggested that there is a balance between AR and β-catenin/Tcf-4 signalling and that this might have a role in normal prostate development and PCa progression. The inhibition of β-catenin/Tcf-4 by AR was ligand-dependent, suggesting that β-catenin is being recruited to the activated AR. However, the inhibition of AR by β-catenin/Tcf-4 signalling was independent of the interaction between β-catenin and Tcf-4 (Chesire and Isaacs, 2002). Taken together, observations demonstrated in this work, together with data provided by others, suggest that there is a crosstalk between AR and β-catenin/Tcf-4 signals and also emphasises the importance of taking this into consideration in future studies on AR and PCa progression.

An endogenous complex between β-catenin and AR was detected in LNCaP cells (Figure 11). IP studies also indicated that the interaction between AR and β-catenin is stronger in the presence of androgen, as previously described (Truica *et al.*, 2000; Yang *et al.*, 2002; Verras *et al.*, 2004). However, these results were achieved after several attempts and by optimising the IP method to very specific conditions (for details on IP technique, see section 2.10-2 in Materials and Methods) suggesting that this complex is either unstable or not very abundant in LNCaP cells. The method that was used to detect the endogenous complex involved disruption of the nuclei using a very fine needle, suggesting that at least some of the complexes are present in the nucleus. In support of this, immunostaining studies suggest that AR and β-catenin co-localise in the nucleus in the presence of androgens (Chesire *et al.*, 2002; Pawlowski *et al.*, 2002; Yang *et al.*, 2002). However, most of these studies were conducted in non-prostate cells and by using overexpressed plasmids of AR and β-catenin. Pawlowski *et al.* (2002) used neuronal cells transfected with rat AR and FLAG-β-catenin and showed nuclear translocation of both proteins within 15-30 minutes of treatment with 10 nM DHT. Their conclusion was that a direct interaction

between AR and β-catenin is necessary for their co-localisation. Yang *et al.* (2002) showed nuclear translocation of overexpressed AR and β-catenin in CV-1 cells. Interestingly, Chesire *et al.* (2002), who performed immunostaining studies in PCa cells, were unable to detect nuclear translocation. However, they found that β-catenin is nuclear in 25% of metastatic PCa tumours and can be found in the nucleus in prostate cells of rats that have been castrated and then treated with androgens to induce prostate regrowth. The translocation of AR and β-catenin to the nucleus in my study was observed using non-nuclear and nuclear protein pools from PCa cells (Figure 12). These results indicated that AR translocates to the nucleus upon androgen treatment in LNCaP, LAPC-4 and CWR-R1 cells. β-catenin translocation to the nucleus was less apparent in LAPC-4 and CWR-R1 cells. Although the small increase in nuclear β-catenin in androgen-treated LNCaP cells suggests that β-catenin also enters the nucleus upon androgen treatment, this effect was not as strong as for AR. This might be because β-catenin is very abundant in PCa cells, making translocation of a small pool of β-catenin difficult to detect. Taken together, these results suggest that β-catenin and AR bind each other in PCa cells and that a small proportion of the endogenous complex enters the nucleus upon androgen treatment.

Expression of Wnt1 and Wnt3A in LAPC-4 and LNCaP cells did not significantly affect AR-mediated transcription (Figure 18). Since these Wnts also did not increase endogenous levels of β-catenin (LNCaP cells, Figure 10b) or activate β-catenin/Tcf-dependent transcription (Figure 13), this was not unexpected. However, expression of Wnt1 and Wnt3A also did not significantly affect AR signalling in CWR-R1 cells, even though they activated β-catenin/Tcf-4-dependent transcription (compare Figures 13 and 18). Moreover, ectopic expression of β-catenin did not increase AR activity in CWR-R1 cells (Figure 19b). These results suggest that the AR in CWR-R1 cells cannot be activated further by a Wnt-dependent signal. This might be due to the fact that the high levels of endogenous β-catenin in these cells already regulate AR. Interestingly, a recent report conducted in LNCaP cells indicated that Wnt3A-conditioned medium can activate AR-mediated transcription in the absence of ligand and in the presence of low concentration of androgens (Verras *et al.*, 2004). Moreover, this group also documented that AR/β-catenin nuclear complexes are more abundant in the presence of androgens. However, Wnt3A-conditioned medium induced the formation of AR/β-catenin nuclear complexes and LNCaP cell growth

only in the absence of androgens (Verras *et al.*, 2004). It is likely that the effect of the Wnt3A-conditioned medium on the cells is more immediate and possibly more physiological than expression of a Wnt3A DNA plasmid. The latter must first be transcribed in the nucleus, then be translated and secreted from the cells before it can bind to its receptors and trigger a downstream cellular signal. In addition, the differences in our results might account for the different dose of androgens used in these experiments. PCa cells in our study were treated with 10 nM R1881, whereas Verras *et al.* observed an effect on AR activity in very low levels (0.1-1 nM DHT) or in the absence of androgens. Moreover, DHT is known to be less stable compare to R1881, suggesting that, in fact, the DHT-treated cells were exposed to androgens for a shorter length of time (Moeller *et al.*, 1983). The fact that Wnt3A-conditioned medium mainly enhanced AR activity and the ability of AR to interact with β-catenin in the absence of hormone suggests that Wnt3A affects AR signalling in a ligand-independent manner.

The effects of β-catenin depletion on AR-dependent transcription revealed that the endogenous β-catenin, although highly expressed in PCa cell lines, is not a transcriptional coactivator for endogenous AR. Surprisingly, AR activity increased rather than decreased in response to β-catenin depletion in PCa cells (Figures 20b and 20c). In contrast, the siRNA experiments performed in HCT116 cells expressing w.t AR, showing that AR activity can be reduced upon depletion of β-catenin, supported results from overexpression studies obtained by others (Truica *et al.*, 2000; Mulholland *et al.*, 2002; Pawlowski *et al.*, 2002). However, these experiments also raise the question as to whether the different responses of ectopically expressed w.t. AR and endogenous AR to depletion of β-catenin might result from mutations in endogenous AR in LNCaP and CWR-R1 cells. Interestingly, results obtained in our laboratory using HCT116 cells expressing the w.t and the LNCaP form of AR (T877A) indicated that both AR forms responded similarly to the depletion of β-catenin. These observations highlight the importance of confirming results obtained using ectopically expressed proteins with reagents that address the roles of the endogenous proteins. Clearly, further experiments are necessary to determine the function of endogenous β-catenin in the regulation of AR transcriptional activity. In addition, further experiments are required to determine the effects of depletion of β-catenin on other pathways, such as cell-cell adhesion, which might indirectly affect

AR activity in different ways in the colon cancer and PCa cell lines. Taken together, these results suggest that the regulation of AR transcriptional activity by β-catenin can vary according to whether it is measured using endogenous or transfected proteins.

4.2 Regulation of AR transcriptional activity by GSK3β

In order to confirm results obtained from the RNAi studies, β-catenin was depleted using another approach. However, this time the tool that was used allowed specific degradation of the cytosolic pool of β-catenin in order to avoid effects on other pathways that β-catenin is involved in. Ectopic expression of Axin is sufficient to inhibit Wnt/β-catenin signalling and is often used to inhibit endogenous β-catenin function (Kishida *et al.*, 1998; Ross *et al.*, 2000; Hsu *et al.*, 2001; Kishida *et al.*, 2001; Reya *et al.*, 2003). For example, Reya et al (2003) showed that expression of Axin increases β-catenin degradation and leads to inhibition of haematopoietic stem cells (HSC) growth *in vitro*, which is normally promoted by β-catenin. I chose to use CWR-R1 cells as a model cell line for my studies. These cells, derived from the CWR22 xenograft model for PCa, express endogenous AR (Gregory *et al.*, 2001b), high levels of β-catenin (Chesire and Isaacs, 2002)(Figure 8), and, most importantly, respond to Wnt stimulation (Figure 13). Additionally, CWR-R1 cells have a very good hormone response. The required concentration of DHT for growth stimulation in CWR-R1 cells is four orders of magnitude lower than that required for the AD PCa cell line, LNCaP (Gregory *et al.*, 2001b). In addition, CWR-R1 cells were also found to have high transfection efficiency. The results obtained in section 3.3 confirm the work described above and lead to two important observations. The first is that increasing the level of Axin inhibits AR activity in CWR-R1 cells (Figure 22). This finding is consistent with studies in which β-catenin overexpression has been shown to activate AR activity (Truica *et al.*, 2000; Chesire *et al.*, 2002; Mulholland *et al.*, 2002; Pawlowski *et al.*, 2002; Yang *et al.*, 2002). However, Axin inhibited AR activity independently of β-catenin (Figure 22b). The GAX1Δβ-catenin mutant, which inhibits AR activity, was particularly useful, as it cannot indirectly bind to β-catenin through endogenous APC (Hinoi *et al.*, 2000), thereby eliminating any possibility of an interaction with β-catenin. Interestingly, the GSK3β-binding domain

of Axin was found to be necessary for the repression of AR activity by Axin, since a point mutation in this domain prevented this repression (Figure 22a). Deletion of the GSK3β-binding domain of Axin (GAX1ΔGSK3) did not completely prevent AR inhibition by Axin (Figure 22b), possibly because this construct can still bind GSK3β weakly (Hinoi *et al.*, 2000). The second important observation is that expression of the GSK3β-binding domain of Axin alone is sufficient to repress AR activity, and the repression of AR by this domain can be rescued by co-expression of w.t GSK3β (Figure 23b).

In support of the hypothesis that the effects of Axin are mediated through GSK3β, FRAT, another GSK3β-binding protein that directly binds and blocks GSK3β activity (Li *et al.*, 1999; Franca-Koh *et al.*, 2002), was also able to repress AR (Figure 24b). This inhibition required the GSK3β-binding domain in FRAT. Expression of FRAT-1 stimulates β-catenin/Tcf signalling because it sequesters GSK3β from the β-catenin degradation complex (Li *et al.*, 1999). Possibly, in a similar way, FRAT sequesters GSK3β from the AR and this results in repression of AR activity. Interestingly, a mutant of GSK3β that cannot bind to FRAT accumulates in the nucleus, suggesting that FRAT is also involved in the nuclear export of GSK3β (Franca-Koh *et al.*, 2002). If GSK3β regulation of AR occurs in the nucleus, this might suggest that FRAT binding to GSK3β leads to nuclear export of the latter and inhibition of AR transcriptional activity. However, further studies are required at this stage in order to clarify whether GSK3β regulates AR signalling in the cytosol or the nucleus, or both.

Ectopic expression of GSK3β activated AR in LNCaP and PC3 cells (Figures 25b and 25c, respectively). In LNCaP cells, the response of AR to GSK3β overexpression might be easier to detect because endogenous GSK3β in these cells is inactive as a result of S9 phosphorylation (Salas *et al.*, 2004; Wang *et al.*, 2004). In PC3 cells, the PTEN mutation that is known to be present in these cells might lead to low endogenous activity of GSK3β (Stambolic *et al.*, 1998; Sun *et al.*, 1999; Persad *et al.*, 2000). However, in most cell lines GSK3β is highly active (reviewed in Doble and Woodgett, 2003). This might explain why GSK3β overexpression did not have any effect on AR activity in 22Rv1 cells (Figure 25a). It is unclear why expression of the kinase-inactive form of GSK3β, K216R, which in other systems can act as a

dominant-negative mutant (Franca-Koh *et al.*, 2002) did not reduce AR activity in 22Rv1 cells. One possible explanation is that the amount of kinase-inactive GSK3β expressed was not sufficient to overcome the high levels of active endogenous GSK3β. To summarise these findings, in support of the RNAi experiments, they show that endogenous β-catenin is not a transcriptional coactivator for AR, but GSK3β, independently of β-catenin, positively regulate AR transcriptional activity.

4.3 Inhibition of AR activity and PCa cell growth by GSK-3 inhibitors

The use of GSK-3 inhibitors confirmed the previous findings suggesting that GSK3β activates AR activity and further supported a model where GSK3β plays a positive role in PCa cell growth. The results of these experiments confirmed that GSK3β activity was required for maximal AR transcriptional activity and proliferation in PCa cells.

