# Differential protein expression on the cell surface of normal epithelial and prostate cancer cells.

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## Abstract

Prostate cancer is the most frequently diagnosed cancer in men in Western countries. Clinically localized disease can be cured with surgery or radiotherapy, but once the disease has advanced or spread, there are no curative treatments. The aim of this thesis is to compare protein expression in a pair of normal epithelial and prostate cancer cells derived from the same patient and to determine the effect of interferon γ on this expression pattern. Proteins that are differentially expressed or change in expression in response to γ interferon might be of clinical value as biomarkers or therapeutic targets.

Biotin labelling followed by avidin chromatography was used to obtain membrane protein enriched lysates from a pair of cell lines derived from normal epithelium (1542 NPX) and prostate cancer (1542 CP3TX) from the same patient. These proteins were resolved and identified using SDS PAGE, coomassie staining and mass spectrometry. The same protocol was used to identify proteins differentially expressed on stimulation with interferon γ.

The proteins identified were subject to further analysis, particularly annexin II (AII), which was interferon-regulated. The expression of this protein was down regulated in the cancer cell line within four hours of interferon γ stimulation. This expression pattern was found to be cell surface specific as the total cellular expression of AII remained unchanged, as confirmed by immunofluorescence. AII down regulation also reduced the invasive potential of the cancer cells. Re-introduction of AII into LNCaP cells, which do not express the protein, led to an increase in their invasive capacity.

The mechanism regulating this cell surface specific effect was explored. It was discovered that inhibition of the ATP binding cassette transporter ABCA1 prevented surface AII expression. Protection of ABCA1 from calpain mediated degradation, maintained AII expression even in the presence of interferon γ. These findings led to the development of a model of interferon γ action on AII involving phosphorylation of ABCA1 as a signal for targeted degradation by calpain II.

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# Chapter 1

# Introduction

## 1.1 Cancer biology

Cancer, no other word in the English language has such an instant impact on our psyche. The actual definition of the word is "uncontrolled division of cells" (Soanes, 2003) with no reference to disease, which has led to many misunderstandings in clinical use of the term. Taken literally the word actually gives some indication of the origins of cancer, the normal process of cell proliferation. This realization indicates why cancers are so difficult to treat. Due to their common origin, the differences between normal and neoplastic cells are often so minimal that they are almost impossible to detect, monitor and exploit therapeutically. This has resulted in the treatment situation at present, where the majority of cancer therapeutics cause damage to normal tissue as well as the target cancerous tissue. A wealth of research activity is focussed on defining and exploiting these minimal differences between normal and cancerous cells to create discriminating therapeutics for clinical use.

To date medical science has achieved much in combating human morbidity in terms of communicable diseases, however, cancer remains one of the major causes of death in the developed world, affecting one in three people (Lewin 2000). Despite the prevalence of cancer, a concrete definition of what the disease actually constitutes remains elusive. The importance of clearly delineating such a definition cannot be underestimated, as substantial progress in the treatment or prevention of cancer cannot occur until researchers have identified the nature of the disease.

The potential for uncontrolled growth is a key feature of the diseases encompassed under the heading cancer (King, 2000). In normal tissue there is a harmonic balance between cell death and proliferation, with balanced regulatory interactions between stroma and cells and little to no competition between neighbouring cells (Bronchud, 2002). Genetic changes, however, can lead to inequalities in this balance subsequently resulting in uncontrolled growth. Such growth resulting in an expansion of cells without invasion into surrounding tissues occurs in both malignant and non-malignant

states such as benign prostate hyperplasia (BPH). Clinically, uncontrolled cell proliferation in solid tissue is classified in a number of ways (See Figure 1.1).

In addition to uncontrolled growth, cells must possess further attributes to be determined malignant and thus set apart from the other forms of tissue growth. One of the key attributes of cancerous lesions is the ability to invade and metastasize to other tissue. This is thought to be due to further genetic damage leading to a breakdown in the regulation of territoriality. In this situation the cancer cells are no longer in harmonic balance with neighbouring cells and begin to compete for space and nutritional resources (Bronchud, 2002). Clinically, cancer can be defined therefore, as a set of diseases characterized by unregulated growth leading to the invasion of surrounding tissues and potentially metastasis to other parts of the body (King, 2000). This definition is by no means all encompassing, but aids in the delineation of the prognosis expected with the clinical diagnosis of cancer. There is, however an obvious problem with using the clinical definition of cancer as a basis for scientific research into the origin of the disease, in that it defines the cancerous state by its end point. To define what makes a cell cancerous at a much earlier stage, where the widest treatment options are available, it is important to identify the processes that occur within the cancer cell which lead to a malignant end point.

| | |
|---|---|
| Hyperplasia | Increase in the number of cells in a tissue or organ without gross morphological changes. |
| ↓ | |
| Dysplasia | Disordered growth, changes in size, shape and differentiation. |
| ↓ | |
| Adenoma | Ordinarily benign neoplasm of epithelial tissue in which tumours form gland like structures. |
| ↓ | |
| Carcinoma | A malignant neoplasm of epithelial cells |

