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Title : The interaction of wnt-11 and signalling cascades in prostate cancer

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THE INTERACTION OF WNT-11 AND SIGNALLING CASCADES IN PROSTATE CANCER.

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THE INTERACTION OF WNT-11 AND SIGNALLING CASCADES IN PROSTATE CANCER

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Abstract

Castration resistant prostate cancer proposes an array of issues in terms of treatment options. It is therefore necessary to decipher the underlying mechanism involved in androgen independent prostate cancer and neuroendocrine differentiation, which is associated with malignant and metastatic disease. Wnt-11 has been previously shown to be associated with the more malignant version of the disease by being involved in the cellular proliferation and differentiation of the cancerous cells. The methodology adopted to identify signalling pathways triggered upon Wnt-11 activation were inhibition of the JNK, PKA, PI3K and mTOR pathway with various concentrations of inhibitors, proceeding this proliferation, migration and gene expression experiments were carried out three times, each experiment containing a triplet of each condition. Results collected were significant in all experiments excluding the proliferation results involving the PKA pathway. A preliminary mechanism was established between Wnt-11 and the mentioned pathways with neuroendocrine differentiation. The experiments carried out along with the correlated data were novel and brings research one step closer to understanding the mechanism of androgen independent prostate cancer, which in turn can hopefully relay to new therapy options which are currently absent on the market.

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Chapter one: Introduction

Issues surrounding detection, management and prevention of prostate cancer have driven many debates amongst scientists across the world. The main objective for current prostate cancer (PCa) researchers is to define treatment options that are successful for both localised and metastatic disease.

The hot topic of prostate specific antigen (PSA) testing becoming legal within the UK still remains in question. According to the U.S. Food and Drug Adminstration, 4.0 ng/ml is described as the upper limit of PSA for PCa (Makarov *et al.*, 2009). An increased PSA level although it can be indicative of PCa, can also be linked to benign prostatic hyperplasia, urinary tract infections and prostatitis. This leads to the assumption that the PSA test is more sensitive than specific. On the other hand, the PSA test can neglect diagnosing PCa and cannot distinguish between aggressive and non-aggressive forms. A modification to the PSA test includes monitoring the PSA levels over a period of time. This is defined as PSA velocity, which is the change in PSA levels per year (ng/ml/year). There is therefore a need for more reliable biomarkers to generate more efficient and effective methods to screen and monitor the progression of PCa. An example of a biomarker that has been well characterized in PCa is the chromosomal rearrangement namely, the TMPRSS2-ETS gene fusion. The TMPRSS2 gene is an androgen responsive promoter, which when fused with the transcriptional factor from the ETS family can drive tumour progression under androgen influences. According to Makarov *et al.*, (2009) this gene fusion was present in 79% of 29 PCa cases. Although this biomarker has been statistically proven to be correlated with PCa, the time at which is presents itself it still unclear, (Makarov *et al.*, 2009).

Nonetheless, PCa still remains a major health risk for men especially over the age of 65 and according to (Wilt and Ahmed, 2013) the yearly death rate owing by PCa is more than 100,000 cases.

1.1 Pathogenesis of prostate cancer

Androgens are vital for the normal development of the prostate gland. Testosterone is primarily synthesised in the testes, however the adrenal gland also contributes to a small proportion of the total androgen amount. Androgens are metabolised by 5α-reductase, to dihydrotestosterone (DHT). It is DHT, which acts as the ligand and binds to the androgen receptor (AR) with a 5 fold stronger affinity that receptor than testosterone. The androgen receptor belongs to the steroid receptor family, made of four domains; N-terminal domain, central DNA binding domain, the hinge region and the C-terminal ligand-binding domain (Girling *et al.*, 2007).

Following interaction between AR and DHT, phosphorylation and homo-dimerisation of the AR occurs. The heat shock protein also dissociates from the AR, allowing the translocation of the AR towards the nucleus. Within the nucleus are androgen response elements to which the AR binds promoting androgen sensitive gene transcription. For transcription to occur, two activation function domains are essential, namely AF-1 and AF-2 (Girling *et al.*, 2007). The AR is unique in that the AF-1 domain can be activated in the absence of the hormone, which is supported by studies showing that hormone refractory cells were still activated with the presence of AF-1, even in the company of anti-androgens (Girling *et al.*, 2007).

The term hormone independent PCa cells may be misleading, as it implies the AR is no longer essential for growth. However, statistics show that more than 80% of hormone refractory PCa tumours still express the AR, and it's the AR overexpression in these cells that can be activated by adrenal androgens (Girling *et al.*, 2007). It is this phenomenon that causes the substantial levels of DHT which still remain after surgical or medical castration in PCa patients. Along with overexpression of the AR, over 70 point mutations have been identified in PCa tumours; these occur predominantly in the ligand binding domain (Girling *et al.,* 2007). These mutations can therefore allow a multitude of non-specific ligands such as oestrogen and anti-androgens to activate the AR. This causes the downfall of anti-androgen therapies and accordingly more specific treatment measures need to be investigated.

For the transcriptional activity of the AR, both co-activators and co-repressors are required. Upon ligand and AR interaction, the co-activator also binds to this complex; this causes the catalysis of histone acetyl-transferase, allowing a more open chromatin structure. This is essential for the transcriptional machinery to bind. P300 and Tip60 are two known co-activators the AR, which have been said to be up-regulated during androgen ablation. This could account for the transition to hormone independence (Girling *et al.*, 2007).

The ligand independent androgen receptor activation is the main concern for PCa treatment. Activation of the AR without a ligand induces its phosphorylation and acetylation producing a conformational change followed by transactivation. The main pathways which have been said to be involved in this process are the mitogen activated protein kinase (MAPK) and the phosphoinositol 3-kinase (PI3K) pathways. Hormones such as insulin like growth factor (ILGF) and epidermal growth factor (EGF) can instigate both pathways. ILGF can cause AR activation with a similar amount as to DHT. This may be due to the dephosphorylation of Serine 650 inhibiting AR nuclear transport (Girling *et al.*, 2007). Additionally, the loss of the tumour suppressor gene PTEN, is associated with cancer relapse and the inability to response to androgen ablation.

Interleukin 6 has also been studied, and can function to activate both MAPK and PI3K pathways, and its implications have been seen in PCa (Chang *et al.*, 2014). Up-regulation of this extracellular protein was seen in both prostate tissue and patient serum of hormone refractory disease (Chang *et al.*, 2014).

PCa stem cells have been implicated in PCa pathogenesis. They have been described to be negative for the AR, incurring resistance to androgen ablation. The survival mechanism of these stem cells allows them to proliferate causing an increase in tumour burden. The heterogeneity of cancer stem cells accounts for the different molecular characteristics of each individual tumour, therefore for successful treatment, the cancer stem cells need to be targeted. CD133 has been identified as a beneficial cancer stem cell marker, however the small

population of cancer stem cells limits the amount of marker profiling that can be carried out (Lawson *et al.*, 2005).

All the processes mentioned are unlikely to act independently and it's the heterogeneity of PCa that leads one to assume it's a multi-step process, involving multiple factors, which leads to the onset of PCa.

1.2 Prostate cancer treatment

The current treatments available on the market for PCa are suited mainly to the androgen dependent disease. First active surveillance is an option for those who have no signs or symptoms and have an indolent cancer and most likely contained within the prostate gland. Chemotherapeutic agents approved for the treatments of PCa include Docetaxel, Prednisone and Cabazitaxel. The most common treatment for PCa is the use of androgen ablation. This can take three forms. Firstly the entire testicular region can be removed via surgery to remove the main bulk of the androgen production. Secondly, anti-androgens can be administrated such as Flutamide and Bicalutamide, that bind to the AR, hold it in an inactive state and cause the recruitment of co-repressors, halting translation of androgen responsive genes. Thirdly, Leutinizing hormone antagonists can be used which acts on the hypothalamus pituitary axis, preventing the stimulation of androgen production.

In terms of castration resistant, Abiraterone Acetate can be used which inhibits the CYP17 enzyme which is responsible for the production of androgens (Kenneth *et al.*, 2009). Enzalutamide known as a hormone treatment blocks the signalling cascade which is activated once the androgen receptor is in contact with a ligand (Kenneth *et al.*, 2009).

1.3 Wnt proteins and their functions

Embryogenesis and adult homeostasis are just two of the many functions that cysteine rich glycoproteins, also known as the Wnt proteins carry out. However when aberrant Wnt signalling occurs, tumourgenesis can arise, an example being PCa. Two Wnt signalling pathways have been identified, namely the canonical and non-canonical pathway. A hallmark of the canonical pathway is the activation and stabilisation of a signalling pool of β-catenin. This leads to the transcription of many cancer genes in the nucleus by binding to a number of transcriptional factors. The

regulation of β-catenin gene expression is mediated by interaction of Wnt with the frizzled receptor and the co-receptor low-density lipoprotein receptor related protein (LEF-1). β-catenin can then translocate to the nucleus with both T cell specific transcriptional factor and lymphoid enhancing binding factor-1 (TCF/LEF-1), which are both part of the transcriptional factor family, to regulate target genes such as *c-myc* and *c-jun*. In the absence of Wnt signalling, a low level of β-catenin in the cell is maintained by ubiquitination and degraded by the destruction complex. The destruction complex constitutes adenomatous polyposis coli protein (APC), glycogen synthase kinase 3 β. (GSK3β), casein kinase 1 and finally the axin proteins. However, when initiation of Wnt signalling occurs, the destruction complex is inactivated thus accumulation of β-catenin occurs. This signalling pathway is demonstrated in the figure below.

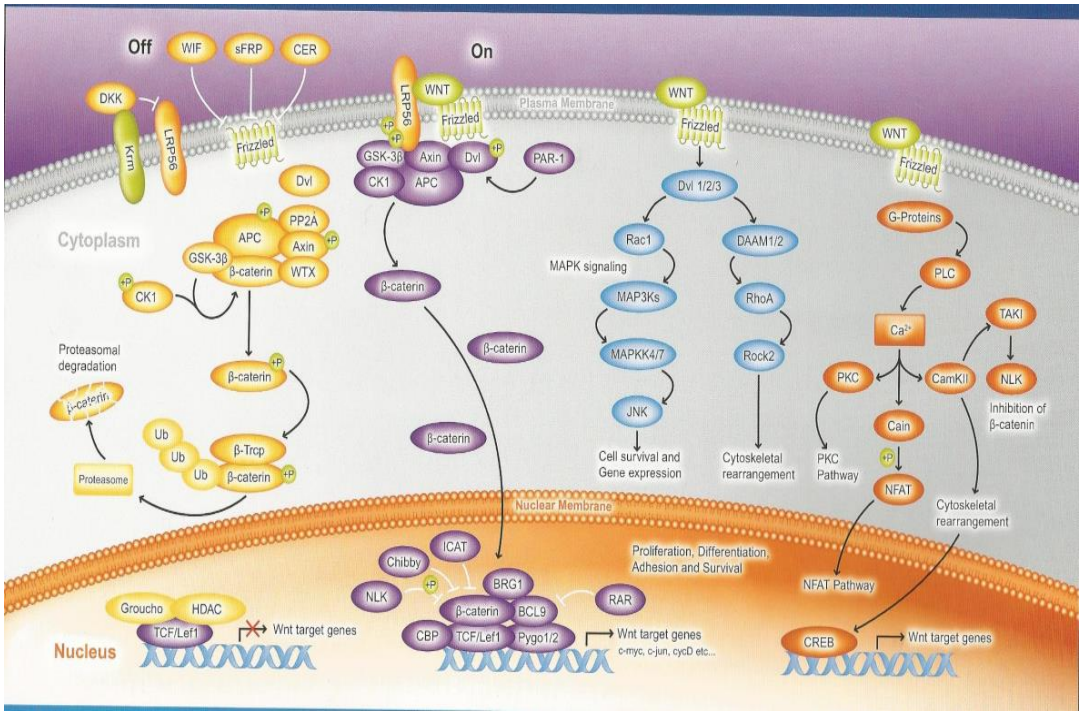

Figure 1: The inactive and active Wnt pathways. Image adopted from Abcam 2014.

1.4 Wnt-11 and prostate cancer

Wnt-11 is one of the 19 known Wnt proteins and belongs to the non-canonical signalling pathway, which involves initiation of other signalling cascades such as the planar cell polarity and the protein kinase C (PKC) pathway. In some cases, non-canonical pathways can antagonise the canonical pathway. The non-canonical pathway is less defined than the canonical pathway, thus more research is crucial.