LiCl is one of the first GSK-3 inhibitors used to study the role of GSK-3 in cells (Klein and Melton, 1996; Stambolic *et al.*, 1996). However, since LiCl has other effects on cells besides inhibiting GSK-3, SB415286 and SB216763, which are more selective for GSK-3 and more potent, were also used in this study. Inhibition of GSK-3 by LiCl has been shown by several groups to stabilise β-catenin (Stambolic *et al.*, 1996; Garcia-Perez *et al.*, 1999; Jones and Veale, 2003). Coghlan et al. (2000) showed that SB415286 and SB216763 are able to induce β-catenin/Tcf-mediated transcription in HEK 293 cells in a dose-dependent manner (maximal effective concentration of 5 μM and 30 μM for SB216763 and SB415286, respectively). Here, in order to examine the inhibitors' effects on β-catenin/Tcf and AR transcriptional activities, the optimal doses for treating CWR-R1 cells were found to be 5 μM and 20 μM for SB216763 and SB415286, respectively. Higher doses of SB415286 seemed to have toxic effects on PCa cells, as measured by the number of cells and cells morphology.

Studies using PCa cells showed that both SB415286 and SB216763 repressed endogenous AR activity, while LiCl had no effect (Figure 27a). Moreover, this result was not restricted to the MMTV promoter, as SB415286 and SB216763 also inhibited the PB-Luc reporter that measures transcription from the AR-responsive probasin promoter (Figure 27b), suggesting this is an AR-specific effect.

Interestingly, Liao *et al.* (2004) found that LiCl repressed AR activity in PCa cells. This group used LNCaP cells, which already have low endogenous GSK3β activity, and a lower concentration of LiCl (10 mM) compared to the dose I used (20 mM) (Liao *et al.*, 2004). LiCl has many additional activities in cells and therefore any effect that LiCl has on AR signalling could be indirect. For example, LiCl inhibits two key enzymes involved in the synthesis of inositol, resulting in reduction of cellular inositol levels. This in turn lowers the concentration of phosphatidylinositol(4,5)P2 (PtdIns(4,5)P2), the PI3K substrate (Williams and Harwood, 2000). There have been several reports describing a link between the AR and PI3K signalling pathways (Li *et al.*, 2001; Manin *et al.*, 2002; Sharma *et al.*, 2002; Liao *et al.*, 2003). One report suggests that PI3K stimulates AR activity (Sharma *et al.*, 2002). Therefore, in a scenario where LiCl leads to a reduction in PtdIns(4,5)P2 cellular levels, this in turn can affect the ability of PI3K to activate AR transcriptional activity in these cells.

The results obtained examining the effects of the GSK-3 inhibitors on β-catenin and on Wnt signalling in PCa cells suggest that all three inhibitors activate β-catenin/Tcf-dependent signalling albeit in different ways (Figure 26): LiCl and SB415286 stabilised cytosolic β-catenin in 22Rv1 cells, but SB216763 did not appear to do so (Figure 26a). The reason for this is not known, but the fact that SB216763 did increase β-catenin/Tcf-dependent transcription suggests that this drug is working (Figure 26c). It might be necessary to use a higher dose of this inhibitor to detect β-catenin stabilisation in 22Rv1 cells. Additionally, LiCl slightly increased S21 phosphorylation, whereas SB216763 and SB415286 reduced GSK-3 tyrosine phosphorylation (Figure 26b). Possibly, the effects of the inhibitors on GSK-3 phosphorylation status differ as a result of the mechanism by which they inhibit GSK-3. SB216763 and SB415286 inhibit GSK-3 by competing with the ATP-binding pocket, whereas LiCl competes with the magnesium-binding site of GSK-3 (Klein and Melton, 1996; Coghlan *et al.*, 2000; Ryves and Harwood, 2001; Smith *et al.*, 2001). Since LiCl is known to have non-specific effects, and since SB216763 and SB415286, which are more specific for GSK-3, reduced GSK-3 tyrosine phosphorylation, these observations might suggest that the ability of GSK-3 to regulate AR activity involves tyrosine phosphorylation rather than serine phosphorylation of GSK-3. Interestingly, Liao *et al.* (2004) showed that androgen

treatment in LNCaP cells leads to Y216 phosphorylation in GSK3β (Liao *et al.*, 2004). In addition, these experiments suggest that the effects of these inhibitors on the phosphorylation state of GSK-3 in 22Rv1 cells are stronger for GSK3α rather than GSK3β. Interestingly, SB216763 and SB415286 were originally identified as leads from a screen of compounds that inhibit rabbit GSK3α. Later, the inhibitors were found to be equally effective at inhibiting GSK3α and GSK3β although these data were not shown (Coghlan *et al.*, 2000). Since most of the experiments described in this thesis were carried out using GSK3β reagents, any conclusions I have drawn here are based on GSK3β only. Clearly, further studies required in order to determine which isoform of GSK-3 is more important for the regulation of AR activity. It would be interesting to determine the roles of GSK3α and GSK3β in the regulation of AR activity, for example by using siRNA constructs that downregulate the cellular expression levels of both GSK-3 isoform in PCa cells.

Cell growth assays implied that GSK3β not only increases AR transcriptional activity but also positively regulates PCa cell growth. Here, only SB216763 and SB415286 were used as the effects of LiCl on AR activity in PCa cells were thought to be indirect. The inhibitory effects of SB216763 on CWR-R1 cell growth were maximal at 3 μM (Figure 28a), which is the same concentration that is optimal for the ability of this inhibitor to protect neurons from apoptotic cell death (Cross *et al.*, 2001). However, in order to match the dose used in the transcription assays, SB216763 was used at 5 μM in cell growth assays. The inhibitory effects of SB415286 on CWR-R1 cell growth increased with dose up to the maximal dose tested (50 μM, Figure 28b), although, owing to the toxic effects of this inhibitor, the chosen dose for use in growth assays was 20 μM.

Both SB216763 and SB415286 reduced CWR-R1 cell growth over a period of 6 days (Figure 29). The repressive effect that SB415286 had on CWR-R1 cell growth was greater than that of SB216763. This might result from GSK-3-independent effects of SB415286, as this drug was toxic at higher doses. Therefore, I focused on SB216763 for the majority of the work. The fact that SB216763 reduced the growth of AR-positive PCa cells supports the idea that AR is required for the growth inhibitory response (Figure 30a). However, SB216763 also inhibited hormone-independent growth of 22Rv1 cells to a certain extent (Figure 30b), suggesting that there is also an effect on AI cell growth. Since GSK-3 inhibitors did not inhibit the

growth of the AR-negative lines, PC3 and DU145, these observations might indicate that inhibition of AI growth still involves the AR. Indeed, studies revealed that even in AI cases the AR signalling pathway and the AR itself remain important in most cancer cells. There are several routes by which alterations of the AR signalling pathway or abnormalities in AR can lead to the progression of AI PCa. These include amplification, mutation, increased sensitivity of the AR, changes in its glutamine repeats length and alterations in coregulators or other interacting pathways (for mechanisms of AI PCa development, see section 1.5 in Introduction). In contrast to these results, Wang *et al.* (2004) used a CWR22R-derived cell line with inducible expression of constitutively-active GSK3β to show that GSK3β suppresses AR activity and androgen-induced growth (Wang *et al.*, 2004). It is unclear why these results contrast directly with ours. It is possible that the unregulated high activity of GSK3β in the induced cells is detrimental to cell growth. In addition, this group measured cell growth using the MTT assay, a method that determines cell number as a function of mitochondrial dehydrogenase activity in living cells. This could lead to unexpected results since GSK-3 is known to affect the activity of mitochondrial dehydrogenases (Hoshi *et al.*, 1996). In my studies, cells were counted using either an automated counter or a haemocytometer, depending on the number and aggregation characteristics of the cells. These techniques are based on physical counting of cells in samples with single cell suspensions, which facilitates measurement of the number of viable cells in a sample.

The idea that GSK3β positively regulate PCa cell growth implies that the regulation of AR signalling by GSK3β might be crucial for PCa progression. Moreover, it suggests that GSK-3 inhibitors could potentially be used to repress tumour progression in PCa therapy. Further studies on the possible therapeutic application of GSK-3 inhibitors and their design as drugs that could be used in PCa treatment would be highly valuable.

4.4 GSK3β associates with the AR

IP studies confirmed that GSK3β and AR can form a complex *in vivo* (Figures 32 and 33). This complex was disrupted when AX2 was overexpressed, demonstrating the ability of Axin to interfere with the association between AR and GSK3β (Figure

34). Interestingly, the association between GSK3β and AR appeared to be stronger when AX2P was overexpressed compared to when an empty vector was overexpressed. Possibly, AX2P affects unknown cellular proteins or reactions that indirectly contribute to GSK3β association with AR. An endogenous complex between GSK3β and AR was detected in 22Rv1 cells, however this was not an easy task to achieve and required specific conditions (for details on IP technique, see section 2.10-2 in Materials and Methods). This might suggests that the endogenous complex between AR and GSK3β is either unstable or present at very low levels (Figure 33). The amount of complex detected did not increase in the presence of androgens. This could possibly be a cell-specific effect or might result from insensitivity of the IP technique. Supporting the IP results, Wang *et al.* (2004) also detected an endogenous complex between AR and GSK3β in LNCaP cells growing in their normal medium (Wang *et al.*, 2004). Taken together, these results support a model in which the association between AR and GSK3β is required for the enhancement in AR transcriptional activity, and in which Axin inhibits AR transcriptional activity by preventing interaction between GSK3β and AR.

Further studies suggested that the activation of AR by GSK3β inv olves the regulation of AR protein stability. GSK-3 inhibitors reduced AR levels in CWR-R1 cells (Figure 31a). However, the GSK-3 inhibitors were not able to diminish the androgen-mediated increase in AR levels, which was observed in non-nuclear and nuclear extracts (Figure 31b and 31c). Therefore, these results suggest that GSK3β plays a role in AR protein stability mainly when AR is in the unliganded state. Together with the finding that GSK3β regulates AI PCa cell growth in AR-expressing cells, these observations suggest that GSK3β regulation of AR signalling might be important in the absence of hormone as well as when the ligand is present.

Previous reports have shown that GSK-3 regulates the stability of a number of proteins (reviewed in Frame and Cohen, 2001; Woodgett, 2001; Doble and Woodgett, 2003). In the majority of cases, phosphorylation by GSK-3 promotes degradation of its target substrate (examples include β-catenin, cyclin D1 and c-myc). However, there are also examples where phosphorylation by GSK3β promotes protein stability, such as Axin (Yamamoto *et al.*, 1999). GSK3β can also have both positive and negative effects on protein stability; for example, it stabilises NFκB/p105 under resting conditions and primes p105 for degradation upon TNF-α

treatment (Demarchi *et al.*, 2003). Moreover, although GSK-3 inhibits the transcriptional activity of many nuclear proteins (Frame *et al.*, 2001; reviewed in Woodgett, 2001; Doble and Woodgett, 2003), it has also been shown to activate at least one transcription factor, CREB, by direct phosphorylation (Salas *et al.*, 2003).

To conclude, protein and IP studies in this work demonstrated that GSK3β can interact with AR, and the inhibitor studies suggested that GSK3β plays a role in AR protein stability mainly in its unliganded state. Therefore, it is very likely that the repression of AR transcriptional activity by inhibition of GSK3β results from a reduction in AR protein stability. One possibility is that AR is a substrate for GSK3β and phosphorylation of AR by GSK3β increases its stability. The observation that AR and GSK3β proteins can be co-immunoprecipitated supports these possibilities.

4.5 GSK3β regulation of AR transcriptionl activity through phosphorylation

AR has been previously shown to be a phosphoprotein; and exposure to androgens increases AR phosphorylation and leads to appearance of a slightly heavier isoform of AR (van Laar *et al.*, 1991; Jenster *et al.*, 1994; Wang *et al.*, 1999; Gioeli *et al.*, 2002). Phosphorylation on AR occurs predominantly on serine residues (Jenster *et al.*, 1994; Kuiper and Brinkmann, 1995; Zhou *et al.*, 1995; Yeh *et al.*, 1999; Wen *et al.*, 2000; Lin *et al.*, 2001; Gioeli *et al.*, 2002). GSK-3 is known to phosphorylate its substrates on serine and threonine residues and one of its consensus sites for phosphorylation is S/T P. This proline-directed site has been found in three major phosphorylation sites in AR, S81, S94 and S650 (Jenster *et al.*, 1994; Zhou *et al.*, 1995; Yeh *et al.*, 1999; Wen *et al.*, 2000; Lin *et al.*, 2001; Gioeli *et al.*, 2002). These sites were previously reported to be involved either in AR transcriptional activity (S650) and/or are phosphorylated after addition of hormone (S81 and S650) (Zhou *et al.*, 1995; Gioeli *et al.*, 2002). Therefore, a mutational analysis was conducted in order to begin to address the possibility that GSK3β phosphorylates AR to activate AR signaling. In order to be consistent with previous experiments, initially, the H874Y AR, which is the form of AR found in CWR-R1 cells, was mutated at S81, S94 and S650. Additionally, to investigate further the role of S94, and to compare the behavior of the S94A mutation in the w.t and the H874Y forms of AR, this mutation was created in w.t AR. In parallel, a S650A mutation was also created in w.t AR. All

five mutants created (w.t- and H874Y-based AR mutants) were expressed in COS7 cells. The results of western blotting and transcription assays indicated that the mutants were synthesised as functional proteins (Figures 35a, lower panel, and 37a). Interestingly, the w.t-based S94A and S650A AR mutants migrated slightly more slowly than the non-mutated w.t AR (Figure 37a). This could possibly indicate that these mutations caused a protein conformational change. PC3 cells were chosen as the model cell line to examine the effect of the AR mutations on GSK3β-dependent AR transcriptional activity since they are a PCa cell line that does not express endogenous AR. The AR plasmid and hormone were titrated in order to determine the optimal amounts to use for GSK-3-dependent enhancement of AR activity. These were found to be 8 ng of AR and 0.15 nM R1881, the latter being close to the Kd of AR for R1881 (Gregory *et al* , 2001b).