**Figure 1.1** Classification of tissue growth (King, 2000).

Despite a lack of concrete definition, over the last 50 years a wealth of research has examined the molecular basis of what makes a cancer cell different from its normal neighbour. Epidemiological studies examining the association between cancer susceptibility and age have indicated that between 4 and 7 mutations are needed for the development of malignant disease (Hanahan and Weinberg, 2000). This research began with the observation that with many solid tumours, disease incidence is proportional to age and that the slope of such a graph could be used to quantify how many mutations were required for carcinogenesis to occur. An example of this can be seen in an epidemiological study by Armitage and Doll where a graph of death rate versus age suggested that six mutations were required before carcinogenesis of the large intestine occurred (Armitage and Doll, 2004). Vogelstein and Fearon attributed this to sequential genetic events leading from colorectal adenoma to carcinoma and indicated that 5-7 mutations were required for such a transition (Fearon and Vogelstein, 1990). The results of these epidemiological and molecular studies were further verified by research into chemical carcinogens, which identified a long lag phase between exposure to a carcinogen and the appearance of a tumour. This lag phase can be observed clinically in epidemiological studies into human exposure to carcinogens such as asbestos, where people exposed form birth to the carcinogen had a mean diffuse malignant pleural mesothelioma appearance time of 55 years (Metintas et al., 1999).

Genetic changes or mutations, such as those required to initiate carcinogenesis, are a natural occurrence, constituting the driving force behind evolution. They are the result of a number of factors, both environmental and endogenous, with every organism having its own background rate of mutation due to errors in cell replication and additional genetic changes due to exposure to environmental mutagens (King, 2000). The prevalence of mutation in natural history and the research cited above confirm that it is not single mutational events that prove to be lethal through malignancy, but that malignant tumour formation is the consequence of several cumulative changes in the genomic DNA sequence (Hanahan and Weinberg, 2000). The acquisition of multiple mutations, needed for a cell to become malignant would be difficult to acquire in the timescale of human life, if the process was truly random (Hanahan and Weinberg, 2000). So how do such multiple mutations occur in a reasonably short timescales? How do these mutations bypass rigorous cell cycle checkpoints? The

answer to these questions is thought to lie in malfunctions of certain DNA caretaker pathways leading to increased genomic mutability.

Despite the many causative factors, genetic mutation remains a relatively rare event and it has long been argued that the high number of mutations seen in malignant cells cannot be accounted for by the low mutation rates of somatic cells (Bindra and Glazer, 2005). As early 1953 Slaughter et al proposed that the likelihood of the occurrence of mutations required to initiate carcinogenesis was greater in a clonal group of cells with a high rate of genetic alteration, than in a background of low somatic mutation, this was named the "field cancerization model" (Slaughter et al., 1953). This research led to the development of the genetic instability theory, which proposed that early changes in "caretaker genes", responsible for maintaining genetic integrity, initiated a level of genetic instability which increases the likelihood of further mutagenic events. Against this background of genetic instability it was proposed that mutations causing an increase in activity of certain promoters of carcinogenesis, so called "gate keeper genes" such as stimulators of proliferative activity, would lead to a cycle of unregulated proliferation and therefore the initiation of carcinogenesis (Bronchud, 2002).

### 1.1.1 Tumour suppressor genes

Tumour suppressor genes are key "caretaker genes" involved in malignant progression. These genes in their normal state halt the processes initiated by promoters of carcinogenesis (such as oncogenes), by suppressing cell growth and division and promoting programmed cell death. Commonly, both alleles of the tumour suppressor gene must be mutated to cause an effect, they are therefore, termed recessive loss of function mutations. An example of such a tumour suppressor is p53, identified in 1979 as a cellular protein which accumulated in the nuclei of cancer cells and was originally thought to be a transcription factor with weak oncogenic activity (Lane and Crawford, 1979). During the 1980's however it transpired that researchers were observing a mutant form of the p53 gene extracted from neoplasms and not the wild type gene (Hofseth et al., 2004). The main function of wild type p53 as suggested in the "guardian of the genome model" was to act as a sensor for DNA damage (Lane, 1992). Wild type p53 was found to be activated on induction of DNA

damage and once activated led to the transcription of G1 cell cycle arrest factors, halting cell cycle progression at G1 if DNA damage was present (Hofseth et al., 2004, Lewin 2000). Following cell cycle arrest wild type p53 was found to mediate DNA repair or apoptosis depending on the cellular microenvironment (Lane and Hupp, 2003). However since the conception of the guardian of the genome model, wild type p53 activation has been demonstrated in response to a number of cellular stressors including hypoxia, oncogene activation, spindle damage, temperature change and ribonucleotide depletion (Lane and Hupp, 2003, Hofseth et al., 2004). Wild type p53 has been found to perform a variety of functions including regulation of transition into S phase and of the spindle checkpoint, G2-M phase transition, apoptosis and angiogenesis (Quinn et al., 2005). In many tumours however, p53 is functionally inactivated therefore the sentinel for the monitoring of DNA damage, hypoxia and oncogene over expression is lost (Hanahan and Weinberg, 2000).