The more clearly defined non-canonical signalling pathway is the Wnt/planar cell polarity signalling. Wnt-11 interaction with the Frizzled receptor can activate Dishevelled (Dvl) which is a cytoplasmic scaffold protein to the plasma membrane. This causes downstream signalling to occur which activates different features of cytoskeleton reorganisation in cell movement and polarity to occur. For example Daam1 and RhoA are activated which can lead to the activation of JNK (Wang, 2009). The PCP signalling pathway has been implicated in the metastasis of cancer and the formation of angiogenesis, thus its importance weighs heavily in cancer research.

The role of Wnt-11 in PCa is complex. For example, for cellular proliferation in tumours to occur androgens are essential, however androgens decrease Wnt-11 expression, yet a considerable amount of tumours still express high levels of Wnt-11 (Uysal-Onganer and Kypta, 2012). This could be the result of the fusion gene TMPRSS2-ERG formation. With regards to other cell lines, Wnt-11 has been shown to increase the survival of MCF-7, breast cancer cells and Chinese hamster ovary cells (Uysal-Onganer *et a*l., 2010).

According to Zhu *et al.,* (2004), there is a positive correlation between the expression of Wnt-11 and the malignancy of the disease. It has been reported that numerous *Wnt* genes were expressed in both normal prostate cells and cancerous prostate cells indicating that these genes play a role in the 'house-keeping' of the prostate gland (Zhu *et al*., 2004). However it has been said that expression of Wnt-11 alone is not sufficient to progress PCa cells to androgen independence and therefore other aberrations alongside Wnt-11 expressions are needed for this phenomenon to occur. Numerous theories surround the fact that Wnt-11 expression is increased in androgen-depleted LNCaP cells. For

example Wnt-11 may take part in the termination of cell growth following androgen depletion by the means of a signal produced by Wnt-11. If this is the case, the specific signal has yet to be identified. Another theory is that the increase in Wnt-11 expression in androgen depleted LNCaP cells is due to their differentiation. Following on from this, research carried out by Uysal-Onganer *et al.*, (2010) demonstrated that the transfection of LNCaP cells with Wnt-11 caused the morphology to undergo NED.

1.5 Neuroendocrine differentiation (NED)

Neuroendocrine cells constitute a population of cells found in the epithelial compartment of the prostate gland that are able to secrete factors to stimulate the survival and metastatic potential of cancerous prostate cells. They partake in the differentiation and growth of epithelial cells in the presence of androgens. The origin of cancerous NE cells still remains unknown however one theory states that they originate from the intermediate stem cells (Yuan *et al.*, 2007). Normal NE cells express cytokeratin 5, a basal cell marker. On the other hand NE like cancer cells expresses prostatic acid phosphastase and K18, both markers of luminal secretary cells. The current therapy of androgen ablation has been noted to increase the incidence of NED that is associated with poorer prognosis for PCa. As mentioned earlier, Wnt-11 expression inactivates androgen receptor expression in LNCaP cells; grouping this information with the fact that NED also leads to a loss of androgen receptors, one can assume that Wnt-11, whilst promoting NED, diminishes the androgen receptor indirectly. However this information is only relevant to the LNCaP cell line. The function of Wnt-11 in the onset of NED has been said to be restricted to PCa cells only (Uysal-Onganer *et al.*, 2010), as the ectopic expression of Wnt-11 in the RWPE-1 cell line, which is non-tumorigenic and exhibit characteristics similar to prostate progenitor cells, did not induce any NED. The commencement of NED is through a PKA dependent manner, thus the inhibition of this pathway theoretically should inhibit NED.

To summarise, Wnt-11 is involved in progression of PCa cells, migration into surrounding tissues and differentiation of these cells in neurone like cells.

1.6 Wnt pathway antagonists

Dickkopfs and sclerostin have been identified as inhibitors of the LRP co-receptor, meaning they are specific antagonists of the canonical pathway. Secreted molecules that inhibit both the canonical and non-canonical pathways are the Wnt inhibitory factor and the secreted frizzled related protein which both bind to Wnt in the extracellular space (Kypta and Kawano, 2003). However recently, both molecules have been said to be not pure antagonists of the Wnt signalling, and can actually participate in receptor activation (Kypta and Kawano, 2003).

1.7 Prostate cancer and the protein kinase C (PKC) pathway

The binding of the Wnt ligand to the Frizzled (Fz) receptor leads to a sudden increase in the concentration of intracellular signalling molecules such as inositol 1,4,5-triphosphate (IP3), 1,2 diacylglycerol (DAG), and Ca^{2+}. Both IP3 and DAG are resultants from the action of phospholipase C on the membrane bound phospholipid, phosphatidyl inositol 4,5-bisphosphate (Garg *et al.*, 2012). This results in the diffusion of IP3 through the cytosol towards the endoplasmic reticulum, where calcium channels are found causing a release of calcium ions. Both DAG and calcium ions activate two calcium sensitive proteins PKC and calmodulin dependent kinase II (CamKII) which then activate nuclear transcription factors such as NFAT, NFκB and CREB that transcribe downstream regulatory genes. As you can see, PKC is one of the main components of the non-canonical pathway involved in the Wnt/Ca^{2+} signalling and has been found to take part in promoting invasiveness and malignancy in human cancers (Garg *et al.*, 2012). Using small hairpin RNA to disorder individual PKC isozymes both *in vitro* and *in vivo* experiments demonstrated that depending on cell types and signalling pathways, PKC isozymes are either pro-apoptotic or anti-apoptotic. It is noted that tumour cells display an altered balance in PKC isozyme expression. For example the isoform PKCϵ has been seen to be elevated in high grade prostate tumours and is involved in the progression of the

tumour and the transition from an androgen dependent state to an androgen-independent state in LnCaP cells (Garg *et al.*, 2012). Interestingly, activation of JNK downstream of TNFβ was increased in PKCϵ depleted cells, whereas overexpression of PKCϵ decreased JNK activation, suggesting some kind of interlink between the two pathways. Therefore pharmacological targeting of PKCϵ within prostate tumours where PKCϵ is overexpressed can be a potential treatment.

1.8 Prostate cancer and c-jun N-terminal kinases (JNK) pathway

A member of the mitogen activated protein kinases (MAPKs) family is c-jun N-terminal kinases (JNKs). MAPKs can activate a variety of processes including cellular proliferation, tumourgenesis, differentiation and apoptosis. JNKs mode of action is to phosphorylate serine 63 and 73 at the N-terminal domain of c-jun. This in turn stimulates protein-1, which carries out transcriptional activity. C-jun is essential for the transition of the G1 phase to the remainder of the cell cycle. It also carries out a protective role against UV-induced apoptosis. At present there are only three distinct spliced JNK genes, JNK1, JNK2 and JNK3. JNK3 is primarily expressed throughout the nervous system, as opposed to JNK1 and JNK2 which are ubiquitously expressed. JNKs become activated during cellular stress due to exposure to UV, hypoxia, osmotic pressure and variation in cytokines and growth factors. Moreover, JNKs play a vital role in the inflammatory signalling network; therefore the hyper-activation of JNKs is not surprisingly associated with oncogenic transformation (Saadeddin *et al.*, 2009).

Reports indicate that less than an hour of JNKs activation relates to cell survival whereas more sustained activation of JNKs causes pro-apoptotic signalling. Moreover JNK participates in mRNA stabilization, cell migration, and integrity of the cell skeleton (Saadeddin *et al.*, 2009). Members of the JNK family are robustly involved in the regulation of certain transcriptional factors such as ATF2, stat3, c-myc and members of the Bcl-2 family. There is gathering evidence to show that JNK is involved in carcinogenesis and the blocking of JNK signalling with small molecule JNK inhibitors can be beneficial in the treatment of cancer. TGFβ-1 has been identified as a key activator of JNK1, which in turn

phosphorylates p21, a cell cycle regulator, and up-regulates its stability through a SMAD independent mechanism (Saadeddin *et al.*, 2009).

1.9 Prostate cancer and NFκB pathway

There is gathering evidence that NF-κB has a key function in tumour progression and chemotherapy resistance. Its function is to regulate numerous cytokines such as TNF-β, IL-8 and IL-1, cell adhesion molecules such as VCAM and cell cycle genes such as *c-myc* and *p53*. Interestingly, it has been shown that NF-κB can regulate gene expression involved in cell motility and invasiveness, an example being MMP-9. The activation of NF-κB can take two pathways: classical or alternative. The classical pathway is triggered by either viral or bacterial infections. The alternative pathway is activated by the members of the tumour necrosis factor family (Garg *et al.*, 2012). It has been reported that the isoform PKCϵ mediates the activation of NF-κB in PCa cell lines and there is an established correlation with the overexpression of PKCϵ and the constant activation of NF-κB in PCa cells. A transgenic PCa mouse model of PKCϵ overexpression triggered pre-neoplastic lesions with noteworthy hyper activation of NF-κB. With the use of interfering RNA (RNAi) targeting PKCϵ, the translocation of NF-κB to the nucleus was consequently depleted, impairing NF-κB transcription. Furthermore PKCϵ aids the assembly of the tumour necrosis receptor-1 signalling complex, which in turn activates NF-κB. Studies carried out by Garg *et al.*, (2012), demonstrated a molecular link between NF-κB and PKCϵ which play a pivotal role in PCa progression. On another note, the link between NF-κB expression and AP-1 in PCa cells may have something to do with the differences in sensitivity to TNF-induced apoptosis in PCa cell lines. To investigate, two cell lines were used: androgen dependent LNCaP cells and androgen independent DU145 cells. Results indicated that DU145 cells always expressed the activate form of NF-κB and AP-1, and thus were resistant to TNF induced apoptosis, whereas LNCaP cells were seen not to express NF-κB, and, therefore were susceptible to TNF induced apoptosis (Garg *et al.*, 2012).

1.10 Prostate cancer and the cAMP/protein kinase A pathway

Wnt11 has been reported to activate PKA and its interaction with cyclic AMP (cAMP). cAMP is a second messenger present in every cell and can direct a variety of functions such as cell growth, differentiation, ion channel activation, gene expression and apoptosis. The cAMP signalling pathway also interlinks with other intracellular signalling such as Ca^{2+} and Ras-mediated MAP kinase. cAMP best known mode of action is interaction with cAMP dependent protein kinases (PKA) (Caretta and Caretta, 2011). During pathological conditions such as PCa, the composition of the PKA holoenzyme or its localisation within the cell may change prompting abnormal effects. An important role of PKA is regulation of cellular proliferation by acting on transcriptional factors, an example being the prevention of the interaction between Ras and c-Raf, thus hindering its activation. The cAMP pathway is concurrent with Ras-activation through a negative feedback loop involving phosphorylation of phosphodiesterases, which diminishes cAMP concentrations within cells (Caretta and Caretta, 2011).

1.11 Prostate cancer and the Rho signalling pathway

The family of Rho proteins regulate the transition between an active GTP bound and an inactive GDP bound conformation. Both Rho and Rac are the most studied members of this family with Rho regulating the assembly of actin and myosin filaments while Rac initiates the actin polymerisation at the periphery of the cell. Therefore one can say Rho-GTPases are ideally positioned for non-canonical signalling such as the polarised reorganisation of the actin cytoskeleton, leading to cell movement. One of the best models providing genetic evidence for Rho GTPase participation in non-canonical Wnt signalling is in the organisation of the Drosophilia wing. In the wing, activation of the Fz receptor results in localised Rho activation. Alongside this, Rac activation by either the Fz receptor or Dsh causes JNK activation, initiating gene expression (Schlessinger *et al.*, 2009).

1.12 Prostate cancer and the phosphoinositide 3-kinase (PI3K) pathway

The phosphoinositide 3-kinase pathway plays a fundamental role in the initiation of malignancy and its progression. The initial signalling is activated by tyrosine kinase receptors or non-tyrosine kinase receptors' interaction with a ligand that then goes onto engaging with PI3K. PI3K then proceeds to convert phosphatidylinositol (4,5)-bisphosphate (PIP2), which is membrane bound to phosphatidylinositol (3,5)-bisphosphate (PIP3). This in turn activates and phosphorylates Akt. Akt translocates to the cytoplasm and nucleus, activating downstream targets (Sarker *et al.*, 2009). Akt has a multi-functional purpose in promoting cell survival regulating pathways that take part in the cell cycle, resistance to apoptosis, and interaction with a host of proteins including GSK3 (glycogen synthase kinase 3) and mTOR (mammalian target of rapamycin). mTOR is a serine threonine kinase which is involved in cellular proliferation and cell growth (Roccaro *et al.*, 2010). There are two known functional complexes of mTOR, mTOR1 and mTOR2. mTOR1 is said to be rapamycin sensitive whereas mTOR2 is not. PTEN, (phosphatases and tensin homolog) a tumour suppressor gene, is an obligate negative regulator of the PI3K/AKT/mTOR signalling pathway and is a crucial therapeutic target for cancer when signalling cascades out of control. Deregulation of this pathway can arise through a series of events. These include loss of function of PTEN through mutation, gene deletion or epigenetic silencing or mutation of AKT/PKB (Sarker *et al.*, 2009).