All five mutants were stabilised by androgen treatment and maintained their hormone response at the transcriptional level. This suggests that the mutagenesis did not have any negative effects on domains in the receptor that are involved in the receptor response to hormone, such as the ligand binding pocket, the transactivation domain (AF-2) in the LBD, and the C/N-terminal domain interaction (Figures 35b and 37b). In fact, in some experiments the mutants responded better than the non-mutated AR (for example, S94A mutant in figure 37b). It is possible that the introduction of a serine to alanine mutation in AR led to a conformational change that improves the receptor accessibility and/or affinity to the hormone.

Zhou *et al.* (1995) generated a similar panel of phosphorylation-site mutants in the w.t. AR and compared their activities in CV-1 cells using the MMTV-Luc reporter. They found that the S650A AR mutant was 30% less active than the w.t AR, suggesting a role for this site in AR transcriptional activity (Zhou *et al.*, 1995). The differences between this report and our observations might be related to the use of different cell lines, amounts of plasmid or transfection assay. For example, this group did not normalise their data for transfection efficiency. In support of results obtained here, Gioeli *et al.* (2002) reported that AR transcriptional activity was unaffected in AR phosphorylation site mutants in CV-1 cells; their results were described as data not shown (Gioeli *et al.*, 2002).

The use of an antibody that recognises AR phosphorylated on S81 confirmed that, as expected, the S81A mutant was not phosphorylated upon androgen treatment,

whereas, H874Y and S650A AR were (Figure 35a, upper panel). However, the detection of S81 phosphorylation on AR using this antibody was only achieved once, possibly due to antibody batch problems. Gioeli *et al.* (2002) have previously demonstrated androgen-mediated S81 phosphorylation of AR in LNCaP cells. Their results were obtained using metabolically labelled LNCaP cells followed by peptide mapping and mass spectrometry. They also showed that phosphorylation on S16, S256, S308, S424, and S650 was elevated in response to R1881. In contrast, S94 was constitutively phosphorylated (Gioeli *et al.*, 2002), suggesting that S94 does not play a role in androgen-mediated AR activity. In support of their results, results obtained here showed that the S94A mutation does not affect androgen-mediated AR activity, however, more importantly, S94 does appear to have a role in the GSK3β-dependent increase in AR activity (Figures 36, 37b and 37c). These results suggest that S94 is a strong candidate for being a GSK3β-dependent phosphorylation site in AR.

Several protein kinases have been suggested to phosphorylate the AR. Induction of the MAPK pathway by IL-6 leads to an enhancement in AR activity (Ueda *et al.*, 2002; Yang *et al.*, 2003). In addition, *in vitro* studies suggested that MAPK phosphorylates S515 (Yeh *et al.*, 1999), although this was not reproducible in LNCaP cells (Gioeli *et al.*, 2002). AR was found as an Akt substrate in two studies, however this group only examined the overall AR phosphorylation status (Wen *et al.*, 2000; Lin *et al.*, 2001). In contrast, inhibition of Akt in LNCaP cells did not affect AR phosphorylation (Gioeli *et al.*, 2002). Gioeli *et al.* (2002) performed *in vivo* studies and suggested that PKA, EGF and PKC signalling can affect AR phosphorylation on S650 (Gioeli *et al.*, 2002). In another study, the effects of a panel of kinase inhibitors on AR transcriptional activity were examined using LNCaP cells to determine the pathways involved in AR signalling (Liao *et al.*, 2004). This group found that the GSK-3 inhibitor, LiCl, and the PI3K inhibitor, LY294002, repressed AR activity. These findings suggested a role for GSK-3 and PI3K in AR activity regulation, although further studies from this group suggested that PI3K does not play a role in AR signalling. Liao *et al.* (2004) also showed that the inhibitors for MEK-1 (PD98059), mTOR (Rapamycin), PKA (H89), p38/MAPK (SB203580) and Src kinase (PP2) had no significant effect on AR activity, ruling out major roles for these kinases in AR signalling in this system.

Sharma *et al.* (2002) were the first to show a possible link between GSK3β and AR signalling. Their studies in LNCaP cells indicated that inhibition of PI3K using the inhibitor LY294002 led to repression of AR activity through regulation of GSK3β and decreased accumulation of β-catenin. Their conclusion was that PI3K signalling stimulates AR activity through inhibition of GSK3β in PCa cells (Sharma *et al.*, 2002). However, this group did not specifically show that GSK3β mediates the effects of LY294002 on AR activity. Recently, three other groups reported results from experiments examining the effects of GSK3β on AR signalling. Salas *et al.* (2004) showed that GSK3β phosphorylates AR and inhibits AR transcriptional activity in transfected COS-1 cells (Salas *et al.*, 2004). Another group showed that GSK3β binds and phosphorylates the AR N-terminal domain *in vitro*, and can inactivate AR in COS-1 and LNCaP cells (Wang *et al.*, 2004). According to this group, GSK3β directly influences AR activity since it affects the AF-1 activity of AR, and not AF-2, which is involved in the regulation of AR by β-catenin. In contrast, Liao *et al.* (2004) showed that inhibition of GSK-3 using either chemical inhibitors or GSK3β siRNA reduces AR transcriptional activity in PCa cell lines (Liao *et al.*, 2004). This group also showed an increase in GSK3β Y216 phosphorylation in androgen-stimulated LNCaP cells. Results described in this thesis using chemical inhibitors of GSK-3 and the GSK3β-binding proteins Axin and FRAT are in agreement with the report from Liao *et al.* Interestingly, Liao *et al.* (2004) also found that depletion of β-catenin in LAPC-4 cells by siRNA slightly increased AR transcriptional activity, in agreement with the results of the siRNA studies described here. It is likely that the use of different systems for transcription assays (endogenous AR in PCa cell lines as opposed to transfected AR in COS-1 cells) accounts for the different results obtained by the other two groups.

To summarise, the use of AR phosphorylation-site mutants revealed a role for S94 in the activation of AR by GSK3β. Although recent reports have published contradicting reports regarding the role of GSK3β in AR signalling, the observations made in this study contribute to the debate and support the idea that S94 is a candidate for being a GSK3β-dependent phosphorylation site in AR.

4.6 Possible mechanisms for the regulation of AR signalling by GSK3β

GSK3β might potentiate AR activity by several mechanisms. The findings that S94 is a candidate for a GSK3β-dependent phosphorylation site in AR and that the two proteins interact *in vivo*, support a model where GSK3β phosphorylates AR directly. Subsequently, this phosphorylation would activate AR-mediated transcription. Another possibility is that AR is being phosphorylated only when GSK3β is tethered to AR by another protein. In fact, since the overexpression of GSK3β and a kinase-inactive form of GSK3β did not greatly affect AR in some cell lines (Figure 25), it is possible that other proteins are required. It would be interesting to identify GSK-3-binding proteins in PCa cells involved in the GSK3β-dependent activation of AR. This could be obtained by performing more protein studies or transcription assays in cells expressing candidate GSK-3-binding proteins.

Although our study clearly demonstrated an association between GSK3β and AR *in vivo* (Figures 32 and 33), the exact binding domain by which GSK3β interacts with AR remains to be found. Identification of the GSK3β-binding site in AR could shed light on the effects that GSK3β binding and/or phosphorylation of AR have on AR conformation and possibly on its ability to translocate to the nucleus. Wang *et al.* (2004) reported that GSK3β interacts both with the N and the C-termini of AR in GST pull-down assays (Wang *et al.*, 2004). If GSK3β will be further found to play a role in AR translocation to the nucleus, this could be important for the understanding of the mechanism by which GSK3β activates AR. However, this will need to be confirmed in PCa cells.

Overexpression studies using the GSK3β-binding proteins Axin and FRAT demonstrated that these proteins can have a negative effect on GSK3β regulation of AR (Figures 22, 23, 24b, and 34). This suggests that in the normal situation the ability of GSK3β to bind and activate AR might depend on the balance between the expression levels of different GSK3β-binding proteins in cells and the ability of GSK3β to interact with AR.

It is still unclear whether GSK3β phosphorylation of AR, if it occurs, is ligand-dependent. Van Laar *et al.* (1991) demonstrated AI AR phosphorylation in cytosolic extracts from LNCaP cells (van Laar *et al.*, 1991). Black *et al.* (2004) detected AD phosphorylation on S81 in w.t AR expressed in COS7 cells (Black *et al.*, 2004). Gioeli *et al.* (2002) showed that AR phosphorylation on several serine residues, but

not S94, which is constitutively phosphorylated, increases in the presence of androgens (Gioeli *et al.*, 2002). It is possible that AD and AI phosphorylation on S94 is facilitated by different kinases and/or through different signalling pathways. Clearly, GSK3β phosphorylation sites in AR will need to be mapped in order to determine if they are AD or not. This could first be done by performing kinase assays using peptides of AR including the potential phosphorylation sites and purified GSK3β. In this study, the use of a commercial antibody that recognises the phosphorylation of S81 in AR was helpful in confirming that S81 is an AD phosphorylation site in AR. Should antibodies recognising additional phosphorylation sites become available in the future, they could be valuable in confirming which sites in AR are AD and whether or not they are phosphorylated by GSK3β.

The western blotting studies using GSK-3 inhibitors suggest that GSK3β regulates AR stability mainly when AR is not bound to ligand (Figure 31). Furthermore, GSK3β/AR complexes were detected in the absence and presence of androgens (Figure 33). Since unliganded AR is primarily located in the cytosol, these results suggest that the two proteins can interact in the cytosol in resting cells. This could account for the constitutive phosphorylation of AR on S94 (if S94 proves to be a GSK3β phosphorylation site in AR). S94 phosphorylation by GSK3β might stabilise unliganded AR. In order to test this possibility it would be necessary to compare the half-lives of unliganded w.t and the S94A AR in the presence and absence of GSK3β. Binding of AR to its ligand might trigger phosphorylation of AR by GSK3β at unidentified sites that require prior phosphorylation at S94, followed by translocation of AR to the nucleus. In support of this theory, Liao at al. (2004) have reported that androgen treatment of LNCaP cells increases phosphorylation of GSK3β on Y216, suggesting that the kinase becomes activated in the presence of androgens (Liao *et al.*, 2004). Moreover, immunohistochemical staining studies in PCa tissues demonstrated that GSK3β and AR co-distribute both in the cytosol and the nucleus (Salas *et al.*, 2004). Thus, it is possible that, as part of the mechanism by which GSK3β activates AR, GSK3β and AR translocate as a complex to the nucleus.

Gioeli *et al.* (2002) showed that the kinetics of hormone-dependent AR phosphorylation on serine sites using phosphopeptide labelling, is relatively slow, peaking at 2 hours or later (Gioeli *et al.*, 2002). The slow kinetics of AR

phosphorylation is similar to the delayed hormone-dependent phosphorylation in the PR (Zhang *et al.*, 1995). Zhang *et al.* (1995) showed that there is an initial and rapid phosphorylation of PR, followed by a slower increase in phosphorylation, which results in decreased mobility of the receptor on SDS gels. Since AR nuclear localisation is known to be a rapid event, Gioeli *et al.* concluded that AR phosphorylation is not involved in the nuclear localisation of AR or initiation of transcription, but rather in a later event such as AR recycling. One report suggested that the S81 phosphorylation site in AR is involved in nuclear export (Black *et al.*, 2004). AR export mutants are found trapped in subnuclear foci and have reduced AD phosphorylation on S81. There might be sites in AR that are more rapidly phosphorylated, similar to in PR, that are important for GSK3β-dependent effects.