The gene for p53 is mutated in around 50% of human cancer cases worldwide (Hollstein et al., 1991). However these mutations tend to be misssense and not truncations or deletions, as seen in other tumour suppressor genes, indicating that selective pressures during the clonal evolution of the cancer cells have maintained a full length mutant form of p53 (Lane and Hupp, 2003). This conservation indicates that full-length mutant p53 may confer a selective advantage to cancer cells. Studies have indicated that misssense mutants of p53 gain oncogenic activity conferring a selective advantage to the expanding cancer cell population and explaining the initial mistaken identity of p53 as an oncogene (Blandino et al., 1999).

Mutation and functional loss of the caretaker protein p53, leads to genetic instability and may lead to oncogenic activity. The gene for this protein and others in the DNA damage repair pathway have been found to be mutated in many cancers (Hanahan and Weinberg, 2000, O'Byrne et al., 2000, Moul et al., 2002, Hobeika et al., 1998). Wild type p53 is not required for normal cell division, however the instability induced by the functional loss of p53 enables increased variability in daughter cells and hence increases the chance of creating new cells with a selective advantage. This explains the experimental findings observed with p53 knock out mice, which develop normally but have an inherited predisposition to a wide range of tumour types displaying aneuploidy and other genetic changes (Malkin et al., 1990). A lack of functional p53

may also explain the genetic instability seen in cells from patients with Li-Fraumeni syndrome or Blooms disease (Bischoff et al., 1990, Lu and Lane, 1993).

Genetic instability enables cells to acquire the six attributes required for the transition to a cancer phenotype at a much higher rate than would be expected via random chance (Hanahan and Weinberg, 2000). The functional inactivation of p53 is an obvious survival advantage for the tumour cell and it is unsurprising, therefore, that nuclear accumulation of mutant p53 is a common feature in human cancers including breast (Honkoop et al. ,1998), colon (Manne et al., 1998, Aprikian et al., 1994), lung (Mitsudomi et al., 1995) and prostate (Aprikian et al., 1994). Nuclear accumulation of mutant p53 has also been correlated with a poor prognosis in these cancers. In prostate cancer, comparison of cells from the primary site with those derived from metastases, suggest that cells with mutant p53 are clonally expanded in metastatic foci (Borre et al., 2000). Studies have demonstrated that an increased level of p53 nuclear accumulation is observed in more advanced stages of prostate cancer and has been found to be predictive of prostate cancer related death (Borre et al., 2000). This research supports recent findings that p53 not only has a role in early carcinogenesis but also in later, more advanced stages of the disease (Lane and Crawford, 1979).

In addition to p53 several other tumour suppressor genes have been associated with prostate carcinogenesis in both mouse and human models, including the retinoblastoma gene (Rb) and PTEN (phosphatase and tensin homologue deleted on chromosome 10) (Maddison et al., 2004). Rb was the first human tumour suppressor gene to be discovered, its tumour suppressor activity was first demonstrated in the rare paediatric eye tumour from which it derives its name (Friend et al., 1986). The cloning of the Rb gene was first achieved by Knudson, who following analysis of incidence rates in hereditary and non-hereditary retinoblastoma suggested that the disease was caused by mutation of both alleles (two-hit hypothesis) of an anti-oncogene (tumour suppressor gene) (Knudson, 1986). Rb encodes a 110 kD phoshoprotein which, like p53, regulates the cellular transition between G1 and S (Zhao and Day, 2001). The role of Rb at this stage is the transduction of growth inhibitory signals that regulate differentiation and growth, which is achieved through complex formation with transcriptional regulatory factors involved in the suppression or activation of transcription. This process is carefully regulated by cyclin dependant

kinases, through modulation of the phosphorylation state of the Rb protein (pRb) (Zhao et al., 1997). Inactivation of Rb either by genetic mutations, deletions or hypermethylation leads to the avoidance of senescence and therefore the expansion of the cellular lifespan (Jarrard et al., 2002). Inactivation of pRb extends the proliferative capacity of the cell, leading to uncontrolled cell growth. This avoidance of senescence via the inactivation of pRb by viral oncoproteins e.g. human papilloma or adeno viruses, has been exploited to immortalise cell lines for scientific research (Jarrard et al., 1999).