In 42% of primary prostate tumours, mutation of the PI3K/AKT/mTOR signalling pathway and its altered expression has been reported (Bitting and Armstrong, 2013). Androgens have been reported to cause the accumulation of TORC2 complex components and stress-activated protein kinase interacting protein-1 in the nucleus. This promotes the activation of AKT by TORC2 components. The loss of both Akt and PTEN is correlated with a poor clinical outcome for PCa patients, causing resistance to both chemotherapy and radiotherapy. Moreover, a loss of PTEN is associated with a raised Akt phosphorylation, and a higher Gleason grade. This can also promote castration resistant growth and a shorter time for metastasis to occur.

Furthermore, preclinical studies of the PI3K/AKT/mTOR pathway has highlighted its importance in maintaining the population of cancer stem cells population and promotes the transition of PCa cells from the epithelium to the mesenchymal (Bitting and Armstrong, 2013).

The PI3K/AKT/mTOR pathway interacts with other signalling cascades such as androgen receptor signalling and the RAS/RAF/MEK signalling. The human epidermal growth factor 2/3 kinases (HER2/3) inhibit androgen receptor signalling through the PI3K/AKT/mTOR pathway through a negative feedback system. Moreover, PTEN can enhance AR activity by supressing the transcription of c-jun and EGR1 which inhibit AR transcription. Therefore, one can say that the ability of PCa cells to survive in androgen deprived conditions is due to PTEN loss or the activation of the PI3K/AKT/mTOR pathway through targeted inhibition of the AR signalling.

1.13 Aims

This thesis will focus on the role Wnt-11 signalling takes in the onset of PCa, its progression and its malignancy. In particular the focus will be identifying signals activated by Wnt-11; the mechanism of its downstream signal transduction cascades; their interactions with one another, and whether the inhibition of certain pathways will decrease cellular proliferation, migration and neuroendocrine differentiation (NED). This can translate to novel and effective treatment options that can be utilised in a clinical environment.

1.14 Hypothesis

The hypothesis of this thesis is that targeting downstream signalling pathways of Wnt-11 such as the JNK, PKA, PI3K and mTOR pathway will have a negative effect on neuroendocrine differentiation, cell proliferation and migration. Thus providing a novel outlook on androgen independent prostate cancer treatment.

Chapter two: Methodology

2.1 Cell culture

PC-3 and LNCaP cell lines were both obtained from the American Type Culture Collection and were received as a gift from Imperial College London. Both PC-3 and LNCaP cell lines were maintained in RPMI 1640 medium accompanied with 10% fetal bovine serum (FBS) (Invitrogen), 1% penicillin streptomycin supplemented with L-glutamine, and 1% amphotericin B. Cell lines were grown in a Thermo Scientific incubator at 37°C with 5% CO_2.

2.2 Cell viability assays: MTT and SRB

MTT, (3[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide assay); both cell lines were seeded in 24 well plates at 5 x 10^4 cells per well. Cells were left to seed for 24 hours and various inhibitors were added in triplicates. The plates were once again left for 24 hours with the inhibitors and a control. The MTT solution concentration of 5mg per ml of PBS was filtered using a minisart high flow syringe filter. 50 µL of the MTT solution was added to each well and incubated for three hours. The media and solution was then removed and 400µL of DMSO was added to each well. The triplets were then transferred to a 96 well plate where the absorbance could be measured. The optical density was measured at 570nm using a 96 well plate reader.

SRB stain (Sulforhodamine B colormetric stain). Similar to the MTT cells were seeded at a cell number of 5 x 10^4 cells per well in a 24 well plate. 100µL of trichloroacetic acid (TCA) was added to the existing media to fix the cells to the plate and incubated at 4°C for 1 hour. The plate was then washed five times using tap water and oven dried until no standing moisture was seen. The TCA stained cells were then stained with 100µL of the SRB stain for 1 hour on a shaking platform. The dye was then washed three times with 1% acetic acid. The cell bound dye was re-solubilised in 10mM of tris solution, 300µL per well. Solutions were then transferred to a 96 well plate for the absorbance to be read at 570nm using the plate reader.

2.3 Qualitative RT-PCR

RNA was extracted from cells using Isolate RNA Mini Kit. The purity and concentration of RNA (μg/μl) was measured using a nanodrop. The amount of RNA needed to give a concentration of 1μg/1μl was calculated and reverse transcribed using Qiagen One-step RT-PCR kit.

2.4 cDNA Synthesis and PCR

For neuroendocrine markers such as *Neurogenin2*, *NeuroD1* and *DDC* the two step PCR method was adopted. The first step entailed using the BiolineTetro cDNA synthesis kit to create cDNA from various RNA samples. To amplify the desired DNA fragment the BiolineMyTaq Red DNA polymerase kit was used.

2.5 Gel electrophoresis

1% agarose gel was made with TAE buffer. 10μL of each sample was loaded with 2μL of loading buffer. The ladder used was hyperladder 100bp. Samples were run at 70V for 30-45 minutes. All negative blanks performed contained the buffer and water to show no contamination.

2.6 Migration assay

Cells were grown to a confluency of 80%-90% and trypsinised and re-suspended in 10ml of 1% FBS RPMI medium. Cells were counted using a haemocytometer and 10 x 10^5 cells were plated onto a 8μm pore size PET tract-etched membrane insert in a 24 well plate. The cluster well contained 1ml of 10% FBS RMPI with the upper chamber containing the cells and 500μL of 1% FBS RPMI. After 12 hours of incubation, the filters were washed in 1ml of 1X PBS, dried and stained in 1ml of Hoechst solution for 15 minutes and then washed thoroughly with PBS. The membranes were cut with a scalpel and placed onto microscopes. 20μL of DAPI loading buffer was added onto the membranes along with a cover slip. Glass slides were then kept in cold conditions covered in foil. Slides were examined using the Olympus fluorescence microscope.

2.7 Protein extraction

Control cells and cells with various inhibitors were grown to a confluency of 80-90% and incubated for 24 hours. 10% RIPA buffer was made in cold PBS and 10% of phosphatase was added. The cell culture dish was placed on ice and washed with ice cold PBS. The PBS was then drained and 1ml of the lysis buffer was added and kept on ice for 5 minutes. The adherent cells were scraped using a plastic cell scraper and then gently the lysate was transferred to a microfuge tube. This was then centrifuged at 14000g at 4 degrees for 20 minutes.

2.8 Inhibitors

The inhibitor used for the JNK pathway was JNK VIII (Calbiochem) working concentration 10μM. Inhibitors used for the PKA pathway were H89 dihydrochloride (Sigma-Aldrich) and KT5720 (Sigma-Aldrich) working concentration 10μM. The inhibitor used for the PI3K pathway was Ly294002 hydrochloride (Sigma-Aldrich) working concentration 1μM. The inhibitor used for the mTOR pathway was Rapamycin (Sigma-Aldrich) working concentration 1μM.

2.9 Statistical analysis

All methods were independently repeated three times, each experiment entailed a triplet of each condition. Error bars represent the standard error of mean. A paired student t-test was performed on all results and significance was measured as a p value <0.05.

Chapter three: The effect of different pathways on prostate cancer cell proliferation, gene expression and migration

3.1 Proliferation results for the PC-3 cell line

To assess whether inhibitors of the PKA, PI3K, mTOR and JNK pathway had an effect on cellular proliferation, initially two methods were adopted; the SRB stain and MTT. Table 1 presents the averaged normalised data over three experiments along with the standard deviation and standard error of mean for the SRB stain. Figure 2 illustrates the accumulation of three experiments to gain an average for the optical density of PC-3 cells without any treatment, PC-3 cells with a 10μM concentration of the PKA inhibitor KT5720 and PC-3 cells with 10μM of JNK inhibitor JNK VIII. Cells were plated at a cell number of 5×10^4 for each assay.

Conditions	PC-3 no treatment	PC-3 + 10μM of JNK VIII	PC-3 + 10μM of KT5720
Normalised value:	1.00	0.78	0.8
Standard deviation:	0.05	0.05	0.09
Standard error of mean:	0.03	0.03	0.05

Table 1: Normalised data collected for the SRB stain. Inhibitors used were JNK VIII for the JNK pathway and KT5720 for the PKA pathway.

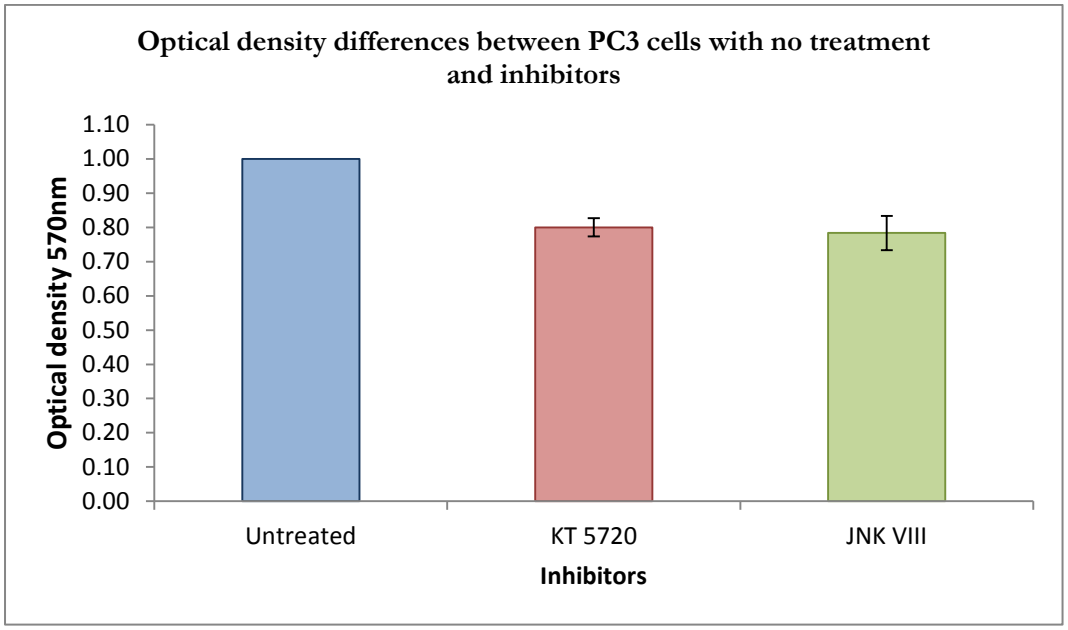

Figure 2: Optical density obtained from the SRB assay. (n=3, KT5720 20% reduction, p>0.05 ±0.05, JNK VIII 22% reduction ± 0.05).

Data collected did not give a p value less than 0.05, therefore the data was not significant. Therefore the SRB assay was changed to the MTT assay.

However when the MTT assay was adopted and the averages from three independent experiments were collected, a higher decrease in optical density was seen between the control and the addition of inhibitors. Table 2 presents the averaged normalised data for the MTT assay. Figure 3 illustrates the MTT data for the PC-3 cell line.