Recent reports show that several nuclear receptors are targets for modifications that contribute to nuclear receptor regulation. For example, GR, PR, PPARgamma2 and AR, are conjugated to SUMO-1 (small ubiquitin-like modifier-1) (Poukka *et al.*, 2000; Abdel-Hafiz *et al.*, 2002; Le Drean *et al.*, 2002; Tian *et al.*, 2002; Callewaert *et al.*, 2004). The SUMO conjugation sites in AR have been mapped to lysines 386 and 520. Mutation of both sumoylation sites results in enhanced AR transcriptional activity (Poukka *et al.*, 2000; Callewaert *et al.*, 2004), indicating that SUMO-1 modification plays a negative role in modulating AR activity. Interestingly, studies with phosphorylation-defective mutants of PPARgamma2 suggested that phosphorylation at S112 promotes K107 sumoylation on PPARgamma2, which exerts repressive effects on the transactivating function of the receptor (Yamashita *et al.*, 2004). This suggests that nuclear receptor phosphorylation might have a role in sumoylation-dependent inhibition of the receptor's transactivity.

AR is also a target for ubiquitination and destruction by the 26S proteasome (Sheflin *et al.*, 2000). The proto-oncogene Mdm2 catalyses AR ubiquitination and proteolysis *in vivo*, suggesting it functions as an E3 ligase for the AR (Lin *et al.*, 2002). Moreover, inhibition of the proteasome by MG132 abolishes productive rounds of AR-mediated transcription of the PSA gene by preventing release of AR from the promoter (Kang *et al.*, 2002). The degradation of most nuclear hormone receptors occurs predominantly after ligand-binding but the trigger for this degradation is unknown. Interestingly, mutation of MAPK phosphorylation sites in PR reduces hormone-dependent degradation, suggesting that phosphorylation of

nuclear receptors can influence their degradation (reviewed in Nawaz and O'Malley, 2004). These reports provide evidence for a mechanism of nuclear receptor turnover in which hormone-dependent degradation and receptor-transactivation are linked processes that act together in order to maintain efficient transcription of target genes. Post-translational modification of the receptor possibly triggers hormone-dependent degradation following the receptor transcriptional activation in the nucleus.

Interestingly, a recent report demonstrates a link between AR acetylation and AR phosphorylation (Fu *et al.*, 2004). Direct acetylation of AR contributes to its full ligand-dependent activity (Fu *et al.*, 2000; Gaughan *et al.*, 2002). The AR acetylation site, K630, is an important regulator of coactivator/corepressor binding: mutations in AR that mimic acetylation (K630Q, K630T) enhance p300 binding and reduce corepressor binding, whereas charged residue substitution (K630R) reduces p300 binding and enhances corepressor binding (Fu *et al.*, 2002; Fu *et al.*, 2003a). Studies conducted by Fu *et al.* (2004) suggest that the conserved AR acetylation site plays a role in coordinating AR phosphorylation: mutation of the lysine residues at the AR acetylation site reduces activation by the HDAC inhibitor, TSA (histone deacetylase inhibitor trichostatin A) and ligand-induced phosphorylation of AR. Of particular relevance, mutation of S94 reduces transactivation by TSA and the histone acetyltransferase p300. Therefore, S94 not only plays a role in AR activation by GSK3β, but also in AR activation by acetylation. One possibility, therefore, is that GSK3β activates AR by promoting its acetylation. Fu *et al.* (2004) also correlated AR acetylation with inhibition by PKA and by Akt. Since both PKA and Akt can repress GSK-3 (Li *et al.*, 2000; Fang *et al.*, 2002; Tanji *et al.*, 2002), GSK3β might mediate both these responses. Clearly, it will be important to determine the relationship between GSK3β and acetylation of AR. One simple scenario is that GSK3β maintains basal phosphorylation of AR at S94 and that this is a pre-requisite for activation by acetylation. Ligand-dependent activation of GSK3β could lead to further phosphorylation of AR at novel sites or to phosphorylation of proteins that regulate AR acetylation.

4.7 Summary

The investigation into the role of the Wnt signalling pathway in PCa has led to a number of interesting discoveries. First, most PCa cell lines were found to express high endogenous levels of β-catenin, an observation also made by other groups. However, in contrast with previous reports, our study suggests that endogenous β-catenin is not a transcriptional coactivator of the AR. Second, this work presents a novel role for GSK3β in activating AR signalling, independently of β-catenin. The use of protein and chemical inhibitors of GSK-3 demonstrated that GSK3β activity is required for maximal activity of the AR. Third, western blotting and IP studies indicate that GSK3β can interact with AR and plays a role in AR protein stability. Moreover, these studies suggest that Axin represses AR by disrupting the GSK3β/AR complex. Fourth, the enhancement of AR transcriptional activity by GSK3β is likely to involve S94 in AR. The exact mechanism of AR regulation by GSK3β in PCa cells remains to be revealed and more studies are required in order to completely understand the crosstalk between AR signalling and GSK3β in PCa cells. Importantly, the observations that inhibition of GSK3β leads to a reduction in AR activity, protein level and PCa cell growth suggest a novel therapeutic application for GSK-3 inhibitors, which are already under development for diabetes and neurological diseases, in the treatment of PCa.

5. References

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6. Appendix

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Inhibition of glycogen synthase kinase-3 represses androgen receptor activity and prostate cancer cell growth

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The transcriptional activity of the androgen receptor (AR) is regulated by interaction with various coregulators, one of which is β-catenin. Interest in the role of β-catenin in prostate cancer has been stimulated by reports showing that it is aberrantly expressed in the cytoplasm and/or nucleus in up to 38% of hormone-refractory tumours and that overexpression of β-catenin results in activation of AR transcriptional activity. We have examined the effect of depleting endogenous β-catenin on AR activity using Axin and RNA interference. Axin, which promotes β-catenin degradation, inhibited AR transcriptional activity. However, this did not require the β-catenin-binding domain of Axin. Depletion of β-catenin using RNA interference increased, rather than decreased, AR activity, suggesting that endogenous β-catenin is not a transcriptional coactivator for the AR. The glycogen synthase kinase-3 (GSK-3)-binding domain of Axin prevented formation of a GSK-3-AR complex and was both necessary and sufficient for inhibition of AR-dependent transcription. A second GSK-3-binding protein, FRAT, also inhibited AR transcriptional activity, as did the GSK-3 inhibitors SB216763 and SB415286. Finally, inhibition of GSK-3 reduced the growth of AR-expressing prostate cancer cell lines. Our observations suggest a potential new therapeutic application for GSK-3 inhibitors in prostate cancer.

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Introduction

Prostate cell growth and development are mediated by androgens and the androgen receptor (AR), a member of the nuclear receptor superfamily. The transcriptional activity of AR is regulated by interaction with various coregulators (reviewed in Cheshire and Isaacs, 2003; Cronauer *et al.*, 2003), one of which is β-catenin. Evidence that increased levels of β-catenin lead to activation of AR transcriptional activity come largely from studies in which β-catenin is overexpressed (Truica *et al.*, 2000; Chesire *et al.*, 2002; Mulholland *et al.*, 2002; Yang *et al.*, 2002). However, the level of endogenous β-catenin is already very high in prostate cancer cells and stable expression of mutant β-catenin does not alter their proliferative response to androgen (Chesire *et al.*, 2002). Therefore, it is important to determine how endogenous β-catenin affects AR transcriptional activity in prostate cancer cells. Here we describe experiments addressing this question using Axin and RNA interference. Our results suggest that glycogen synthase kinase-3 (GSK-3), rather than β-catenin, is an important endogenous regulator of AR transcriptional activity.

GSK-3 is a serine/threonine kinase best known for its roles in glycogen metabolism, the Wnt signalling pathway and in neurological disorders (reviewed in Frame and Cohen, 2001; Grimes and Jope, 2001; Woodgett, 2001; Doble and Woodgett, 2003). GSK-3 has been shown to be active in most resting cells and is subject to negative regulation by external stimuli. In response to growth factor stimulation, for example, kinases such as Akt inhibit GSK-3 by phosphorylation on serine 9 (Stambolic and Woodgett, 1994; Cross *et al.*, 1995). In some instances, GSK-3 has been shown to be activated by agents that promote phosphorylation on tyrosine 216 (Bhat *et al.*, 2000). GSK-3 can also be regulated by binding to the proteins Axin, FRAT (frequently rearranged in advanced T-cell lymphomas)/GBP and the Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen (Ikeda *et al.*, 1998; Yost *et al.*, 1998; Fujimuro *et al.*, 2003).

GSK-3 has numerous substrates, including a number of transcription factors such as c-Jun, c-*myc*, C/EBPs (CCAAT enhancer-binding proteins) and NF-ATc (nuclear factor of activated T cells). The effects of phosphorylation by GSK-3 tend to be inhibitory and include promotion of degradation and enhancement of nuclear export (for references see Frame and Cohen, 2001). Thus, inhibition of GSK-3 often results in increased gene expression. However, there are examples where GSK-3 positively regulates gene expression, such as through CREB phosphorylation (Salas *et al.*, 2003). Here we show that GSK-3 positively regulates AR transcriptional activity. Furthermore, GSK-3 inhibitors

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inhibit the proliferation of prostate cancer cells, suggesting that these drugs might be useful in the treatment of patients with prostate cancer.

Results

Inhibition of AR transcriptional activity by Axin

Axin inhibits the Wnt signalling pathway by acting as a scaffold protein, bringing together a number of proteins, including β-catenin, APC and GSK-3, and thereby promoting phosphorylation and degradation of β-catenin (for references see Kikuchi, 2000; Gregory *et al*., 2001). Ectopic expression of Axin is sufficient to inhibit Wnt/β-catenin signalling and is therefore often used as a tool to inhibit endogenous β-catenin function (Ross *et al*., 2000; Hsu *et al*., 2001; Reya *et al*., 2003). In order to determine whether endogenous β-catenin functions as a coactivator for the AR in prostate cancer cells, we expressed Axin in CWR-R1 cells. This is a cell line derived from the CWR22 xenograft model for prostate cancer that expresses endogenous AR (Gregory *et al*., 2001) and high levels of β-catenin (Chesire and Isaacs, 2002). For these studies we used a luciferase reporter plasmid driven by the MMTV promoter, which contains AR-binding sites, R1881 (a synthetic ligand for the AR) and a panel of previously characterized GFP-Axin expression constructs (Orme *et al*., 2003) (Figure 1a); GFP was used as a negative control. As expected, compared with cells expressing GFP and treated with carrier (Figure 1b, lane 1), addition of R1881 resulted in an increase in AR transcriptional activity (Figure 1b, lane 2). Expression of GFP-Axin resulted in a reduction in AR transcriptional activity (Figure 1b, lane 3). This was consistent with studies in which β-catenin overexpression has been shown to activate AR (Truica *et al*., 2000; Chesire *et al*., 2002; Mulholland *et al*., 2002; Pawlowski *et al*., 2002; Yang *et al*., 2002). Mutation of a conserved proline residue in the GSK-3-binding domain of Axin (GFP-AxinP), which prevents binding to GSK-3 and also reduces binding to β-catenin (Smalley *et al*., 1999), prevented the inhibition of AR transcriptional activity (Figure 1b, lane 4). To determine the importance of the β-catenin-binding domain in Axin for repression of AR, we used a mutant form of Axin that lacks both the β-catenin and the APC-binding domains, GFP-Axin$^{\Delta APC/\Delta\beta}$. This mutant is useful because it cannot indirectly interact with β-catenin through endogenous APC (Hinoi *et al*., 2000). GFP-Axin$^{\Delta APC/\Delta\beta}$ inhibited AR transcriptional activity (Figure 1b, lane 6) to the same extent as a mutant lacking only the APC-binding domain, GFP-Axin$^{\Delta APC}$ (Figure 1b, lane 5) and GFP-Axin itself. These results suggest that the inhibition of AR transcriptional activity by Axin is independent of β-catenin and that the loss of inhibitory activity in GFP-AxinP results from its inability to bind GSK-3.