Loss of pRb expression has been found to be an indicator of poor prognosis in a number of cancers including non small cell carcinoma of the lung and bladder carcinoma (Quinn et al., 2005). In prostate cancer loss of pRb expression is seen in approximately 30% of tumours, however reports of loss of heterozygosity at the Rb locus in prostate cancer extend to 60% of clinical cases (Maddison et al., 2004, Quinn et al., 2005, Jarrard et al., 2002). Increased loss of Rb expression is correlated with increasing stage and grade in prostate cancer, in early disease 1-45% cases show loss of pRb, rising to 20-60% in later more advanced disease (Quinn et al., 2005). A recent study explored the role of pRb in prostate carcinogenesis and found that a single somatic allelic deletion of Rb in epithelial cells within a mouse model led to the development of focal hyperplasia, indicating that loss of Rb initiated proliferative prostatic disease (Maddison et al., 2004). The increase in loss of pRb with advancing stage in prostate cancer suggests that inactivation of Rb, like that of p53, has a role not only in carcinogenesis but also in later stages of the disease. A potential role for Rb in the regulation of apoptosis has been described in prostate cancer, although the mechanism is unclear (Zhao et al., 1997). It is proposed that a loss of pRb would allow prostate cancer cells to avoid apoptosis and as tumourigenesis is regulated in part by the balance of cell growth and death, this would lead to further unchecked proliferation (Zhao and Day, 2001). In metastatic disease a four-fold increase in proliferation is observed, when compared to the primary prostate tumour, which coincides with a loss of pRb expression in 34% cases. This suggests that loss of pRb expression is advantageous in the selection for aggressive clonal phenotypes that occurs in metastatic disease (Jarrard et al., 2002).

In addition to the loss of Rb expression, loss of PTEN (MMAC1) expression in prostate cancer is also correlated with advancing disease. The tumour suppressor activity of PTEN was indicated following genetic analysis of a section of chromosome 10 commonly found to be deleted in 90% human glioblastomas, in this study 60% of glioma and 33% prostate cancer cell lines possessed mutations in the PTEN gene (Steck et al., 1997). Subsequently PTEN deletion has been observed in a number of solid tumours such as those of the breast (Li et al., 1997) and endometrium (Tashiro et al., 1997). Confirmation of the functional tumour suppressor role of PTEN came from the in vitro observation that over expression suppresses tumour colony formation and the in vivo observation that over expression suppresses tumour formation in nude mice (Di Cristofano et al., 1998). In addition, it was discovered that patients with cancer predisposition syndromes such as Cowden's disease or Bannayan-Zonana syndrome had germ line mutations of PTEN (McMenamin et al., 1999). The consequences of germ line inactivation of PTEN were analysed using PTEN knock out mice which develop tumours similar to those found in Cowden's disease including breast, thyroid and prostate tumours (Di Cristofano et al., 1998). PTEN is thought act as a tumour suppressor by inhibiting cell cycle progression and inducing arrest at G1 through negative regulation of the P-I-3 kinase/Akt signalling pathway (Furnari et al., 1998). The loss of PTEN expression, like many other tumour suppressors is associated with mutation, deletion or hypermethylation of the gene (McMenamin et al., 1999).

Although much is now known of the tumour suppressor role of PTEN, little is known of the role in metastasis suppression it appears to display in prostate cancer. Recently PTEN was found to interact with Drg-1, the product of which has been found to have a metastasis suppressor activity in colon and prostate cancers (Bandyopadhyay et al., 2004). Both molecular and immunohistological findings suggest that PTEN and Drg-1 expression are inversely correlated with prostate and breast cancer progression and that in combination these markers are a significant predictor of patient survival (Bandyopadhyay et al., 2004). Traditionally loss of expression of a tumour suppressor protein is considered an early event in carcinogenesis, however in contrast with the findings of germ line PTEN mutation in mouse models, loss of PTEN appears to be a relatively late event in prostate cancer. Immunohistological studies discovered that PTEN was expressed in all prostate intraepithelial neoplasias (the alleged precursor of

prostate cancer). In addition 64.2% of prostate cancer specimens showed heterogeneous staining and loss of PTEN expression was found to correlate significantly with the presence of high-stage disease (Gleason score >7) (McMenamin et al., 1999). Loss of PTEN expression has also been correlated with other indicators such as PSA and Gleason grade, to assess the suitability of PTEN loss as a prognostic indicator. In this study, 86% of advanced cases displayed a loss of PTEN expression, compared to 61% of cases with localised disease and 58% of cases with benign prostate hyperplasia. Statistical analysis of these results suggested that loss of PTEN could be utilised as an independent marker to follow the progression of prostate cancer (Koksal et al., 2004).

### 1.1.2 Oncogenes

Certain key "gate keeper genes" involved in controlling cell division have been isolated that when mutated, promote malignant progression. Proto-oncogenes (called oncogenes in their altered state) code for proteins associated with the promotion of regulated growth in their normal state, this group includes cell division, growth receptor and cell cycle regulatory proteins. Mutations within these genes often confer on the cell the ability to initiate growth without the appropriate stimulus leading to a cycle of deregulated growth. Such changes need only occur within one copy of the gene to have effect and are therefore, called dominant gain of function mutations. There are four main functional groups of oncogenes, protein kinases (src, erbB), nuclear signal transducers (myc, fos), regulators of cell growth (sis, fms) and guanine nucleotide binding proteins (ras gene family) (Shih et al., 1986).