Conditions:	PC-3 no treatment	PC-3 + 10μM of Jnk VIII	PC-3 + 10μM of KT5720	PC-3 + Rapamycin 1μM	PC-3 + Ly294002 1μM
Normalised value:	1.00	0.6	0.7	0.61	0.47
Standard deviation:	0.07	0.15	0.13	0.107	0.05
Standard error of mean:	0.04	0.09	0.07	0.06	0.03

Table 2: Averaged normalised data collected for the MTT assay

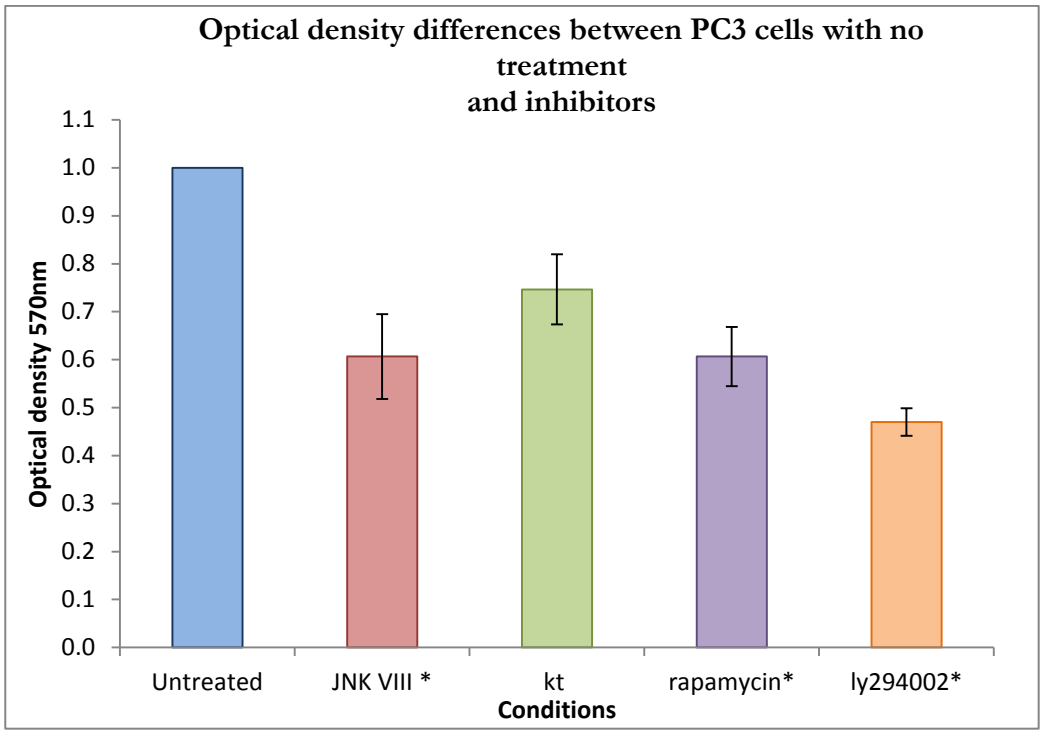

Figure 3: Optical density obtained from the MTT assay. N=3. (JNK VIII 40% reduction, p=0.02, ±0.09, KT 5720 30% reduction, p>0.05, ±0.07, rapamycin 40% reduction, p=0.03 ±0.06, ly294002 50% reduction, p=0.0004 ±0.03).

Figure 3 illustrates PC-3 cells plated at 5 x 10^4 cells under four conditions. The first condition was PC-3 cells incubated with 10μM of JNK VIII for 24 hours. This inhibitor gave a 40% reduction in PC-3 proliferation with a p value of 0.02. The next condition was PC-3 cells incubated with 10μM of the PKA inhibitor KT5720 that gave a 30% in PC-3 proliferation. As the p value was greater than 0.05 this particular experiment was not significant. The next condition was PC-3 cells incubated with 1μM of the mTOR inhibitor Rapamycin, which gave a 40% reduction in PC-3 proliferation and had a highly significant P value of 0.03. The final condition that gave the best decrease in PC-3 proliferation was PC-3 incubated with 1μM of Ly294002 (p=0.0004).

The MTT assay was also performed on the LNCaP cell line. The inhibitor, which was not significant when compared to the normalised data of the PC-3 cell line, KT5920 was replaced with another PKA

inhibitor, H89 when used on the LNCaP cell line. The table below presents the averaged normalised data collected for the MTT assay and the accompanying figure 4 illustrates the MTT results in a graph form.

3.2 Proliferation results for the LNCaP cell line

Conditions:	LNCaP no treatment	LNCaP + 10µM of Jnk VIII	LNCaP + 10µM of H89	LNCaP + Rapamycin 1µM	LNCaP + Ly294002 1µM
Normalised value:	1.00	0.6	0.57	0.39	0.31
Standard deviation:	0.26	0.14	0.13	0.07	0.08
Standard error of mean:	0.14	0.08	0.07	0.04	0.04

Table 3: Averaged normalised data collected for the MTT assay on the LNCaP cell line.

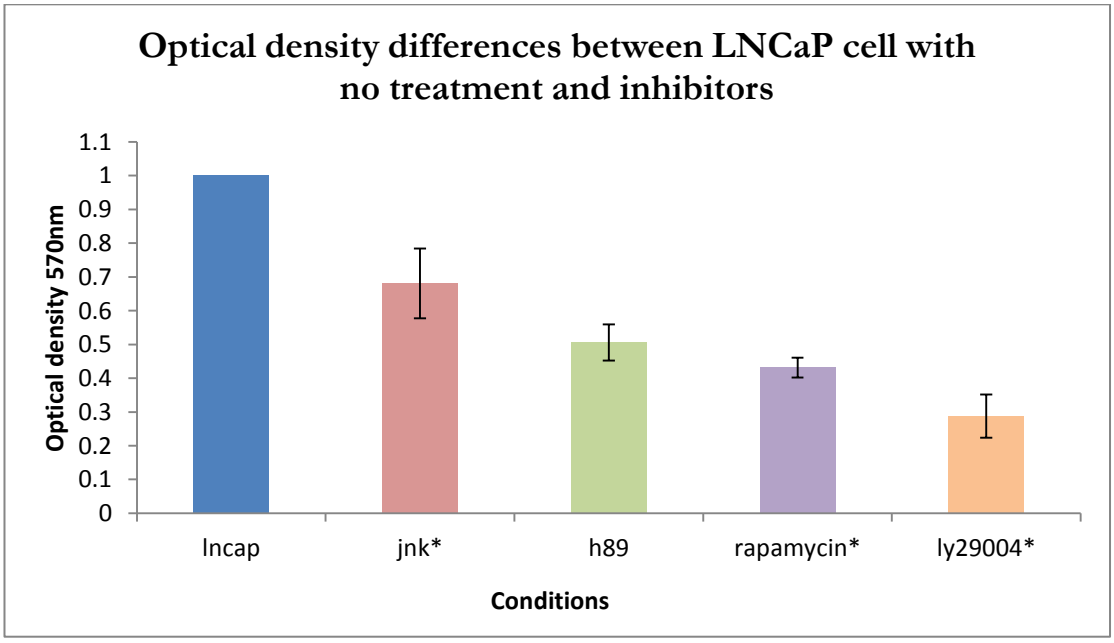

Figure 4: Optical density obtained from the MTT assay. N=3. (JNK VIII 32% reduction p value=0.003 ±0.08, H89 50% reduction, p value>0.05 ±0.07, Rapamycin 57% reduction, p value=0.01 ±0.04, Ly294002 72% reduction, p value=0.006 ±0.04).

Figure 4 illustrates the normalised data collected for the LNCaP MTT assay assessing the effect each pathway had on the proliferation status of these cells. First the JNK pathway inhibitor JNK VIII gave a 32% reduction in the LNCaP cell proliferation; this result was significant as the two-tailed t test provided a p value of 0.003. Next the PKA inhibitor H89 gave 50% reduction in the cell proliferation, however the p value did not produce a value less than 0.05 therefore that experiment was not significant. Rapamycin produced a 57% reduction in the LNCaP cell proliferation and this was significant as the p value was 0.01. Finally similarly to PC-3 cells, Ly294002 produced the best inhibition of LNCaP cell proliferation by 72% reduction with a p value of 0.006.

The inhibitors screened and used for the PKA pathway were KT5720, H89, pki-(myr-14-22)-amide and RP-cAMPS. Both pki-(myr-14-22)-amide and RP-cAMPS are not displayed in the graphs and an initial screening of pki-(myr-14-22)-amide and RP-cAMPS with the SRB stain provided an optical density higher than the control at the working concentration of 10μM. The inhibitors used for the JNK pathway were JNK VIII and SP600, both used at a working concentration of 10μM. However SP600 did not give a significant reduction in optical density when compared to the control, therefore SP600 was not used in further experiments. The inhibitors used for the PI3K pathway ly294002, working concentration 1μM and rapamycin for mTOR, working concentration 1μM.

3.3 Gene expression in the PC-3 cell line

As PCa cells have been previously shown to express high amounts of *Wnt-11* and the NED marker, neurone specific enolase (*NSE*), the gene expression of both these were assessed in control cells and cells treated with the various inhibitors. Cells were grown to 80% confluency, incubated with inhibitors and the RNA was extracted. The RNA was reversed transcribed using a one-step RT-PCR kit and the product was identified using gel electrophoresis.

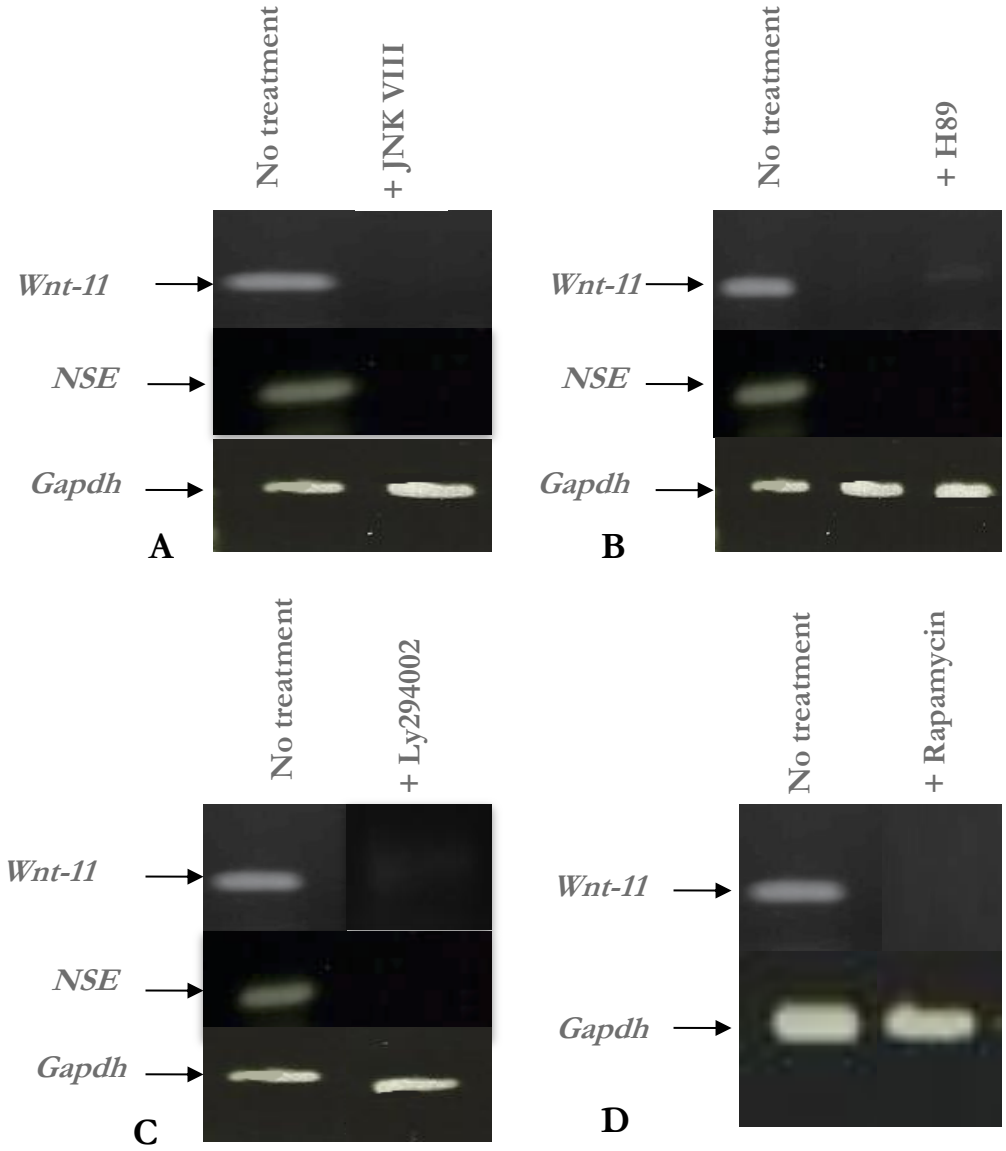

Figure 5A-D: RT-PCR gene expression in PC-3 cells. n=3.