In order to determine if the GSK-3-binding domain of Axin is sufficient for the inhibition of AR activity, we expressed a construct of Axin comprising only the GSK-

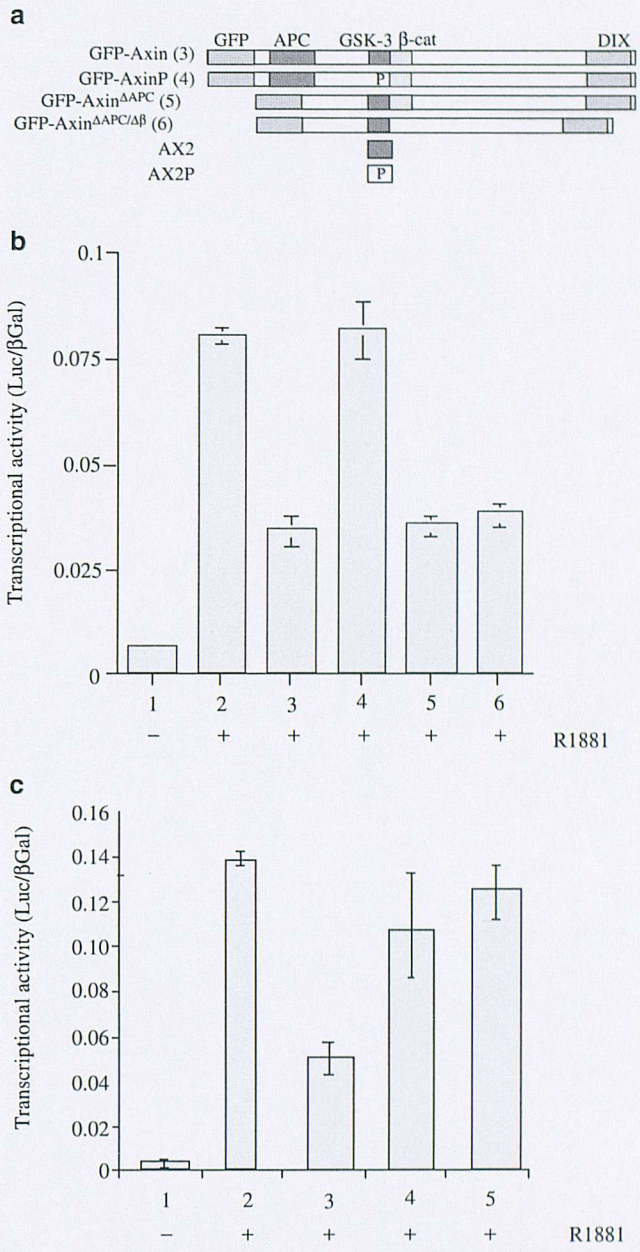

Figure 1 Inhibition of AR transcriptional activity by Axin. (**a**) Cartoon of Axin constructs used. P denotes the L521P mutation that disrupts binding of Axin to GSK-3. The numbers indicate the constructs used in (**b**). (**b**) Requirement of the GSK3-binding domain (but not the β-catenin- or APC-binding domains) for inhibition of AR activity by Axin. CWR-R1 prostate cancer cells were transfected with GFP (1 and 2), GFP-Axin (3), GFP-AxinP (4), GFP-Axin$^{\Delta APC}$ (5) or GFP-Axin$^{\Delta APC/\Delta\beta\text{-catenin}}$ (6), MMTV-Luciferase and RSV-β-Gal. AR transcriptional activity was determined in extracts from cells grown in hormone-depleted medium in the absence (−) or presence (+) of 10 nM R1881. (**c**) The GSK-3-binding domain of Axin is sufficient for inhibition of AR activity. CWR-R1 cells were transfected with empty vector (1 and 2), AX2 (3), AX2P (4) or AX2 plus pMT23 GSK-3 S9A (5), MMTV-Luciferase and RSV-β-Gal. Empty vector (pMT23) was included in transfections 1–4 to allow direct comparison with transfection 5. AR transcriptional activity was determined in extracts from cells grown in hormone-depleted medium either in the absence (−) or presence (+) of 10 nM R1881. All experiments were done three or more times in triplicate. The error bars indicate standard deviation

3-binding domain, AX2 (Smalley *et al.*, 1999). Relative to empty vector (Figure 1c, lane 2), AX2 inhibited AR transcriptional activity (Figure 1c, lane 3). As a control we used AX2 with a mutation in the conserved proline residue required for GSK-3 binding (AX2P), and we found that AX2P did not inhibit AR activity (Figure 1c, lane 4). Moreover, coexpression of constitutively active GSK-3 with AX2 rescued the inhibitory effects of AX2 on AR transcriptional activity (Figure 1c, lane 5). Taken together, these results indicate that GSK-3, rather than β-catenin, is involved in the inhibitory effects of Axin on AR transcriptional activity.

Depletion of endogenous β-catenin does not inhibit endogenous AR transcriptional activity in prostate cancer cells

Our results using Axin suggest that endogenous β-catenin in prostate cancer cells does not affect AR activity. In order to test this possibility, we used a second approach to determine the effect of removing endogenous β-catenin on AR transcriptional activity. For these studies, we used a well-characterized β-catenin siRNA expression vector that has been shown to reduce β-catenin protein levels and inhibit β-catenin/Tcf transcriptional activity (van de Wetering *et al.*, 2003). We first used HCT 116 colon cancer cells, which have a stabilizing mutation in β-catenin, and pOT-Luciferase, a reporter plasmid with Tcf/LEF-1-binding sites. As expected, β-catenin siRNA inhibited β-catenin/Tcf-dependent transcription in HCT 116 cells (Figure 2a). β-catenin siRNA expression did not affect the activity of pOF-Luciferase, which comprises the pOT promoter with mutations in the Tcf-binding sites (data not shown). β-catenin siRNA expression also inhibited β-catenin/Tcf-dependent transcription in CWR-R1 cells, LNCaP cells and 22Rv1 cells (Figure 2a and data not shown). Next, AR was expressed in HCT 116 cells together with either control siRNA expression vector or β-catenin siRNA expression vector and MMTV-Luciferase. As predicted from β-catenin overexpression studies, depletion of β-catenin resulted in a reduction in the transcriptional activity of transfected AR in HCT 116 cells (Figure 2b, lanes 1 and 2 and Figure 2c). A second control siRNA expression vector (Control 2) had no effect on AR transcriptional activity, and the inhibitory effect was not observed in the absence of hormone (Figure 2c).

In order to determine if the effects of β-catenin siRNA resulted from a reduction in the level of expression of cotransfected AR, Western blots were conducted after transfection (Figure 2e). Expression of β-catenin siRNA led to a significant reduction in β-catenin protein (Figure 2e, upper panel, lanes 3 and 4). The depletion of β-catenin is likely to be more efficient than suggested by the Western blot since the extracts also contained β-catenin from untransfected cells, which comprise more than half the cell population. In the same extracts the expression level of AR was unaffected by expression of β-catenin siRNA (Figure 2e, lower panel), indicating that the inhibition of AR after depletion of β-catenin did not result from a reduction in AR protein levels.

We also examined the effects of depletion of β-catenin on endogenous AR activity in prostate cancer cells. The low transfection efficiency of the prostate cancer cell lines made it difficult to detect changes in β-catenin protein levels after expression of β-catenin siRNA. Therefore we used the reduction of β-catenin/Tcf transcriptional activity (Figure 2a) as a measure of the efficiency of the β-catenin siRNA. Depletion of β-catenin did not inhibit endogenous AR transcriptional activity in CWR-R1 cells, LNCaP cells (Figure 2b) or 22Rv1 cells (Figure 2d); in fact AR activity was significantly increased. These results suggest that the regulation of endogenous AR transcriptional activity by endogenous β-catenin differs from what has been observed in experiments where one or both of these proteins are ectopically expressed.

GSK-3 increases AR transcriptional activity

Axin deletion analysis suggested an important role for GSK-3 in the regulation of AR activity. Therefore, we assessed the effects of overexpressing GSK-3 on AR transcriptional activity. For these studies we used wild-type GSK-3, a constitutively active form of GSK-3 that has a mutation at serine 9 (S9A), the inhibitory phosphorylation site, and a catalytically inactive form of GSK-3 (K216R). AR transcriptional activity was not significantly affected by expression of any of these constructs in 22Rv1 cells (Figure 3a); GSK-3 S9A expression did result in a small increase in AR transcriptional activity in CWR-R1 cells (data not shown).

We reasoned that the weak effect of GSK-3 on AR activity might be because endogenous GSK-3 is already active in these cell lines. Therefore, we examined the effects of GSK-3 expression on AR transcriptional activity in LNCaP cells, in which GSK-3 is known to be inactive as a result of phosphorylation at serine 9 (Salas *et al.*, 2004). When expressed at high levels, wild-type GSK-3 significantly increased AR transcriptional activity in LNCaP cells (Figure 3b, lane 6). Constitutively active GSK-3 increased AR transcriptional activity both at low and high levels of expression (Figure 3b, lanes 8 and 10, respectively). These results suggest that wild-type GSK-3 is inhibited by phosphorylation at serine 9 in LNCaP cells. Catalytically inactive GSK-3 did not affect AR transcriptional activity (Figure 3b, lane 12). Taken together, these results support a hypothesis in which GSK-3 positively regulates AR transcriptional activity in prostate cancer cells.

Inhibition of GSK-3 reduces AR transcriptional activity

In order to determine whether the inhibition of AR transcriptional activity by Axin was specific to the effects of Axin on GSK-3 and could not be elicited by other means, we used two further approaches. First, we expressed the proto-oncogene FRAT, which activates the Wnt signalling pathway by binding and sequestering