The ras family of oncogenes were among the first to be discovered, found following research into the cellular homologues of the transforming genes of Harvey and Kirsten retroviruses (Bar-Sagi, 1989). The ras gene family encodes a group of closely related 21kD proteins with an affinity for guanine nucleotides and are therefore part of a larger family of guanine binding proteins (G proteins). G proteins are commonly involved in signal transduction, Ras proteins are thought to have a functional role in the transduction and mediation of growth control signals (Shih et al., 1986). The first mutationally activated, oncogenic versions of the ras genes were identified in human tumours in 1982 (Goldfarb et al., 1982, Pulciani et al., 1982, Shih and Weinberg,

1982). Subsequently, members of the ras gene family have since been found to be mutated in many cancers (Lewin 2000, Hanahan and Weinberg, 2000). The three most commonly mutated ras genes are HRAS, KRAS and NRAS with almost 50% of cancers showing mutation of one or more of these genes (Cox and Der, 2002, Stearns et al., 2005). ras genes are highly conserved, the amino acid sequence of human HRAS is identical to is murine homologue and similar to the form found in yeast (Shih et al., 1986). This high degree of sequence conservation suggests a critical cellular role for the ras family of proteins, this has been confirmed by analysis of single point mutations at key amino acid residues which decrease the oncogenic activity of ras in a number of human tumours (Shih et al., 1986).

Mutations of HRAS, KRAS and NRAS are observed in 10% of prostate cancers (Stearns et al., 2005). The functional significance of attenuation of ras expression in prostate cancer has been explored in the androgen receptor positive cell line LNCaP. In this cell line expression of mutationally activated Ras increased the proliferative response of the cells to androgens, thus decreasing their androgen dependence (Bakin et al., 2003b, Bakin et al., 2003a). In xenografts of LNCaP cells carrying a non–oncogenic mutant of ras, androgen sensitivity was restored and complete regression of the tumour could be achieved through castration of the animal in 80% of cases with the remaining 20% exhibiting stasis in tumour growth (Bakin et al., 2003a). Androgen independence is an almost inevitable consequence following treatment of prostate cancer with castration or anti-androgens. Increases in autocrine and paracrine growth factors such as EGF, TGF-α and IGF-1 are among the most commonly reported changes in the progression of localized prostate cancer to androgen insensitive and/or metastatic disease (Culig et al., 2002). ras activation is a component of the signalling cascade for virtually all the growth factors found to be up regulated in prostate cancer. It is thought that ras acts as an intersection point for the diverse receptors and ligands involved in growth signal transduction (Weber and Gioeli, 2004). In agreement with the view that as prostate cancer advances, growth signalling increases, a recent study found that an increase in expression of growth factor receptors and indicators of ras activity correlated with increasing stage and grade of tumour (Weber and Gioeli, 2004). The indication from this and other studies is that mutationally activated Ras proteins heighten both growth factor and androgen receptor activity decreasing the dependence of prostate epithelial cells on androgen for proliferative growth. This is

confirmed by clinical observations of androgen dependent tumours with little to no indication of ras activity, becoming positive for ras activity following disease recurrence after hormonal treatment (Weber and Gioeli, 2004, Bakin et al., 2003a).

In addition to its proposed role in androgen independence in prostate cancer, mutationally activated ras has also been implicated in the enhancement of the pro-angiogenic response to VEGF. In a recent study activated HRAS has been found to enhance VEGF production in response to IGF-1 in sufficient amounts to significantly increase endothelial migration (Stearns et al., 2005). The above studies suggest that molecular cooperation between mutationally activated ras proteins and various cellular receptors mediates a hypersensitivity to both pro-growth and pro-angiogenic signals, both of which are selectively advantageous in tumourigenesis.

### 1.1.3 The multifactoral nature of cancer

Oncogenes and those genes involved in tumour suppression have an important role in malignant progression, there are, however, other changes which must occur for a cell to obtain tumourigenic potential. Research into the genetic changes required for a cell to become cancerous, has led to the characterisation of key attributes acquired by cells during this progression to a malignant status. It has been indicated that a complex collection of cancer genotypes can be characterised by six common alterations that collectively dictate malignant growth. The progression of pre-malignant cells to possession of all six traits is not the same in all cancers but they share a common end point (Hanahan and Weinberg, 2000) see Figure 1.2.

The first of these traits characterised is the ability of cancer cells to obtain self-sufficiency growth signalling (Hanahan and Weinberg, 2000). For a cell to progress from a quiescent state to an active state (G1) where growth and division can occur mitogenic growth signals (GS) are required. Tumour cells, by gaining autonomy in GS, decrease their dependence on the extra cellular environment, this can be achieved in a number of ways. In sarcomas and some glioblastomas, for instance, the extra cellular environment is altered by secretion of their own growth signals (Tysnes and Mahesparan, 2001). A more common strategy in malignant cells is the deregulation of membrane receptors that bind extra cellular GS. This is exemplified in breast tumours

where the epidermal growth factor receptor (EGFR) is up regulated increasing the cell's sensitivity to ambient levels of this growth factor (Morris, 2002). Another common autonomous growth signalling strategy observed in cancer cells is the alteration of extra cellular matrix (ECM) attachment proteins favouring those that transmit pro-growth signals e.g. integrins (Stewart et al., 2004). Alterations in the growth signalling can also occur within the cell, proteins responsible for changes in gene expression following the detection of cellular abnormalities (e.g. SOS-Ras-Raf-MAPK cascade), are mutated in many types of cancer. The main targets for alteration in this pathway are members of the ras gene family which when mutationally activated can transmit pro-growth signals without upstream stimulus. The alteration of this intracellular signalling cascade has multiple effects the main advantage of which is to promote survival of the cell (Hanahan and Weinberg, 2000).