Figure 5A illustrates the gene expression of *Wnt-11*, *NSE* and *Gapdh* in PC-3 control cells however when 10μM of JNK VIII was incubated with the PC-3 cells for 24 hours the band for *Wnt-11* and *NSE* is not seen. This suggests that the JNK pathway is involved in the mRNA expression of both *NSE* and *Wnt-11*. *Gadph* is used as a control throughout the RT-PCR experiments to ensure the mRNA levels are constant in all conditions. Figure 5B illustrates that with the addition of 10μM of H89 for 24 hours again the expression of both *NSE* and *Wnt-11* is diminished. The same applies for figure 5C whereby the addition of 1μM of Ly294002 for 24 hours also diminishes the expression of both *Wnt-11* and *NSE*. Finally with regards to figure 5D Rapamycin was used

only to assess the expression of *Wnt-11* and indeed it reduced the expression of *Wnt-11* compared to the control. These results do indeed suggest that the identified pathways do play a role in the expression of *Wnt-11* and *NSE*.

As targeting the PI3K pathway with Ly294002 has been successful for proliferation and gene expression analysis, a migration assay was taken forward.

3.4 PI3K involvement in PC-3 cell migration

The PC-3 cell line displays highly aggressive and metastatic characteristics, thus the approach to assess the involvement of the PI3K pathway in the migration of these cells was carried out. Initially experiments were carried out using various concentrations of ly294002 until the appropriate concentration was found. PC-3 cells were seeded at 10 x 10^5 cells per well and incubated for 12 hours. Duplicate trans-well filters were assessed for each condition. Analysis of the membranes with the fluorescence microscope included capturing 5 fields of view per membrane. Cells in each field of view were counted and averaged.

Preliminary results assessing the effect of 1μM of Ly294002 on the migrating PC-3 cells did not give a significant result, thus the concentration was increased to 2μM. An average of three experiments was carried out. The final inhibitory effect on the migration of PC-3 cells with the addition of 2μM of ly294002 over a 12 hour incubation period was 78%. Figure 6 below illustrates three random fields of view of PC-3 cells with no treatment and three random fields of view of PC-3 cells with 2μM of ly294002. The pictures were taken using a fluorescent microscope at a magnification of 10X and DAPI.

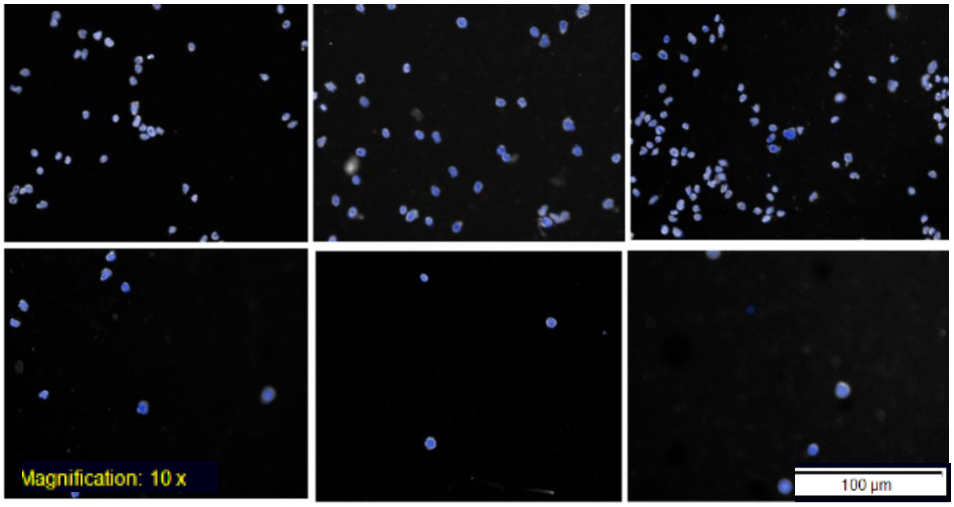

Figure 6: PI3K inhibitor, Ly294002 reduced cell migration of PC-3 cells after 12h incubation by 78%. Three random fields of view of migrating PC-3 cells for each condition were taken. The top three pictures show PC-3 cells with no treatment and the bottom three illustrates PC-3 cells incubated with 2µM of ly294002 for 12 hour. n=3.

To assess the proliferative effect of ly294002 at 2µM, a MTT assay was repeated with the same cell number over a 12 hour incubation period. Figure 6 illustrates that the difference in proliferation over 12 hours with this inhibitor is not significant therefore an ideal candidate for inhibiting migrating PC-3 cells.

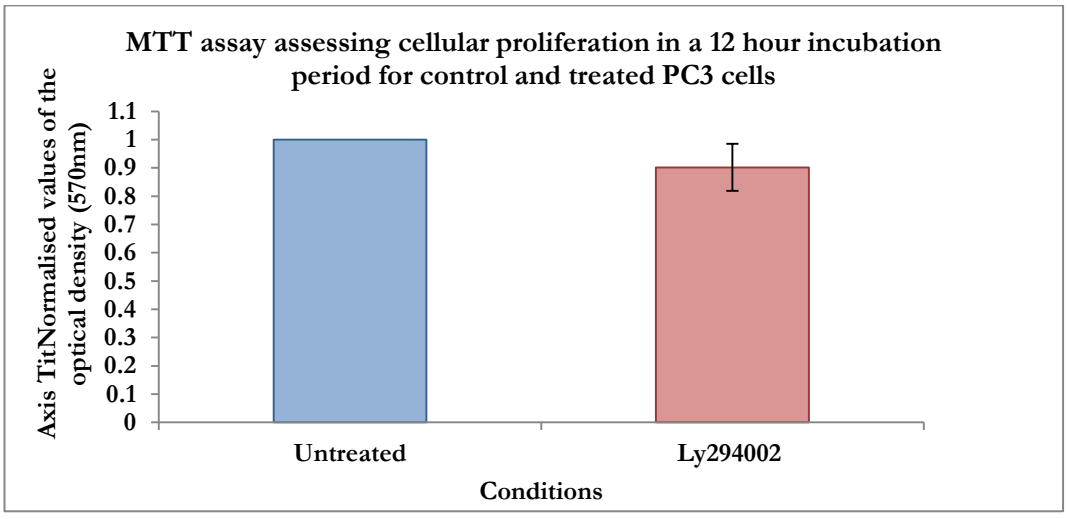

Figure 7: Cellular proliferation of PC-3 cells over a 12 hour incubation. Results are not significant. Ly294002 (9.8% reduction ±0.08, n=3)

3.4 Gene expression of Wnt-11 in the LNCaP cell line

Theory states that under normal conditions by which LNCaPs cells are cultured in 10% FBS RPMI, they do not express *Wnt-11*, as ectopic expression of *Wnt-11* can halt their growth and differentiation. It is only when LNCaPs are serum starved for 24 hours or more; *Wnt-11* expression is up-regulated. This is demonstrated in figure 8 below.

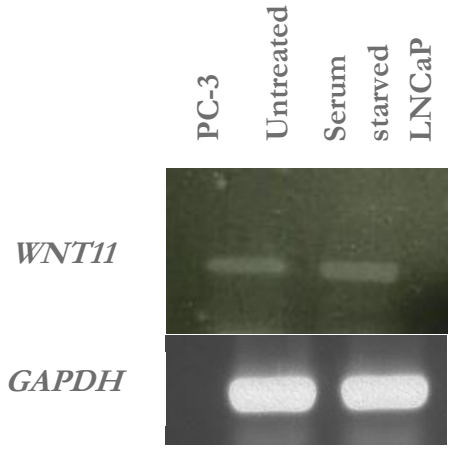

Figure 8: Gene expression in serum starved LNCaP cells and PC-3 cells. n=3.

3.5 Gene expression of Wnt-11 in the LNCaP cell line

To assess further the involvement of the PKA, JNK and PI3K pathway in neuroendocrine differentiation, three neuroendocrine markers' expression were assessed. The three markers are as follows; *Neurogenin2*, *NeuroD1* and Dopa decarboxylase (*DDC*).

Firstly the expression of Neurogenin2 in PC-3 cells with no treatment, PC-3 cells with 10μM of H89, PC-3 cells with 1μM of ly294002 and PC-3 cells with 1μM of rapamycin was assessed. The conditions of the cells were identical to previous experiments. The only difference was that rather than the one-step PCR reaction kit, a two-step PCR was undertaken, first cDNA was synthesised then the PCR reaction took place.

Figure 9 below illustrates the expression of *NeuroD1* under the 4 conditions.

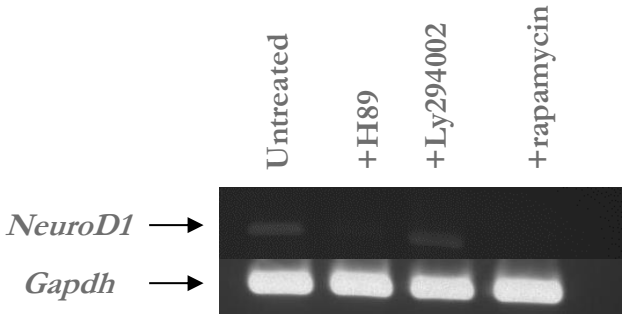

Figure 9: Gene expression of NeuroD1 and Gapdh in PC-3 cells under 4 conditions. n=3.

One can depict that under normal conditions, PC-3 cells express the band for *neuroD1*, however when the inhibitors H89 and rapamycin are added the band is diminished. On the other hand, it appears that the PI3K pathway inhibitor Ly294002 had no effect on inhibiting the expression of *neuroD1*.

Figure 9 below illustrates the expression of *Neurogenin2* under the 4 conditions.

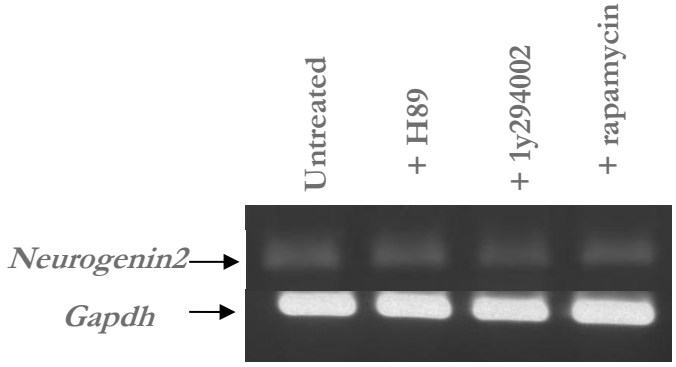

Figure 10: Gene expression of *Neurogenin2* and *Gapdh* in PC-3 cells under 4 conditions. n=3

Figure 10 demonstrates that there is no difference in the expression of *neurogenin2* in PC-3 cells under these four conditions. This may be due to the optimum conditions not being met, or perhaps these specific pathways are indeed not involved in the expression of this neuroendocrine marker.

Finally, the expression of *DDC* in PC-3 cells under the same conditions was assessed; the results are in the figure below.

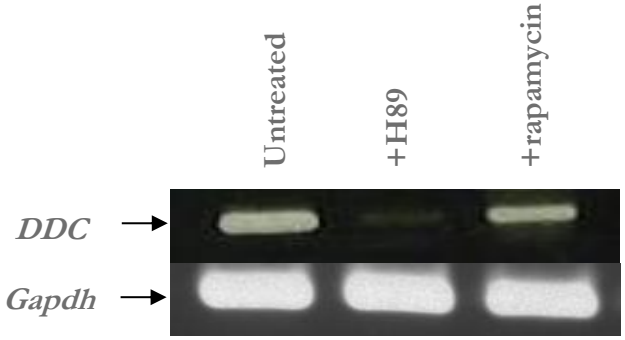

Figure 11: Gene expression of *DDC* and *Gapdh* in PC-3 cells under 3 conditions. n=3.

Figure 11 shows the expression of *DDC* under three conditions, control PC-3 cells, PC-3 cells with 10μM of H89 and PC-3 cells with 1μM of rapamycin. There is a notable reduction in the expression of *DDC* with the addition of both inhibitors, more so with the H89 inhibitor the PKA inhibitor, this agrees with multiply sources that the PKA pathway indeed plays a pivotal role in the onset of neuroendocrine differentiation.