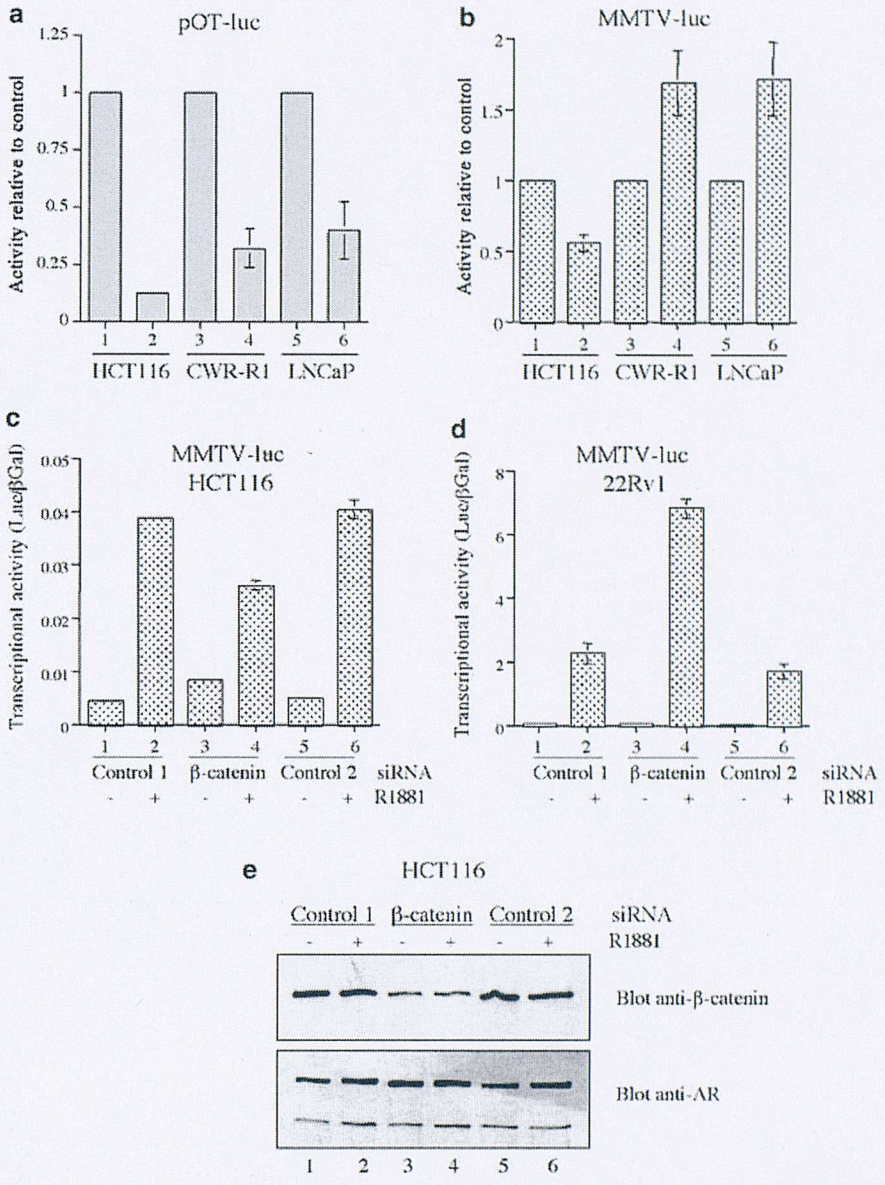

Figure 2 Depletion of endogenous β-catenin does not inhibit endogenous AR transcriptional activity in prostate cancer cells. (**a**) HCT 116 colon cancer cells, CWR-R1 cells and LNCaP cells were transfected with the reporter vector pOT-Luciferase, which measures β-catenin/Tcf transcrptional activity, RSV-β-Gal, and either Control 1 (1, 3 and 5) or β-catenin (2, 4 and 6) siRNA expression vectors. β-catenin/Tcf transcriptional activity was determined in extracts from cells grown in normal growth medium. Results are presented as the activity relative to each cell line transfected with Control 1 siRNA expression vector. (**b**) HCT 116 cells, CWR-R1 cells and LNCaP cells were transfected with MMTV-Luciferase, RSV-β-Gal, pSG5 AR (HCT 116 cells only) and either Control 1 (1, 3 and 5) or β-catenin (2, 4 and 6) siRNA expression vectors. AR transcriptional activity was determined in extracts from cells grown in androgen-depleted medium in the presence of 10 nM (CWR-R1 cells) or 1 nM (HCT 116 and LNCaP cells) R1881. Results are presented as the activity relative to each cell line transfected with Control 1. β-catenin siRNA expression vector significantly increased AR activity in CWR-R1 cells ($P=0.004$) and LNCaP cells ($P=0.003$) and significantly decreased it in HCT 116 cells ($P=0.01$). (**c**) HCT 116 cells were transfected with MMTV-Luciferase, RSV-β-Gal, pSG5 AR and either Control 1 (1 and 2), β-catenin (3 and 4) or Control 2 (5 and 6) siRNA expression vectors. AR transcriptional activity was determined in extracts from cells grown in androgen-depleted medium in the absence (−) or presence (+) of 1 nM R1881. (**d**) 22Rv1 cells were transfected with MMTV-Luciferase, RSV-β-Gal and either Control 1 (1, 2), β-catenin (3, 4) or Control 2 (5, 6) siRNA expression vectors. AR transcriptional activity was determined in extracts from cells grown in androgen-depleted medium in the absence (−) or presence (+) of 1 nM R1881. All experiments were done three or more times in triplicate. The error bars indicate standard deviation. (**e**) HCT 116 cells were transfected with pSG5 AR and either Control 1 (lanes 1 and 2), β-catenin (lanes 3 and 4) or Control 2 (lanes 5 and 6) siRNA expression vectors and grown in androgen-depleted medium in the absence (−) or presence (+) of 1 nM R1881 for 24 h. Extracts were probed for β-catenin (upper panel) and then stripped and reprobed for AR (lower panel, upper band). The faster migrating band in the anti-AR blot is a degradation product of AR

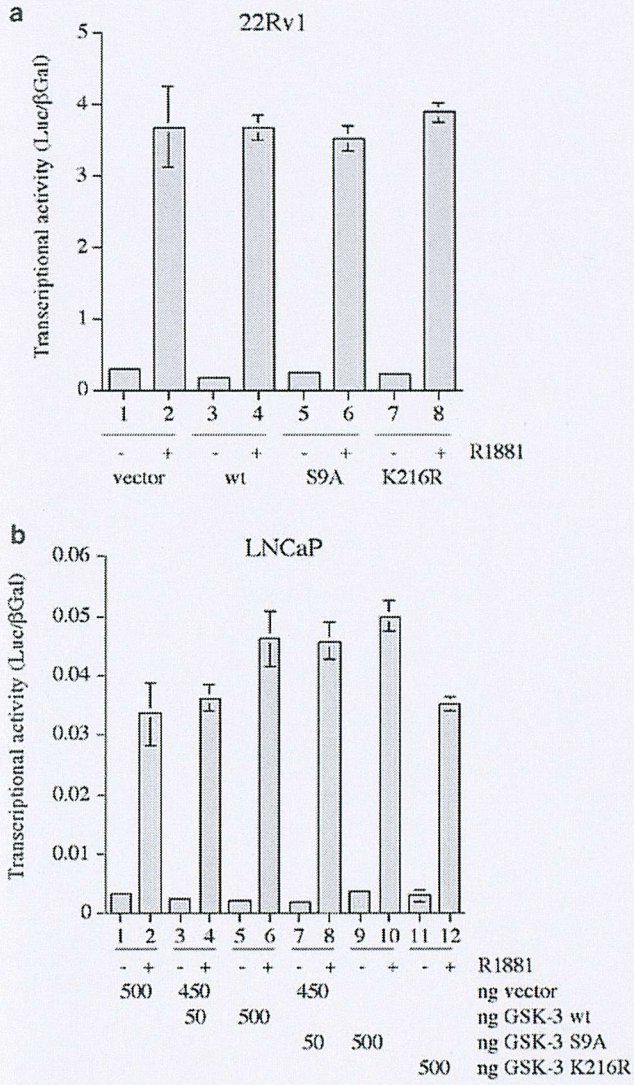

Figure 3 GSK-3 increases AR transcriptional activity. (**a**) 22Rv1 cells and were transfected with empty vector (1 and 2), wild-type GSK-3 (3 and 4), GSK-3 S9A (5 and 6) or GSK-3 K216R (7 and 8) plus MMTV-Luciferase, and RSV-β-Gal. AR transcriptional activity was determined in extracts from cells grown in hormone-depleted medium either in the absence (−) or presence (+) of 1 nM R1881. (**b**) LNCaP cells were transfected with the indicated amounts of empty vector (1 and 2), wild-type GSK-3 (3–6), GSK-3 S9A (7–10) or GSK-3 K216R (11 and 12) plus MMTV-Luciferase, and RSV-β-Gal. AR transcriptional activity was determined in extracts from cells grown in hormone-depleted medium either in the absence (−) or presence (+) of 1 nM R1881. AR activity was significantly increased by wild-type GSK-3 at the higher dose ($P=0.02$) and by GSK-3 S9A at the lower dose ($P=0.0006$) and the higher dose ($P=0.0004$). Experiments were done twice in triplicate and error bars indicate standard deviation

GSK-3 (Yost *et al*., 1998). Consistent with the published data (Li *et al*., 1999; Franca-Koh *et al*., 2002), expression of FRAT increased β-catenin/Tcf-dependent transcription in HEK 293 cells, while expression of a FRAT mutant that cannot bind to GSK-3 (FRATΔC) did not (Figure 4a). Expression of FRAT and FRATΔC did not affect the activity of a reporter with mutated Tcf-binding sites ((Smalley *et al*., 1999) and data not shown). We next determined the effects of FRAT expression on AR transcriptional activity in CWR-R1 cells. As predicted from the experiments using Axin, FRAT inhibited AR transcriptional activity, and the extent of inhibition was significantly reduced when using FRATΔC (Figure 4b). However, FRATΔC did repress AR activity to a certain extent, particularly when expressed at higher levels (data not shown). We interpret this result as a manifestation of an indirect effect on GSK-3, since FRATΔC can associate with dishevelled, which binds to GSK-3 via Axin (Li *et al*., 1999). To summarize, although FRAT and Axin have opposite effects on β-catenin/Tcf transcriptional activity, they both inhibit AR transcriptional activity, and in both cases this requires their GSK-3-binding domains.

In the second approach, we used two commercially available inhibitors of GSK-3, SB415286 and SB216763 (Coghlan *et al*., 2000). First we examined the effects of these inhibitors on β-catenin/Tcf-dependent signalling. CWR-R1 cells were transfected with the reporter vector pOT-Luc and treated with GSK-3 inhibitors for 24 h. Consistent with results in other cell types (Coghlan *et al*., 2000), both inhibitors increased β-catenin/Tcf-dependent transcriptional activity (Figure 4c). In contrast, both inhibitors reduced AR transcriptional activity (Figure 4d), consistent with a model in which endogenous GSK-3 activates AR. Taken together, these results suggest that the inhibitory effects of Axin on AR result from its ability to regulate GSK-3, rather than any function unique to Axin.

Inhibition of GSK-3 reduces prostate cancer cell growth

We next examined the effects of GSK-3 inhibitors on prostate cancer cell growth. We first used CWR-R1 cells, which are hypersensitive to androgens and grow optimally in medium containing 2% FCS, as described previously (Gregory *et al*., 2001). CWR-R1 cells were treated with GSK-3 inhibitors and counted over a period of 6 days (Figure 5a). Both SB415286 and SB216763 repressed CWR-R1 cell growth. The inhibitory effects of SB216763 on cell growth were maximal at 3 μM (Figure 5b), which is the same concentration that is optimal for the ability of this drug to protect neurons from apoptotic cell death (Cross *et al*., 2001). The inhibitory effects of SB415286 increased with dose up to the maximal dose tested (50 μM; data not shown).

In order to determine whether inhibition of GSK-3 specifically inhibited growth of AR-positive prostate cancer cells, we compared the effects of SB216763 on the growth of CWR-R1, 22Rv1 and LNCaP cells, which express AR, and DU145 and PC3 cells, which do not (Figure 5c). 22Rv1 and CWR-R1 cells both derive from the CWR22 prostate cancer xenograft but were selected under different growth conditions (Sramkoski *et al*., 1999; Gregory *et al*., 2001). SB216763 similarly reduced the growth of CWR-R1 cells and 22Rv1 cells. In contrast, SB216763 did not significantly affect DU145 or PC3 cell growth, consistent with the possibility that AR is required for the growth inhibitory response. The

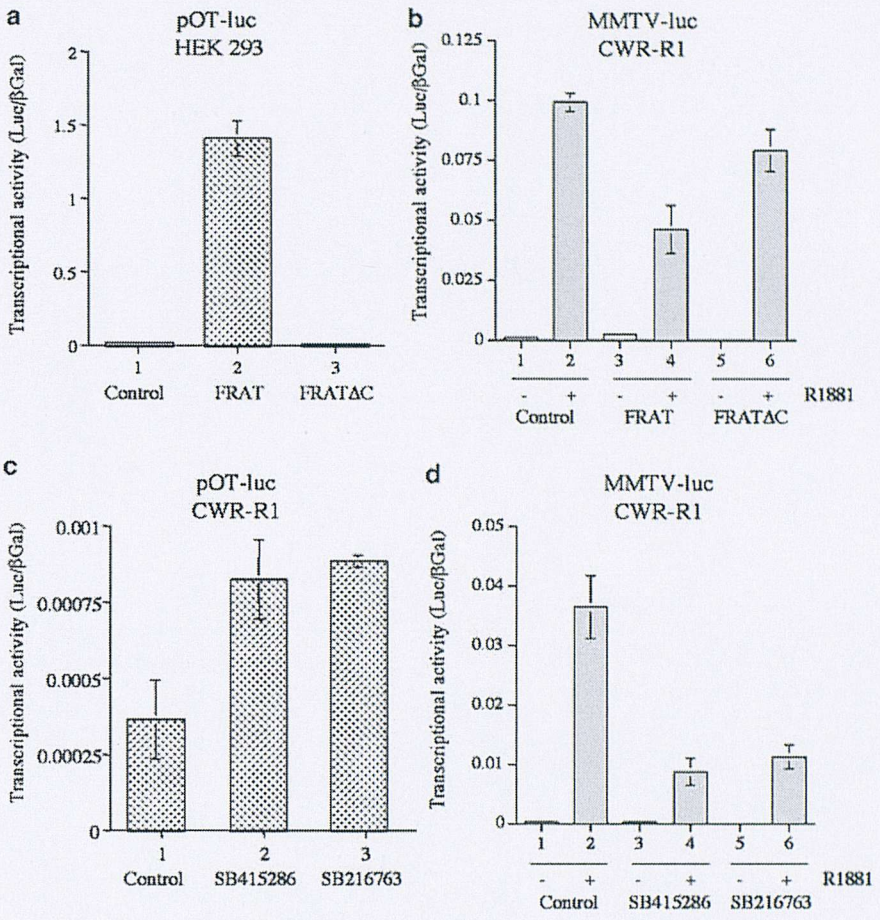

Figure 4 Inhibition of GSK-3 reduces AR transcriptional activity. (**a**) HEK 293 cells were transfected with pOT-Luciferase, RSV-β-Gal and either GFP control vector (1), GFP-FRAT (2) or GFP-FRATΔC (a deletion mutant of FRAT that lacks the GSK-3-binding site) (3). β-catenin/Tcf transcriptional activity was determined in cell extracts from cells grown in normal growth medium 24 h after transfection. (**b**) CWR-R1 cells were transfected with MMTV-Luciferase, RSV-β-Gal and either GFP control vector (1 and 2), GFP-FRAT (3 and 4) or GFP-FRATΔC (5 and 6). AR activity transcriptional activity was measured in extracts from cells grown in hormone-depleted medium in the absence (−) or presence (+) of 10 nM R1881. (**c**) CWR-R1 cells were transfected with pOT-Luciferase and RSV-β-Gal. Cells were treated for 24 h with carrier (1), 20 μM SB415286 (2) or 5 μM SB216763 (3) and β-catenin/Tcf transcriptional activity was determined in extracts from cells grown in normal growth medium. (**d**) CWR-R1 cells were transfected with MMTV-Luciferase and RSV-β-Gal. After transfection, cells were incubated in hormone-depleted medium in the absence (−) or presence (+) of 10 nM R1881 and either carrier (1 and 2), 20 μM SB415286 (3 and 4) or 5 μM SB216763 (5 and 6) for 24 h. AR transcriptional activity was then determined from cell extracts. All experiments were done three or more times in triplicate. The error bars indicate standard deviation