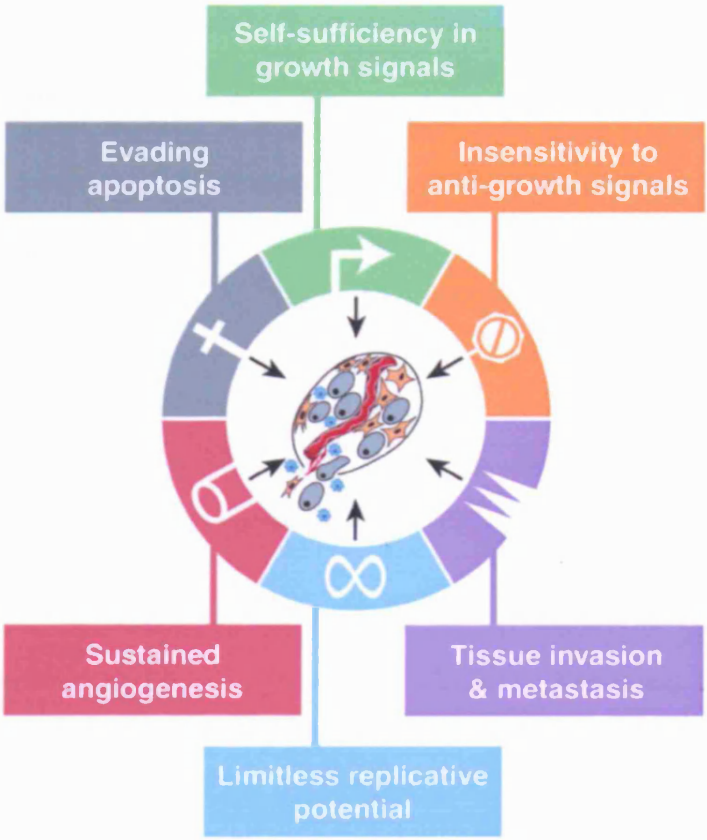



**Figure 1.2.** Acquired capabilities of cancer (Hanahan and Weinberg, 2000).

Autonomy in growth signalling is not the only attribute cells require to begin a cycle of deregulated growth as there are opposing forces to such behaviour in the form of anti-growth factors. To achieve deregulated growth, the malignant cell requires

desensitisation to such anti-growth signals. Anti-growth factors are present as soluble factors in the extra cellular fluid and bound to the ECM. These factors act by forcing cells into quiescence or differentiation. In inducing the quiescence anti-growth factors act through the Rb protein, the phosphorylation state of which decides the fate of the cell in the G1 phase of the cell cycle (Hanahan and Weinberg, 2000). Anti-growth signals induce cells to differentiate by signalling throughout the Myc-Max-Mad pathway. Association of the Myc and Max proteins drives the cell into growth, association of Myc with Mad leads to differentiation of the cell and thus apposes growth (see Figure 1.3).

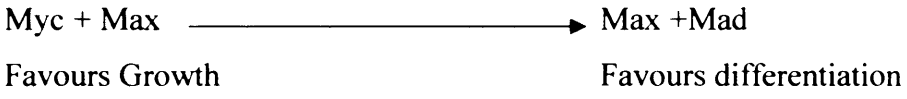



**Figure 1.3.** The balance of growth and differentiation.

Tumour cells however, avoid terminal differentiation by up regulation of c-myc which causes Myc to remain associated with Max, a situation conducive to growth and opposed differentiation (Lewin 2000). Transforming growth factor beta (TGFβ) is an example of an anti-growth factor often deregulated in human tumours such as prostate (Tu et al., 2003), breast (Lebrecht et al., 2004) and colon (Biswas et al., 2004). Tumours often become insensitive to TGFβ signalling by the down regulation or alteration of intracellular transducers of this signal such as TbetaR-I and TbetaR-II (Evangelou et al., 2000), or by the deregulation of proteins downstream of the receptor such as Smad3 (Chipuk et al., 2002).

The pre-malignant cell with its loss of sensitivity to pro and anti-growth signals is able to initiate deregulated proliferation. There are, however, further hurdles in the path to malignant progression. To achieve neoplastic growth, the pre-malignant cell has to overcome the finite replicative potential of normal cells. This limitation is due to the shortening of chromosomes with every round of replication. This shortening of the chromosome ends, or telomeres, means that human cells can undergo only around 50 to 70 doublings, before senescence occurs. Telomeres are created initially by the enzyme telomerase that is switched off in later development of the cell. In 85 to 90 per cent of cancer cells, however, telomerase is up regulated giving the cells increased replicative potential (King, 2000).