Chapter Four: Discussion

4.1 The interaction of Wnt-11 and signalling pathways

The collected results throughout this thesis leads to the assumption there is a connection between the identified pathways, Wnt-11 and NED that is innovative and novel. The proposed mechanism is as follows; the activation of the PI3K pathway upon stimulation via tyrosine receptor kinases and non-receptor kinases leads to the dephosphorylation of GSK3β thus inhibiting its capacity to partake in the destruction complex that destabilises the accumulation of βcatenin. As the definition between the non-canonical and the canonical pathway is rather disjointed one can then go on to say that this disruption of the destruction complex can initiate the interaction of Wnt-11 and its frizzled receptor. This in turn can cause the downstream signalling of the PKA pathway. Accordingly this leads to the onset of NED which numerous literatures supplement towards and also the resulted collected in this thesis. Moreover, there is also documented evidence to suggest that both the PI3K and JNK pathway lead to the onset of NED.

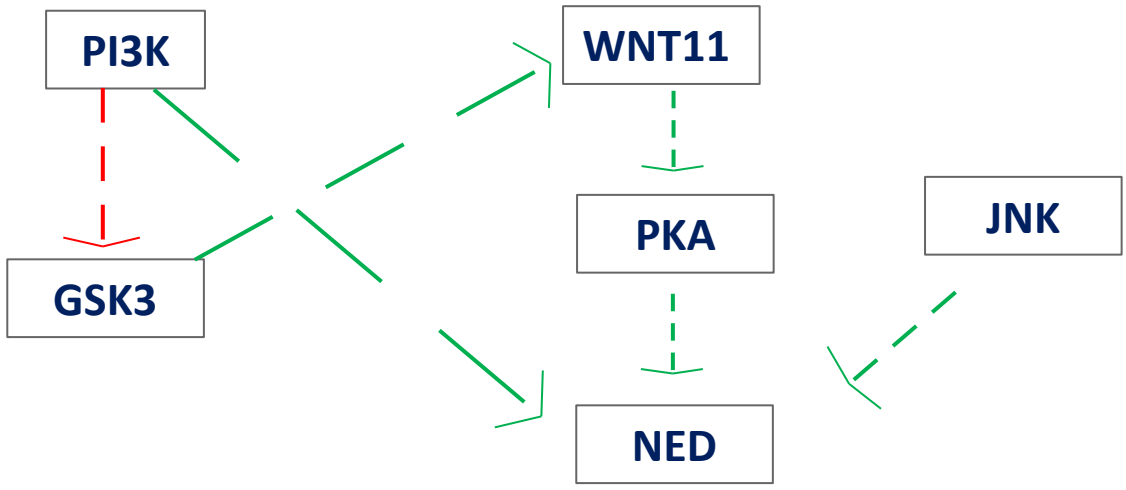

Figure 12: Schematic view of the involved pathways cross talk with Wnt-11 and NED. Red arrow = inhibition. Green arrow= activation. Dotted arrow=unconfirmed.

This preliminary mechanism holds immense importance in unveiling the interaction and cross talk between the studied pathways, Wnt-11 and NED. This could have the potential to stabilise a mechanism and can translate to targeting androgen-independent PCa, which at the moment has limited treatment options.

4.2 JNK pathway and Proliferation and NED

Figures 2-3 illustrate a significant difference in proliferation between the control PC-3 cells and PC-3 cells with10μM of JNK VIII in both cell lines. In fact the p value for the LNCaP cell line experiment was more significant than the p value for the PC-3 cell line experiment. It is well established that the JNK pathway is implicated in tumourgenesis as this data supports. However as JNK has been implicated in both survival and apoptosis its function is still very convoluted. The results collected in this thesis however highlight the importance of the JNK pathway in PCa survival as opposed to apoptosis. The theory to support this statement is that the activation of the JNK signalling cascade causes the transcription of members of the activating protein 1 (AP-1) transcriptional factor family such as *c-jun,* and j*unD*. It is these transcriptional factors that play a role in the proliferation of malignant cells. According to (Das *et al*., 2011) knockout of JNK in murine embryo fibroblasts hindered the proliferation capacity of the cells, thus highlighting the JNK signalling pathway in proliferation. This statement is in agreement with the in vitro experiments carried out in this thesis. What is interesting is that *c-jun* can interact with the AR enhancing its DNA binding ability. This also gives an explanation to why the JNK pathway plays a role in PCa proliferation. A study carried out by (Chen *et al*., 2006) demonstrated that the over expression of a mutant c-jun caused an increase in gene expression, cell proliferation and androgen regulated AR transactivation in the LNCaP cell line. Moreover over expression of si-RNA targeting c-jun diminished the growth of the LNCaPs. Numerous studies, journals, papers and pharmaceutical studies provide evidence that the inhibition of the JNK signalling can benefit therapeutic therapies (Zhang *et al*., 2012). Therefore the data collected regarding the involvement of JNK and PCa proliferation in this thesis is of high importance and can contribute to the developing studies.

Figure 5A illustrates that with the administration of 10μM of JNK VIII the expression of *NSE* is diminished in PC-3 cells. It is known that taking on the characteristics of a neuroendocrine tumour, there is a vast amount of activator and inhibitory receptors that PCa cells will begin to express. Therefore the activation of JNK by cytokine receptors may lead to the downstream event of NED. To emphasis, the data collected also produces a theory that indeed the JNK pathway plays a role in the onset of NED, as this idea is novel it is of great importance to carry this information forward and to further assess the involvement of the JNK pathway in other neuroendocrine marker expression.

4.3 The involvement of the PKA pathway in proliferation and NED

Unfortunately, targeting the PKA pathway with two different inhibitors (KT5720 and H89) did not give significant results with regards to the LNCaP and PC-3 cell line. The KT5720 inhibitor only gave a 30% reduction in proliferation in 24 hours in the PC-3 cells whereas H89 gave a 43% reduction in proliferation for the LNCaP cell line. Perhaps it is merely a case the PKA pathway not being heavily involved in PCa proliferation. On the other hand the concentration of both inhibitors (10μM) may not be optimum to produce a significant effect. PKA possesses two regulatory and two catalytic subunits. The regulatory subunits can be classified into 4 subtypes; RIalpha, RIbeta, RIIalpha and RIIbeta. The expression of each subtype is dependent upon the cell type and environment. For example in prostate carcinoma, a rise in RIIbeta is associated with the inhibition of tumour growth whereas a rise in RIalpha can enhance cell proliferation (Caretta and Caretta). Therefore the inhibitors used to target the PKA pathway may have targeted a regulatory unit which is not associated with PCa proliferation and was not present upon the time the experiment was carried out.

The PKA pathway has been linked to NED, (Deeble *et al.*, 2007) by the means of LNCaP cells acquiring neuroendocrine characteristics by increasing levels of cAMP by agents such as epinephrine and forskolin. More so, androgen ablation can incur an increase in intracellular cAMP. A study carried out by (Deeble *et al.*, 2007) demonstrated that the addition of tetracycline to the LNCaP cell line caused a constant activation of PKA which lead to an enhanced neuroendocrine

morphology and secretion of neuropeptides. Surprisingly, the serum starvation of LNCaP cells induced a 9-fold increase in cAMP levels and this could theoretically happen within a clinical environment with a patient undergoing androgen ablation, increasing the risk of NED. All this information supports the results that figure 5B, 9 and 11 illustrate. As indeed once the PKA pathway was inhibited for 24 hours the expression of important neuroendocrine markers such as *NSE*, *DDC* and *Neurogenin2* were reduced. Interestingly there may be crosstalk between the JNK and PI3K pathways that all contribute to the complete onset of NED, which this thesis aimed to investigate and produced a preliminary novel mechanism.

4.4 The involvement of the PI3K pathway, proliferation and NED

By looking at figure 2 one can see that 1μM ly294002 the PI3K inhibitor had the leading effect on reducing the proliferation of PC-3 cells by 50%. This runs parallel for the LNCaP cell line by which the same inhibitor at the same concentration of 1μM inhibited the proliferation by 70%. The p value was highly significant for the PC-3 cell line (**0.0004)** and for the LNCaP cell line (**0.006)**. These results are completely backed up by numerous studies and have established that PI3K signalling partakes in several processes such as cancer progression and metabolism, survival, growth and motility. Therefore this pathway is an ideal therapeutic target to inhibit these hallmarks of cancer. In terms of isoforms of PI3K, Class 1_A has been the most documented in carcinogenesis. This isoform is activated by stimulation of growth factors through tyrosine kinase receptors or G-protein coupled receptors. It is this isoform; the inhibitor Ly294002 targets. Therefore by targeting this isoform, stimulation of growth factors are inhibited inducing a haut in proliferation. Moreover, the activation of PI3K along with mTOR activation phosphorylates and disables 4E-BP1, which is a repressor of mRNA translation and leads to the stimulation of S6K. According to (Arcaro and Guerreiro, 2007) Akt, which is a downstream target of PI3K induces the inhibition of tuberin-mediated degradation of p27. This leads to an increase in CDK2 activity thus promoting cellular proliferation. Moreover the PI3K pathway plays a role in the cell cycle entry by the means of inactivating *FOXO* genes results in the silencing of

quiescent genes such as *p130* (Arcaro and Guerreiro, 2007). *FOXO* genes and PI3K work in synergy, when PI3K is active, *FOXO* genes are inactivated and vice versa. This leads to a uniform regulation of the cell cycle. Akt also initiates a signal allowing G1/S phase transition of the cell cycle. For example Akt inactivates GSK3β causing downstream inhibition of tumour suppressor tuberin. Akt also employs anti-apoptotic activity by preventing cytochrome C release from mitochondria (Arcaro and Guerreiro, 2007). Therefore mutant PI3K signalling can cause the constant activation of Akt, leading to a constant inactivation of tumour suppressor tuberin, leading to uncontrolled cell cycle progression and cellular proliferation. Therefore by targeting this pathway one can disable disorderly cell proliferation that is shown in figures 3-4.

Figure 5C illustrates that with the administration of 1μM of ly294002, the expression of *NSE* is diminished. This complies with theory as according to (Wu and Huang, 2007) the PI3K pathway is essential for the NED phenomenon occurring in PCa. The transition of androgen dependence to androgen independence is thought to have a connection with NED. Studies support this theory include transgenic mouse models of PCa showing NED to be more prominent in poorly differentiated tumours and after castration. It has been reported that LNCaPs as well as harbouring a point mutation in the *PTEN* gene, over expresses Akt upon androgen withdrawal. Experiments carried out by (Wu and Huang, 2007) illustrated that by targeting key components of the PI3K pathway such as PI3K and mTOR with their respective inhibitors; ly294002 and rapamycin the expression of *NSE* was significantly reduced in serum starved LNCaP cells. This correlates to data collected in the results chapter that show that the gene expression of *NSE* is reduced when 1μM of both ly294002 and rapamycin were added to PC-3 cells.

NE cells do not express the luminal marker of the androgen receptor. Thus with androgen ablation, these NE cells are able to escape therapy and begin paracrine networks leading to a proliferation increase, increasing tumour burden. More so, NE cells have been shown to secrete IL-8 and the non-NE cells express the IL-8 receptor, (Sun *et al.*, 2009). Hence, there is a definite need to target these cells As this thesis has data to demonstrate certain pathways which leads to the onset of

NED, therapeutically targeting them could stop the NE cells escaping therapy and therefore preventing metastasis which could in turn give an improved prognosis for PCa patients.

4.5 PI3K pathway and PC-3 cell migration

Cancer metastasis is due to the migration capacity that cancerous cells undertake. Therefore the idea of disabling the migration capacity of the PC-3 cells is an ideal target to prevent metastasis from the prostate gland. Figure 6 indeed illustrates that the PI3K pathway plays a critical role in the migration of PC-3 cells through a trans-well filter. This is demonstrated by the data analysis providing a 78% reduction in migrating PC-3 cells when 2μM of Ly294002 was administrated. There is documented evidence that the PI3K pathway plays a direct role in migration as its activation can disorganise epithelial polarisation leading to migration. It is the disturbance of this pathway that triggers actin structures alterations, stressing PI3K importance in cell motility. Activation of the PI3K pathway by intergrin initiates Rac activation. Rac is part of the Rho GTPase family and the fate of each Rho GTPase function depends on two factors, tissue specificity and lipid modification. Conferring to (Parri and Chiarugi, 2010), Rho proteins can regulate cell-to-cell adhesion, vesicle transportation, transcription and secretion of proteins. In terms of the process of cell migration, Rac can initiate new actin polymerisation and is an essential component of the lamellipodium extension that is involved in the networking of actin filaments. The PI3K pathway is key in initiating the Rac once this pathway is inhibited Rac activation is also blocked. These stresses the importance that PI3K should be targeted especially if it disables the migrating capacity of the highly aggressive PC-3 cells with a percentage as high as 78%.