growth of LNCaP cells was weakly inhibited by SB216763, consistent with the low GSK-3 activity in this cell line. Taken together, these results suggest that inhibition of GSK-3 reduces the growth of AR-positive prostate cancer cells. To determine if inhibition of GSK-3 specifically affected androgen-dependent cell growth, a similar experiment was conducted using 22Rv1 cells grown in hormone-depleted medium in the absence or presence of R1881 (Figure 5d). Although 22Rv1 cells are able to grow in hormone-depleted medium, their growth can be stimulated by androgens (Sramkoski *et al.*, 1999). We found that R1881 stimulated the growth of 22Rv1 cells and that this was blocked by treatment with SB216763. However, SB216763 also inhibited hormone-independent proliferation of 22Rv1 cells to a certain extent.

Inhibition of GSK-3 leads to a reduction in AR protein levels

As a first step in determining the mechanism by which GSK-3 regulates AR transcriptional activity, we examined the expression level of the AR protein in CWR-R1 cells treated with GSK-3 inhibitors. CWR-R1 cells were treated with GSK-3 inhibitors for 24 h and whole-cell extracts were probed for AR by Western blotting (Figure 6, upper panel). Interestingly, compared with untreated cells (lane 1), the protein level of AR was reduced after treatment with both SB216763 (lane 2) and SB415286 (lane 3). SB415286 appeared to reduce AR protein levels more than SB216763, but reprobing the blot for tubulin (lower panel) indicated that part of this reduction resulted from the effects of this drug on the number of cells. Nevertheless, taking into account

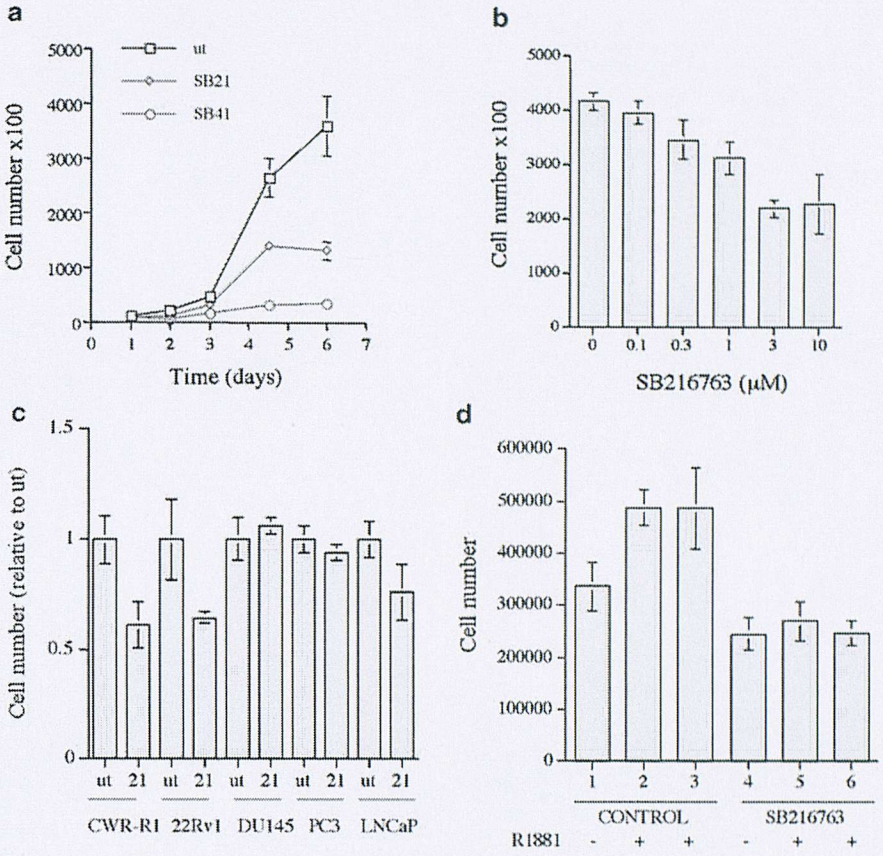

Figure 5 Inhibition of GSK-3 reduces prostate cancer cell growth. (**a**) CWR-R1 cells were grown in the presence of carrier (ut), 5 μM SB216763 (SB21) or 20 μM SB415286 (SB41) for up to 6 days and the number of cells was counted. The experiment was done twice in triplicate and the error bars indicate standard deviation. The difference in the number of cells in untreated and treated cells was statistically significant ($P=0.02$ for SB216763 and $P=0.008$ for SB415286 at day 6). (**b**) CWR-R1 cells were grown for 72 h in complete growth medium in the presence of the indicated concentrations of SB216763 and the number of cells was counted. The experiment was done in triplicate and the error bars indicate standard deviation. The difference in the number of cells in untreated and treated cells was significant ($P=0.0007$ at 3 μM). (**c**) CWR-R1, 22Rv1, DU145, PC3 and LNCaP cells were grown in normal growth medium (DU145, PC3 and CWR-R1 cells) or in hormone-depleted medium (22Rv1 and LNCaP cells) in the presence of 1 nM R1881 either with carrier (ut) or 5 μM SB216763 (21). The number of cells was counted after 72 h (or after 5 days for LNCaP cells). Experiments were done in triplicate and the error bars indicate standard deviation. The number of CWR-R1, 22Rv1 and LNCaP cells was significantly reduced by treatment with SB216763 ($P=0.002$ for LNCaP cells). (**d**) 22Rv1 cells were grown in hormone-depleted medium in the absence of hormone (1 and 4), in the presence of 10^{-12} M R1881 (2 and 5) or 10^{-9} M R1881 (3 and 6) and either with carrier (1–3) or 5 μM SB216763 (4–6). The number of cells was counted after 72 h. Experiments were done in triplicate and the error bars indicate standard deviation

the loading controls, both inhibitors reduced AR protein levels in CWR-R1 cells.

Association between AR and GSK-3 and its disruption by AX2

In order to determine whether the effects of GSK-3 on AR might involve interactions between these proteins, we examined the possibility that GSK-3 and AR form a complex. AR and myc epitope-tagged GSK-3 were coexpressed in COS7 cells and these proteins were then immunoprecipitated and probed by Western blotting (Figure 7). GSK-3 was detected in anti-AR immune precipitates and not in control immune precipitates (Figure 7a), and AR was detected in anti-GSK-3 immune precipitates and not in control immune precipitates (Figure 7b). These results support a model in which GSK-3 increases AR transcriptional activity by forming a complex with AR. To determine a possible mechanism for the inhibition of AR activity by Axin, we expressed AX2 or AX2P in COS7 cells together with GSK-3 and AR (Figure 7c). AR was readily detected in GSK-3 immune precipitates from COS7 cells expressing AX2P. In contrast, we were unable to detect a complex between AR and GSK-3 in cells expressing AX2. This suggests that AX2 inhibits AR transcriptional activity by preventing interaction between GSK-3 and AR and further supports a model in which the association of GSK-3 with AR leads to elevated AR transcriptional activity.

Discussion

Several reports have suggested that β-catenin is a transcriptional coactivator of AR (Truica *et al.*, 2000;

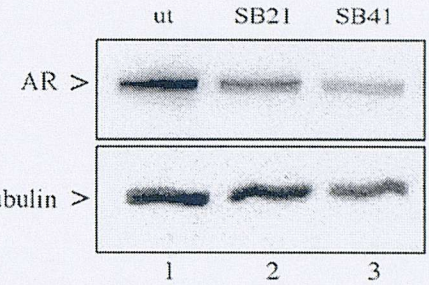

Figure 6 Inhibition of GSK-3 leads to a reduction in AR protein levels. CWR-R1 cells were treated either with carrier (ut, lane 1), 5 μM SB216763 (SB21, lane 2) or with 20 μM SB415286 (SB41, lane 3) for 24 h. Western blots of whole-cell extracts were probed for AR (upper panels) and reprobed for γ-tubulin as an internal loading control (lower panels)

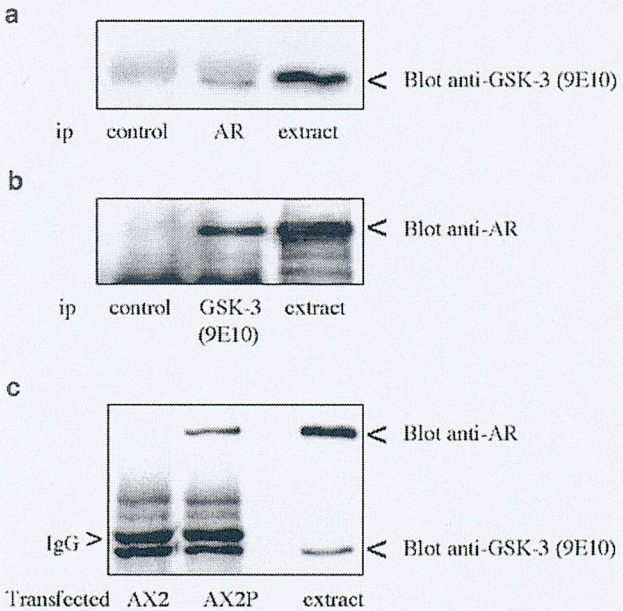

Figure 7 Association between AR and GSK-3 and its disruption by AX2. (**a**) Extracts from COS7 cells transfected with AR and myc epitope-tagged GSK-3 were immunoprecipitated with polyclonal control antibody or polyclonal anti-AR antibody and probed with anti-myc antibody (9E10). The arrow indicates the position of GSK-3 in the cell extract. (**b**) Extracts from COS7 cells transfected with AR and myc epitope-tagged GSK-3 were immunoprecipitated with control mAb or 9E10 antibodies and probed with anti-AR antibody. The arrow indicates the position of AR in the cell extract. (**c**) Extracts from COS7 cells transfected with AR and myc epitope-tagged GSK-3 and either AX2 or AX2P were immunoprecipitated with 9E10 antibodies, probed with anti-AR antibody and then reprobed with 9E10. The upper arrow indicates the position of AR and the lower arrow the position of GSK-3 in from cells transfected with AR and GSK-3 extracts. The band migrating above GSK-3 is IgG recognized by the secondary antibody

Chesire *et al*., 2002; Mulholland *et al*., 2002; Yang *et al*., 2002). However, the results from our experiments using Axin and a β-catenin siRNA expression vector to deplete β-catenin suggest that endogenous β-catenin, although highly expressed in prostate cancer cell lines, is not a transcriptional coactivator for endogenous AR. This is in contrast to the results we obtained using transfected AR in HCT116 cells (Figure 1b). This difference is not due to the AR mutations found in prostate cancer cell lines since we obtained similar results in HCT 116 cells using both wild type and the LNCaP mutant form of AR (unpublished observations). Our observations highlight the importance of confirming results obtained using ectopically expressed proteins by examining the functions of the endogenous proteins. Clearly, further experiments are necessary to determine the function of endogenous β-catenin in the regulation of AR transcriptional activity.