Cells undergoing deregulated proliferation will eventually form large cell masses. Without sufficient vascularisation, the cells and the centre of such structures would therefore depend on diffusion to receive all the essential nutrients. This form of nutrient transport via diffusion would not sustain large cell population, as cells need to be within 100μm of a capillary (Strohmeyer, 1999). Neoplastic growth therefore, requires the formation of new blood vessels (angiogenesis). As with any regulatory system, there are pro and anti-angiogenic signals. The induction of angiogenesis is present at an early stage in cancer and is initiated by a change in the balance of pro and anti-angiogenic factors at transcription level (King, 2000). Vascular endothelial growth factor (VEGF) is an example of a pro angiogenic protein and has been isolated as a target for inhibition in clinical drug trials. The regulation of angiogenesis is an obvious therapeutic target, as limiting angiogenic potential causes nutrient starvation leading to large-scale apoptosis within the tumour (Ziche et al., 2004).

### 1.1.4 Invasion and metastasis

Invasion and metastasis are the result of a co-ordinated balance between cell adhesion and detachment through CAM proteins, which occurs simultaneously with tumour cell-induced remodelling of the basement membrane by endopeptidases (Stewart et al., 2004). This is a multi-step process (See Figure 1.4) and in accordance with the heterotypic model, involves both cancerous and normal cells within the tumour microenvironment.

Tissue invasion and metastasis are the attributes that make cancers malignant and ultimately fatal, contributing to 90 per cent of human cancer deaths (Quaranta and Giannelli, 2003, Hanahan and Weinberg, 2000). Invasion into surrounding tissue alone, decreases treatment options and can be lethal as seen in astrocytomas and head and neck small cell carcinoma (HNSCC). The term invasion suggests the penetration and occupation of neighbouring tissue, this tissue contains nerves and blood vessels, leading to complications such as pain and internal bleeding. Tumours are often surgically staged using the TNM system, which takes into account the spread of the disease in terms of invasion and metastasis (see Appendix 1). The extent of invasion into surrounding tissue can vastly affect the prognosis, in patients with stage I disease where there is no spread to the lymph nodes there is a 92% 5 year relapse free

probability following treatment with irradiation. In comparison patients with stage III or IV disease where there is spread outside the prostate to lymph nodes or outside the pelvis the 5 year relapse free probability is 24% (Pisansky et al., 1997).