It has also been documented that Akt can promote the production of metalloproteinase -9 (MMP-9) from malignant cells thus aiding not only the migration capacity of these cells but their invasion capacity. The PI3K pathway has not only been implicated in PCa, its role in migration has been recognised in breast cancer cell lines also. For instant, a study demonstrated that PI3K is constantly active and regulates the cell motility of breast cancer cells by activating the transcriptional activity of

NF-κB, (Arcaro and Guerreiro, 2007). Urokinase-type plasminogen activator (uPA) which has been shown to be strongly associated with cancer metastasis has a NF-κB binding site and it was exhibited that the extremely invasive MDA-MB-231 breast cancer cell line secreted copious amounts of uPA compared to the weakly invasive MCF-7 breast cancer cell line. Moreover the blockade of the PI3K pathway weakened the migration of the cells and also reduced the amount of uPA. There is increasing evidence that activation of NF-κB play a key role in the mechanism of tumourgenesis by protecting transformed cells from apoptosis. An experiment carried out by (Huber *et al.*, 2004) demonstrated that NF-κB along with oncogenic Ras can protect mammary epithelial cells from TNF-α initiated apoptosis and can induce epithelial to mesenchymal transition in the absence of TNF- α. To sum up the PI3K pathway not only has been evidenced to play a role in the PC-3 cell migration, it also has been documented in other cancers such as breast cancer. Therefore one can say that this pathway is key to be targeted in regards to migration and metastasis.

4.6 The involvement of mTOR pathway and proliferation

Rapamycin which targets mTOR1 had a vast effect on each cell line, 40% reduction for the PC-3 cell line and 60% reduction for the LNCaP cell line which is shown in figures 2-3. The reason to why rapamycin did not have the same effect on the proliferation as ly294002 is due to the difference in sensitivity to rapamycin between the two mTOR complexes, mTORC1 and mTORC2. mTORC2 is known to be insensitive to rapamycin and there has yet to be an identified inhibitor to target this complex due to the functions of mTORC2 not being fully described (Feldman *et al.*, 2009). Theory states that mTORC2 is thought to control growth factor signalling by phosphorylation of Akt. Akt is a key regulator in suppressing apoptosis and enhancing survival, thus the activation of mTORC2 may have the capacity to allow cellular proliferation and survival even in the presence of rapamycin. Upon administration rapamycin enters the cells and binds to its receptor FKB12, this complex then goes onto bind mTOR debilitating its function. The mechanism behind the growth control through mTOR signalling begins with regulating the synthesis of proteins and silencing

autophagy, (Geurtin and Sabatini, 2005). A downstream target of rapamycin sensitive mTOR complex is S6K1 and is thought to run translation by phosphorylating S6, a ribosomal protein. However it is not to say that the mTOR pathway does not play a role in the proliferation of PC-3 cells, as the p values were highly significant. Consequently dual therapy, which targets both the PI3K pathway and mTOR, may produce better-quality results in detaining both LNCaP and PC-3 cell proliferation.

4.7 Neuroendocrine differentiation markers

It is essential to identify improved neuroendocrine differentiation biomarkers within PCa to give a clearer view on the neuroendocrine phenotype, which is highly beneficial for the androgen independent disease. It can also be beneficial to assess tumour aggression and patient survival. Inactivation of these neuroendocrine markers may be hugely beneficial for PCa therapy. This thesis has focused on the expression of four main neuroendocrine markers; *NSE* which has already been covered, *Neurogenin2, NeuroD1* and *DDC*.

Firstly the expression of Neurogenin2 was assessed. Figure 10 demonstrates that control PC-3 do indeed expression *Neurogenin2* however with the administration of all the inhibitors there was no change seen in the mRNA expression. This can indeed be accepted, as the expression of *Gapdh* was constant. This data leads one to assume that the PKA, Ly294002 and mTOR pathway does not play a role in the expression of *Neurogenin2*. Perhaps under these conditions the concentration of each inhibitor was not optimum to produce a significant change in expression of this marker. However as the JNK pathway was not assessed in this particular experiment, perhaps it would be an idea to evaluate the role JNK has in the expression of *Neurogenin2*.

Second, the expression of *NeuroD1* was also assessed. Again, control PC-3 cells in 24 hours do indeed express *NeuroD1*, and with the addition of 10μM of the PKA inhibitor H89 the band has lessened. On the other hand, with the addition of 1μM of Ly294002 the expression of *NeuroD1* does not change. Perhaps indicating the PI3K pathway does not initiate the expression of *NeuroD1*. Finally the expression of *NeuroD1* is affected

by the administration of 1μM of rapamycin as the band is again diminished when compared to the control PC-3 cells. These results can be accepted as the expression of *Gapdh* is constant in all lanes. Therefore to sum up these results, the PKA pathway has been established to play a role in the expression of *NeuroD1,* along with the mTOR pathway. Again this highlights the multitude of roles that these signalling pathways undertake which the thesis focuses on which again are novel ideas.

Neurogenin2 gene encodes for a helix-loop-helix transcriptional factor that can determine the neuronal fate on ectodermal cells. They are indispensable to drive neuronal differentiation. Moreover it is also expressed during the development of the central and peripheral nervous system in neural progenitor cells. Both *neurogenin2* and *neuroD1* belong to the Atonal/Neurogenin family. *Neurogenin* targets multiply genes needed for neurogenesis, and the overexpression of this protein has said to lead cells towards a neuronal lineage in *Xenopus*, chicks and rats (Murai *et al.*, 2011). *NeuroD1* can be considered the central effector of Neurogenin's function and in the *Xenopus* and mouse model has similar transcriptional targets. Downstream signalling from the Notch receptor has been identified as the key pathway involved in maintaining the balance between progenitor safeguarding and progenitor differentiation. Upon activation, Notch initiates its downstream effector, namely members of the *Hes* family, in particular *Hes1* and *Hes5* in mammals. Members of this *Hes* family are negative regulators of neural differentiation as this demonstrated by overexpression of *Hes1* in mice inhibited neuronal formation (Murai *et al.*, 2011). On the other hand, *Hes6* works independently from notch signalling and is downstream of *neuroD1* and induces neurogenesis once overexpressed in mice (Murai *et al.*, 2011). *Hes6* has said to bind to notch regulated *Hes* genes such as *hes1*, thus preventing its inhibitory effect, allowing neurogenesis to take place. Below illustrates a systematic view of the interaction of Notch, *Hes1* and *Hes6* in the involvement of neurogenesis.

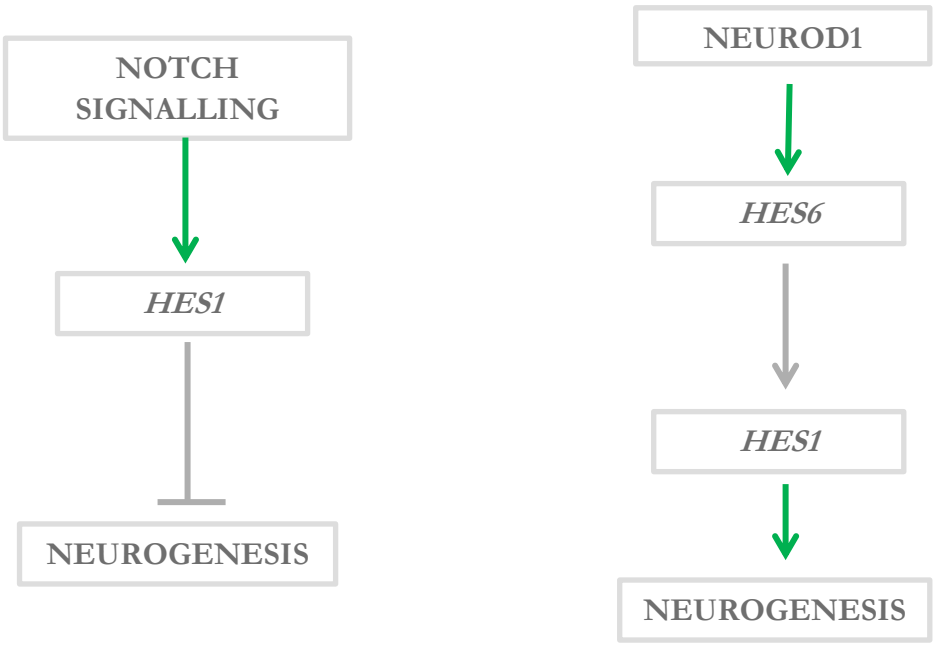

Figure 13: Notch signalling causes the downstream activation of Hes1 and this inhibits neurogenesis. Hes6 is independent from Notch signalling and it is downstream of NeuroD1, interacts with Hes1 disabling its inhibitory effect, allowing neurogenesis to take place.

Third, the expression of *DDC* was assessed. Figure 11 demonstrates that *DDC* is expressed in PC-3 cells, however when 10μM of H89 was administrated for 24 hours the band for *DDC* is reduced, again highlighting the importance of the PKA pathway in NED markers. However 1μM of Rapamycin did not produce a significant effect on the expression of *DDC*. Perhaps the expression of *DDC* is independent of the PI3K pathway.

DDC is considered a biomarker of neuroendocrine malignancies such as small-cell lung cancer and neuroblastoma. It can also be used as a biomarker for PCa as it can predict shorter disease free survival intervals and relapse. The human *L-DDC* gene found on chromosome 7, encodes for a pyridoxal-phosphate dependent enzyme which catalyses the decarboxylation of L-DOPA to dopamine and also 5-HTP to serotonin (Geomela *et al.*, 2012). Importantly, *DDC* is a co-activator of the androgen receptor by modulating the expression of AR regulated genes. *DDC* is involved in PCa cell proliferation under the influence of

androgens by the means of catalysing the production of biogenic amines, which contributes to both proliferation and differentiation (Geomela *et al.*, 2012). This thesis therefore stresses that the successful inhibition of DDC by 10μM of H89 not only can hinder the onset of NED but can also hinder the proliferation of the cancer, which is of great significance. Below figure 14 summarised the mechanism by which the mentioned pathways and the different neuroendocrine markers interaction which this thesis has gathered data for.

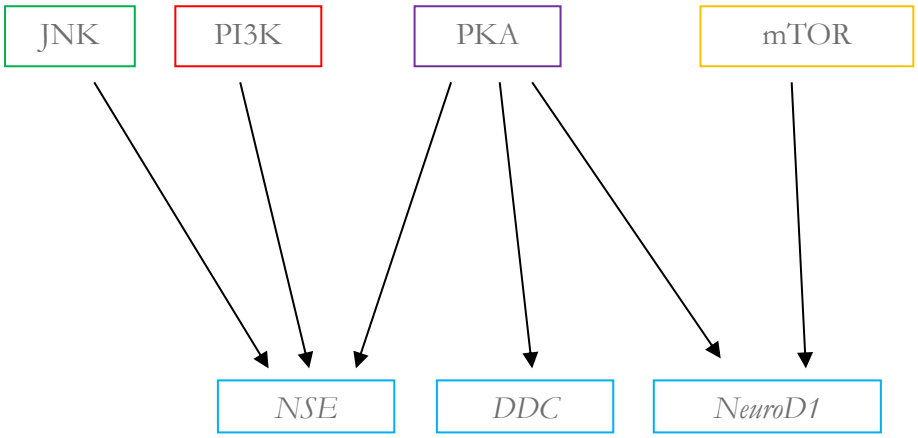

Figure 14: The interaction of the signalling pathways and neuroendocrine markers. Activation of the JNK pathway leads to the onset of *NSE* expression, activation of the PKA pathway leads to onset of *NSE*, *DDC* and *NeuroD1* expression, the activation of PI3K pathway leads to the onset of *NSE* expression. Finally the mTOR pathway leads to the onset of *NeuroD1* expression.

4.8 Androgen dependence to androgen independence

The initial intervention for PCa treatment is androgen ablation, however the downfall with this treatment occurs when the PCa cells transition from an androgen dependent state to androgen independent state. This could be due to several mechanisms due to different signalling cascades that this thesis has focused on. Firstly, PCa cells can survive in a low androgen environment by the means of AR amplification which leads to an improved occupied ligand receptor content. This is confirmed by 30% of androgen independent tumours expressing an amplified AR gene, (Feldman and Feldman, 2001). The survival of hormone refractory PCa cells through proliferation can be due to growth factors and the surrounding chemokine and cytokine milieu which can stimulate

the JNK, PI3K, PKA and mTOR pathway. This has been shown in the proliferation results whereby inhibition of the JNK, PI3K and mTOR pathway reduced the proliferation of two PCa cell lines. Moreover, an increased sensitivity to low levels of DHT could also result in cancerous cells still being able to proliferate in castrated men. Possibly the ability of PCa cells to convert testosterone to DHT by increasing the activity of 5α-reductase could lead to local production of androgens needed for stable proliferation. This was supported by the evidence that only 60% of DHT was reduced after androgen deprivation (Feldman and Feldman, 2001).