Our results using Axin suggest a role for GSK-3 in the regulation of AR transcriptional activity. The repression of AR activity by Axin required an intact GSK-3-binding domain, since a point mutation in this domain prevented repression. Indeed, expression of the GSK-3-binding domain alone was sufficient to repress AR activity, and the repression of AR by this domain was rescued by coexpression of constitutively active GSK-3. Furthermore, the GSK-3-binding domain of Axin prevented the formation of a GSK-3-AR complex. In support of the hypothesis that Axin inhibits AR transcriptional activity by binding to GSK-3, a second GSK-3-binding protein, FRAT, also repressed AR activity. Further work will be required to determine if endogenous Axin or FRAT play a role in the regulation of AR transcriptional activity, or if the observations we have made solely result from their abilities to sequester GSK-3 when they are overexpressed.

The role of GSK-3 in the regulation of AR transcriptional activity and prostate cancer cell growth was addressed further using GSK-3 inhibitors. The results of these experiments confirmed that GSK-3 activity was required for maximal AR transcriptional activity and proliferation in CWR-R1 cells and 22Rv1 cells. Interestingly, treatment of CWR-R1 cells with GSK-3 inhibitors reduced the level of AR protein. One possible interpretation of these data is that GSK-3 directly phosphorylates AR, and that this phosphorylation increases AR stability. GSK-3 has been shown to regulate the stability of a number of proteins (for references see Frame and Cohen, 2001; Woodgett, 2001; Doble and Woodgett, 2003). In the majority of cases, phosphorylation by GSK-3 promotes degradation of its target substrate (examples include β-catenin, cyclin D1 and c-myc). However, there are also examples where phosphorylation by GSK-3 promotes protein stability, such as Axin (Yamamoto *et al*., 1999). Finally, GSK-3 can have both positive and negative effects on protein stability; for example, it stabilizes nuclear factor-κB1/p105 under resting conditions and primes p105 for degradation upon TNF-α treatment (Demarchi *et al*., 2003). To continue this line of reasoning, the decrease in AR transcriptional activity in cells treated with GSK-3 inhibitors would result from a reduction in AR protein levels, as was observed experimentally (Figure 6). Although GSK-3 inhibits the transcriptional activity of many nuclear proteins (for references see Frame and Cohen, 2001; Woodgett, 2001; Doble and Woodgett, 2003), it activates at least one transcription factor, CREB, by direct phosphorylation (Salas *et al*., 2003). It

remains to be seen whether GSK-3 activates the AR by direct phosphorylation, and whether this then leads to increased AR protein levels. However, our observation that AR and GSK-3 can be co-immunoprecipitated supports such a possibility.

While this manuscript was in preparation, three other groups reported results from experiments examining the regulation of AR by GSK-3. The first showed that GSK-3 phosphorylates AR and inhibits AR transcriptional activity in transfected COS-1 cells (Salas *et al.*, 2004). Wang *et al.* (2004) reported similar results using both COS-1 cells and LNCaP cells. This group also showed that the inhibition of AR by GSK-3 is blocked by lithium chloride. In contrast, Liao *et al.* (2004) showed that inhibition of GSK-3 using either chemical inhibitors or GSK-3 siRNA reduces AR transcriptional activity. Interestingly, this group also found that depletion of β-catenin by siRNA increased AR transcriptional activity. Our results, obtained using different chemical inhibitors of GSK-3 and using the GSK-3-binding proteins Axin and FRAT, are in agreement with the report from Liao *et al.* It is possible that the use of different systems for transcription assays (endogenous AR in prostate cancer cell lines as opposed to transfected AR in COS-1 cells) accounts for many of the differences between our results and those suggesting that GSK-3 inhibits AR. However, the results of Wang *et al.* obtained using LNCaP cells also differ from ours; it is possible that they reflect differences in cell passage number, since this influences regulation of AR activity by Akt, a kinase that can inhibit GSK-3 (Lin *et al.*, 2003). Akt inhibits AR transcriptional activity in low passage LNCaP cells but enhances AR activity in high passage LNCaP cells. Our experiments were restricted to low passage LNCaP cells (below 25 passages), consistent with the inhibitory effects of Akt on AR activity.

Another important observation made by Wang *et al.* is that expression of an inducible form of GSK-3 S9A in CWR22 cells (which originate from the same patient as CWR-R1 and 22Rv1 cells) inhibits their growth. This is in contrast to our observations that inhibition of GSK-3 reduces CWR-R1 and 22Rv1 cell growth. The difference might reflect the different methods used to determine cell number (cell counting versus MTT assay).

To summarize, we have used protein and chemical inhibitors of GSK-3 to show that GSK-3 activity is required for maximal activity of the AR. Importantly, our observations that inhibition of GSK-3 leads to a reduction both in AR protein levels and the growth of AR-positive prostate cancer cell lines suggest a novel therapeutic application for GSK-3 inhibitors, which are already under development for diabetes and neurological diseases, in the treatment of prostate cancer.

Materials and methods

Plasmids

GFP-Axin constructs, pOT-Luciferase and RSV-β-Gal have been described (Giannini *et al.*, 2000; Orme *et al.*, 2003). MMTV-Luciferase and pSG5 AR were gifts from Charlotte Bevan (Imperial College, London). pTER and pTERβi (van de Wetering *et al.*, 2003) were generously provided by Marc van de Wetering and Hans Clevers (Hubrecht Lab, Utrecht, the Netherlands). The pTER Control 1 siRNA plasmid expresses an siRNA with no known homology to human genes. It was generated using the following oligonucleotides (5′–3′): GATCCCCTTCTCCGAACGTGTCACGTTTCAAGAGAA CGTGACACGTTCGGAGAATTTTTGGAAA, GGGAAG AGGCTTGCACAGTGCAAAGTTCTCTTGCACTGTGCA AGCCTCTTAAAAACCTTTTCGA-5′. The pTER Control 2 siRNA plasmid expresses an siRNA to a human gene (NM_004626). It was generated using the following oligonucleotides (5′–3′): GATCCCCGGACTCGGAACTCGTC TATTTCAAGAGAATAGACGAGTTCCGAGTCCTTTTT GGAAA and GGGCCTGAGCCTTGAGCAGATAAAGTT CTCTT ATCTGCTCAAGGCTCAGGAAAAACCTTTTC GA.

The annealed oligonucleotides were phosphorylated using T4 polynucleotide kinase and ligated into pTER that had been cut with BglII and HinDIII and dephosphorylated using calf intestinal phosphatase.

AX2 (FlagAx-(501–560)), AX2P, FRAT and GSK-3β constructs (Smalley *et al.*, 1999; Franca-Koh *et al.*, 2002; Fraser *et al.*, 2002) were generously provided by Trevor Dale (Cardiff School of Biosciences, UK).

Cell culture and growth assays

Cell lines were from the American Type Culture Collection (Rockville, MD, USA), except for CWR-R1 cells (Gregory *et al.*, 2001), which were kindly provided by Christopher Gregory (University of North Carolina at Chapel Hill, NC, USA). Cells were grown at 37°C, 5% CO_2. COS7, HEK 293 and HCT 116 cells were grown in DMEM (Invitrogen) with 10% fetal bovine serum (FBS, Invitrogen) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, Sigma). LNCaP, PC3 and DU145 cells were grown in RPMI-1640 medium (Invitrogen) with 10% FBS. CWR-R1 cells were grown in Richter's improved MEM, Zn option (Invitrogen) with 20 ng/ml EGF, 10 mM nicotinamide, 5 μg/ml insulin, 5 μg/ml transferrin, 2% FBS and antibiotics. 22Rv1 cells (Sramkoski *et al.*, 1999) were grown in 1 : 1 RPMI/DMEM with 20% FCS. For experiments using R1881, cells were grown in phenol red-free medium containing 5% (LNCaP, HCT 116 and 22Rv1) or 2% (CWR-R1) charcoal-stripped serum (CSS, First Link Ltd, UK). R1881 (methyltrienolone, DuPont-NEN) was used at 1 nM or 10 nM and control cultures received an equal volume of carrier (ethanol). The GSK-3 inhibitors SB216763 and SB415286 were from Sigma and Biomol Research Labs Inc. (Plymouth Meeting, PA, USA), respectively.

Cell growth assays were conducted according to Gregory *et al.* (2001). Briefly, cells (1.5×10^5/well) were plated in 12-well plates (three wells were used for each condition) and allowed to attach overnight. Carrier or GSK-3 inhibitors were then added and, when indicated, R1881 (or carrier) was added 30 min later. Cells were collected by trypsinization at the indicated times and were counted using a Coulter Counter or using a haemocytometer.

Transfections

All cells were transfected in triplicate in six-well tissue-culture plates. Cells were incubated in serum-free Optimem-1 (Invitrogen) prior to transfection. Cells were transfected using 3.5 μl Plus reagent, 2 μl of lipofectamine and 1 μg DNA per well according to the manufacturers instructions (Invitrogen).

For transcription assays, each well of a six-well plate was transfected with RSV promoter-driven β-galactosidase (200 ng for prostate cancer cell lines, 20 ng for HCT 116 and HEK 293 cells), 300 ng pOT-Luciferase (or pOF-Luciferase, data not shown) or 400 ng MMTV-Luciferase. When necessary, the total amount of DNA was brought to 1 μg using empty plasmid DNA. The amounts of plasmid DNA transfected per well were 200 ng of pSG5 AR (or pSG5 vector as a control), 100 ng of GFP-Axin, GFP-Axin mutants, GFP-FRAT and GFP-FRATΔC (or GFP as a control), 600 ng of AX2, AX2P (or pcDNA3 as a control), 50 or 500 ng of GSK-3β constructs (or pcDNA1 vector as a control). For RNAi experiments, cells were first transfected with 1 μg pTERβi or pTER Control 1 or Control 2, and after 24 h they were transfected with reporter vectors together with 200 ng of pTER plasmids. For GSK3β inhibitor experiments, cells were transfected with the reporter plasmids only. In all transfections, after incubating with transfection reagents, cells were grown in their normal growth medium for 40–42 h, or in hormone-depleted medium for 18 h, after which R1881 or ethanol was added and cells were grown for a further 24 h.

Transcription assays

Cells were rinsed in PBS and lysed using Reporter Lysis Buffer (Promega). Luciferase and β-galactosidase assays were performed using the LucLite Plus (Perkin-Elmer Life Sciences) and Galacto-light Plus (Applied Biosystems) kits, respectively, according to manufacturer's instructions. Plates were read on a NXT TopCount Luminometer (Packard Bioscience) and values shown are luciferase activity normalized to β-galactosidase activity.

Cell extraction, immunoprecipitation (IP) and Western blotting

Cells were grown to 50–70% confluence in 100 mm dishes or six-well plates. Lysates were obtained using the following steps: cells were rinsed in cold TBS, lysed in modified RIPA buffer (0.5% deoxycholate, 1% Triton X-100, 20 mM Tris, pH 8.0, 0.1% SDS, 100 mM NaCl, 50 mM NaF) or Nonidet P-40 buffer (1% NP-40, 20 mM Tris, pH 8.0, 150 mM NaCl, 50 mM NaF) for 10 min and centrifuged for 12 min at 15 000 *g*. Cell extracts were then mixed with an equal volume of SDS sample buffer (Sigma Aldrich) and heated to 95°C for 3 min. For IP assays in transfected COS7 cells, cell extracts were prepared using NP-40 lysis buffer, incubated with primary antibody for 1 h on ice. This was followed by 30 min incubation with 20 μl protein A/G-agarose (Cambridge Biosciences) on a rotating wheel in the cold room. After four washes in lysis buffer and one wash in TBS, the beads were resuspended in 10 μl of SDS sample buffer and heated as above. For Western blotting, extracts and IPs were separated by SDS–PAGE, transferred to nitrocellulose membrane and incubated in blocking solution (3% Fraction V BSA, 1% ovalbumin in TBS-T (20 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween-20) for 1 h. After probing with antibodies and washing in TBS-T, antigens were visualized using chemiluminescence (ECL, Amersham Biosciences) and exposure to film. Each experiment was repeated at least three times and the results presented are representative.

Antibodies

Western blots were probed using antibodies at 1 : 1000 unless stated otherwise. The following antibodies were used for Western blotting: 9E10 mAb (Sigma Aldrich), P111A rabbit anti-AR (Affinity Bioreagents), anti-β-catenin mAb (Transduction Labs) and anti-γ-tubulin mAb (Sigma Aldrich). The following antibodies were used for IP: P110 rabbit anti-AR (Affinity Bioreagents) at 1 : 50, 5 μl anti-GFP polyclonal (Kypta *et al*., 1996) as a control, 2 μg 9E10 and 2 μg anti-GFP mAb (Roche) as a control. HRP-conjugated secondary antibodies (Jackson Laboratories) were used at 1 : 10000 dilution.

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