Some cases of castration resistant PCa have acquired gene mutations that can allow non-specific binding to the ligand binding domain of the AR causing malignant cells to thrive and avoid apoptosis. Non-specific ligands comprise of oestrogen, progestins and anti-androgens such as flutamide. It is thought throughout androgen ablation with the presence of a mutant AR can endure clonal expansion concurring a growth advantage. What's more, mutations can arise in the co-activators of the AR such as SRC1 and TIF2, when combined with AR mutations can lead to lethal AR activation (Feldman and Feldman, 2001). A vital signalling pathway that has been acknowledged for its role in PCa development is the PI3K/AKT/mTOR pathway. The tumour suppressor gene PTEN is often found to be deregulated in PCa thus aberrant PI3K signalling progresses leading to over activation of Akt. Akt is known to disable pro-apoptotic proteins such as procaspase-9, thus apoptosis can be avoided. Moreover Akt is known to phosphorylate over 50 substrates each involved in the hallmarks of carcinogenesis (Feldman and Feldman, 2001).

4.9 Further research

Firstly the protein expression of Wnt-11 and NSE need to be assessed with the addition of the mentioned inhibitors. The reason being is most biological functions are carried out at a protein level rather than an mRNA level. As most genes are regulated at a transcriptional level, other genes are involved with post transcriptional regulation processes such as the initiation of translation and the stability of proteins (Tian *et al.*, 2004). It is essential to understand the complexity of the changing

patterns amongst mRNA and how this correlates to their related protein. Therefore the western blot is an ideal technique to identify differences in protein expression under different cellular conditions. Moreover the phosphorylation status of the PI3K pathway should be assessed by either kinase activity assays or by the use of a phospho-specific antibody. In terms of focusing on the PI3K pathway, several other components of the pathway need to be weighed. For example the phosphorylation of Akt plays a heavy role in PCa progression and hormone independence. Moreover, invasion assays should be carried out to assess the ability of the mentioned inhibitors to destabilise the invasion capacity of PC-3 cells. Q-PCR could be carried out to give quantitative results of gene expression as opposed to only visual data.

4.10 Conclusion

The research carried out during this thesis had the aims to identify the association with the expression of Wnt-11 with the JNK, PKA, PI3K and mTOR pathway and the effects this had on cellular proliferation, migration and gene expression. These aims were successfully achieved and an interaction between *Wnt-11* and the mentioned pathways was identified which is novel and innovative. Moreover, the PI3K pathway had a definite role in aiding the migration of the PC-3 cells along with its proliferation which holds extreme importance especially with regards to PCa metastasis which causes the fatality of many patients. All pathways were seen to take part in the expression of *Wnt-11* and *NSE* collectively, which again is extremely significant as both markers are associated with aggressive and metastatic PCa.

To conclude Wnt-11 has been evidenced to play a role in the progression, differentiation and hormone independence characteristics associated with malignant PCa. There are definite interlinks which have been preliminarily examined between the mentioned pathways and signals produced from these pathways which initiate the phenomenon of NED. Wht-11 is therefore an ideal target for novel androgen independent PCa. Dual therapy will be more effective by targeting both Wnt-11 and one of the mentioned signalling pathways such as the PI3K pathway. This underlines the essence of personalised treatment that is heavily needed for the near future to equip against rare cases of cancers and to stop unnecessary complications with non-specific PCa treatments.

References

Amorino, G., Deeble, P. and Parsons, S. (2007). Neurotensin stimulates mitogenesis of prostate cancer cells through a novel c-SRC/Stat5b pathway. *Oncogene* **26**: 745-756.

Arcaro, A. and Guerreiro, A. (2007). The Phosphoinositide 3-Kinase Pathway in Human Cancer: Genetic Alterations and Therapeutic Implications. *Curr. Genomics* **5**: 271-309.

Bitting, R. and Armstrong, A. (2013).Targeting the PI3K/Akt/mTOR pathway in castration-resistant prostate cancer. *Endocr. Relat. Cancer* **20**: 83-96.

Bode, A. and Dong, Z. (2010). The functional contrariety of JNK. *Mol. Carcinog* **8**: 591-598.

Caretta, A. and Caretta, C. (2011).Protein Kinase A in Cancer. *Cancers* **3**: 913-926.

Chang, P., Wang, T., Chang, Y., Chu. C., Lee, C., Hsu, H., Zhou, T., Wu, Z., Kim, R., Desai, S., Liu, S. and Kung, K. (2014). Autophagy Pathway Is Required for IL-6 Induced Neuroendocrine Differentiation and Chemo-resistance of Prostate Cancer LNCaP Cells. *Plos one* **9**: 1-15.

Chen, S., Cai, C., Fisher, C., Zheng, Z., Omwancha, J., Hsieh, C. and Shemshedini, L. (2006). C-Jun enhancement of androgen receptor transactivation is associated with prostate cancer cell proliferation. *Oncogene* **25**: 7212-7223.

Deeble, P., Cox, M., Frierson, H., Sikes, R., Palmer, J., Davidson, R., Casarez, E., Amorino, G. and Parsons, S. (2007). Androgen-Independent Growth and Tumorigenesis of Prostate Cancer Cells Are Enhanced by the Presence of PKA-Differentiated Neuroendocrine Cells. *Cancer Res* **8**: 3663-3672.

Feldman, B. and Feldman, D. (2001).The development of androgen independent prostate cancer.*Reviews Cancer* **1**: 34-45

Feldman, M., Apsel, B., Uotila, A., Loewith, R., Knight, Z., Ruggero, D. and Shokat, K. (2009). Active-site inhibitors of mTOR target of

rapamycin resistant outputs of mTORC1 and mTORC2. *PloS Biol* **2**: 371-383.

Garg, R., Blando, J., Perez, C., Wang, H., Benavides, F. and Kazanietz, M. (2012).Activation of Nuclear Factor κB (NF-κB) in Prostate Cancer Is Mediated by Protein Kinase C ϵ (PKCϵ). The journal of Biological Chemistry **287**: 37570-37582.

Geomela, P., Kontos, C., Yiotakis, I., Fragoulis, E. and Scorilas, A. (2012). L-DOPA decarboxylase mRNA expression is associated with tumour stage and size in head and neck squamous cell carcinoma: a retrospective cohort study. *BMC Cancer* **12**: 1471-247.

Girling, J., Whitaker, C. and Neal, D. (2007).Pathogenesis of prostate cancer and hormone refractory prostate cancer. *Indian J Urol* **1**: 35-42.

Guertin, D. and Sabatini, D. (2005).An expanding role for mTOR in cancer. TRENDS in Molecular Medicine **6***:* 1471-4921.

Kenneth, P., Pienta, D., David, C. and Smith, M. (2009). Advances in Prostate Cancer Chemotherapy. A new era begins. *Advances in Prostate Cancer Chemotherapy* **55**: 300-318.

Kypta, R. and Waxman, J. (2012).Wnt/ β-catenin signalling in prostate cancer. *Nature Reviews Urology* **9**: 418-428.

Kypta, R. and Kawano, (2003). Secreted antagonists of the Wnt signalling pathway. *J. Cell Sci* **1**: 2627-34.

Lawson, D., Xin, L., Xu, Q., Cheng, D. and Witte, O. (2005). Prostate stem cells and prostate cancer. *Cold Spring Harb Symp Quant Biol* (**70**): 187-196.

Lories, R., Corr, M. and Lane, N. (2013). To Wnt or not to Wnt: the bone and joint health dilemma. *Nat.Rev.Rheumatol* **9**: 328-339.

Luyten, F., Tylzanowski, P. and Lories, R. (2009).Wnt signalling and osteoarthritis.Bone. **44**: 522-527.

Makarov, D., Loeb, S., Getzenberg, R. and Partin, A. (2009). Biomarkers for Prostate Cancer. *Annu.Rev.Med* **60**: 139-151.

Murai, K., Philpott, A. and Jones, P. (2011). Hes6 is required for the neurogenic activity of neurogenin and neuroD1. *Plus one* **11**: e27880.

Parri, M and Chiarugi, P. (2010).Rac and Rho GTPases in cancer cell motility control. *Cell. Commun. Signal* **8**: 1-31.

Roccaro, M,A., Sacco, A., Husu, E., Pitsillides, C., Vesole, S., Azab, A., Azab, F., Melhem, M., PhongQuang, H., Maiso, P., Runnels, J., Liang, M., Wong, K., Lin, C. and Ghobrial, I. (2010). Dual targeting of the PI3K/Akt/mTOR pathway as an antitumor strategy in Waldenstrommacroglobulinemia. *Blood* **115**: 559-569.

Rodriguez-Berriguete, G., Fraile, B., Martinez-Onsurbe, P., Olmedilla, G., Paniagua, R. and Royuela, m. (2011). MAP kinases and Prostate Cancer. *Journal Signal Transduction* **2012**: 2-11.

Saadeddin, A., Jadidi, R., Dene, B. and Nateri, A. (2009).The links between transcription, β-Catenin/JNK signalling and carcinogenesis. *Mol Cancer. Res* **7**: 1189-1245.

Sarker, D., Reid, A., Yap, T. and Bono, J. (2009).Targeting the PI3K/AKT Pathway for the Treatment of Prostate Cancer. *Clin. Cancer. Res* **15**: 4799-4805.

Schlessinger, K., Hall, A. and Tolwinski, N. (2009). Wnt signalling pathways meet Rho GTPases. *Genes & Dev* **23**:265-277.

Sun, Y., Niu, J. and Huang, J. (2009).Neuroendocrine differentiation in prostate cancer. *Am. J. Transl. Res* **2**: 148-162.

Tian, Q., Stepaniants, S., Mao, M., Weng, L., Feetham, M., Doyle, M., Yi, E., Dai, H., Thorsson, V., Eng, J., Goodlett, D., Berger, J., Gunter, B., Linseley, P., Stoughton, B., Aebersold, R., Collins, S., Hanlon, W. and Hood, L. (2004). Integrated Genomic and Proteomic Analyses of Gene Expression in Mammalian Cells. *Molecular & Cellular Proteomics* **3**: 960-969.

Uysal-Onganer,P., Kawano, Y., Caro, M., Walker, M., Diez, S., Darrington, R., Waxman, J. and Kypta,R. (2010). Wnt-11 promotes neuroendocrine like differentiation, survival and migration of prostate cancer cells. *Molecular Cancer* **9**:1186-1476.

Uysal-Onganer,P. and Kypta, R. (2012). Wnt-11 in 2011- the regulation and function of a non-canonical wnt. *Acta. Physiol* **204**: 52-64.

Wang, Y. (2009). Wnt/Planar cell polarity signalling. A new paradigm for cancer therapy.*Molecular Cancer Therapeutics* **8**: 2103-2109.

Wilt, J. and Ahmed, H. (2013). Prostate cancer screening and the management of clinically localised disease. *BMJ* **29**: 325-346.

Wu, C. and Huang, J. (2007). Phosphatidylinositol 3-Kinase-AKT-Mammalian Target of Rapamycin Pathway Is Essential for Neuroendocrine Differentiation of Prostate cancer. *The Journal of Biological Chemistry* **282**: 3571-3583.

Yang.Y., Bost, Frederic, B., Charbono, W., Dean, N., McKay, R., Rhim, J., Depatie, C. and Mercola, D. (2003). C-Jun NH_2 terminal kinase mediates proliferation and tumour growth of human prostate carcinoma. *Clin. Cancer. Res* **9**: 140-156.

Yuan, T. and Veeramani, S. and Lin, M. (2007). Neuroendocrine-like prostate cancer cells: neuroendocrine trans differentiation of prostate adenocarcinoma cells. *Endocrine-Related Cancer* **14**: 531-547.

Zhu, H., Mazor, M., Kawano, Y. , Walker, M., Leung, H., Armstrong, K., Waxman, J. and Kypta, R. (2004). Analysis of Wnt gene expression in prostate cancer: Mutual inhibition by Wnt-11 and the androgen receptor. *Cancer research* **64**: 7918-7926.

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