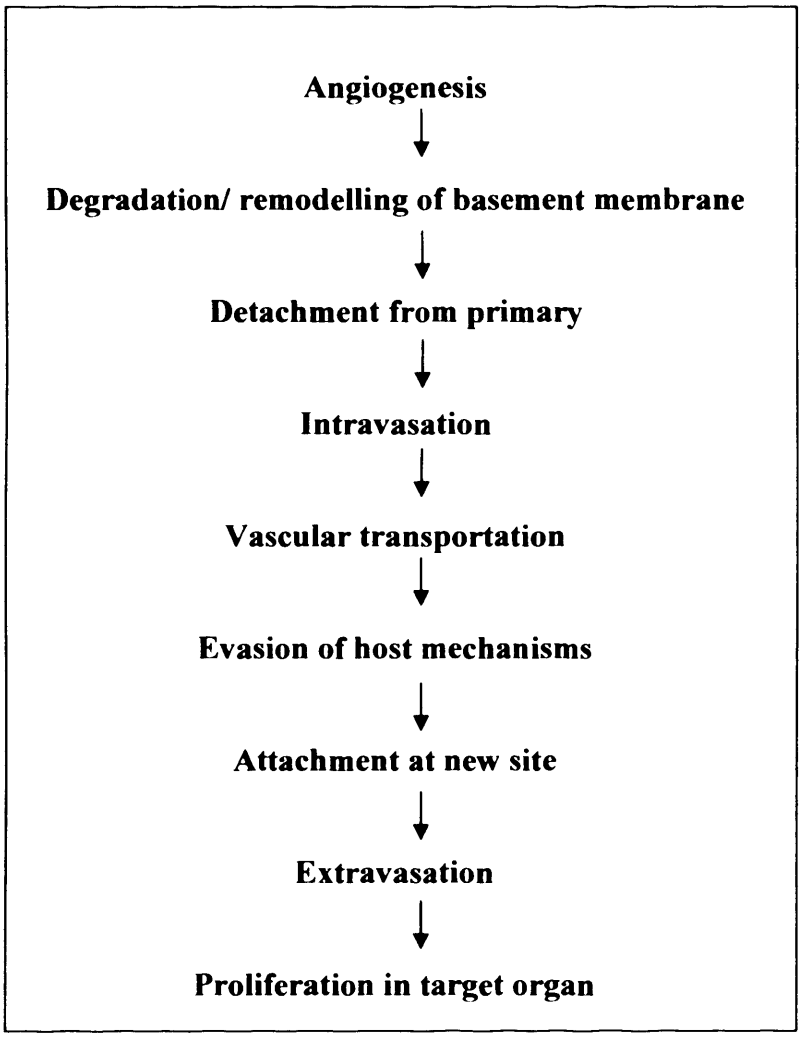



**Figure 1.4.** The multi-step process of invasion and metastasis. Adapted from (Stewart et al., 2004).

The process of invasion generally involves the uncoupling of cells from their surrounding microenvironment and the activation of extra cellular proteases. These enzymes are resident in the transmembrane region of the cell, associated with integrins or bound to receptors. Some proteases are thought to be transported into the tumour area from normal tissue and promote carcinogenesis and invasion by degrading tissues, initiating angiogenesis and pro-growth signalling (Hanahan and Weinberg, 2000). The recruitment of proteases from normal tissue to aid malignant growth is an example of the heterotypic signalling seen in tumour cell masses (De Wever and Mareel, 2003). Certain host elements are present in all cancers; these

include lymphocytes, macrophages, dendritic cells and angiogenic factors. In pancreatic cancers greater than 50% of tumours contain a majority of host cells (Mareel and Leroy, 2003). The idea that other cell types contribute to the growth, proliferation and spread of malignant cells, was proposed in the first edition of the Lancet, termed "the soil and seed model." This model suggests that the microenvironment of each organ (soil) influences the survival and growth of the cancer cell (seed) (Paget, 1889). Recently this model has been termed the heterotypic model of tumour growth and suggests that tumour cells gain an advantage by being able to co-opt the neighbouring cells (Hanahan and Weinberg, 2000).

Uncoupling of cells from their surrounding microenvironment occurs due to the deregulation of cell-cell adhesion molecules (CAMs). The adherence of cells via CAMs also acts to regulate growth, for example, E-cadherin bridges transmit anti-growth signals. The expression of E-cadherin is down regulated in a number of epithelial cell cancers including prostate (Jaggi et al., 2005), head and neck (Bosch et al., 2005) and melanoma (Ikoma et al., 2005) and has also been found to correlate with poor prognosis in these diseases. Reduced E-cadherin expression in mouse cancer models increases metastasis and invasion (Perl et al., 1998, Gingrich et al., 1996). These results indicate that E-cadherin has an important role in metastatic progression. Invasion and metastasis into new microenvironments requires recognition of new ECM proteins by the CAM proteins. In cancer cells this can be achieved through alteration of alpha and beta subunits of integrin molecules (Stewart et al., 2004). Different combinations of these subunits results in greater than 20 subtypes of integrins (Fornaro et al., 2001). Cancer cells are thought to have a selective advantage by switching expression to novel integrin subtypes that recognise the degraded stromal components produced by protease action, allowing the cell to associate with ECM proteins in new microenvironments (Stewart et al., 2004).

Invasion can be regarded as a normal developmental process, most markedly seen at gastrulation where a spatiotemporal balance of promoters and suppressors of invasion participate to form the different cellular layers (Mareel and Leroy, 2003). Invasion is a normal phenomenon occurring in terminally differentiated cells such as endothelial cells, for example leucocytes and macrophages which are extremely invasive (Falcone et al., 2001, Mareel and Leroy, 2003). It is possible that normal cells are affected in a

paracrine fashion by soluble factors secreted by the cancer cells, which could then lead to the induction of pro-invasive factors by the normal cells. The understanding that cancer is not a discrete foreign collection of cells, but derived from an isogenic source and dependent on normal endogenous processes, may explain why curative treatments are so damaging to normal tissue and cause detrimental side effects. However achieving a better understanding of the microenvironment of the tumour should uncover new ways to exploit endogenous mechanisms to limit the spread of the disease, perhaps by restoring the normal restriction of invasion.

Metastasis, or the development of secondary cancer sites distal to the original tumour constitutes the final and often lethal phase of the disease (Quaranta and Giannelli, 2003). Metastasis to a novel tissue environment is advantageous to cancer cells because it allows them to escape space and nutrient limitations. The process of metastasis is distinct from that of invasion in that not only must the cells invade through surrounding tissue, but also detach from the primary tumour, enter a transport vessel (either lymph or blood vessel), be transported to a distal site whilst evading the immune systems defences, attach at a new site, leave the blood vessel and finally proliferate in the target organ (Stewart et al., 2004). This process requires many changes in the surface composition of the cells, which will be discussed further in section 1.3.2.

### 1.1.5 Stem cell theory of carcinogenesis

In normal tissue, cell growth is opposed by differentiation, with a small subset of stem cells dividing to produce transit-amplifying cells that in turn produce the differentiated cell types of the tissue (Lajtha, 1979, Leblond, 1981). As these cells progress to a more differentiated phenotype their ability to replicate is lost, this is in part due to a loss of telomerase, the enzyme responsible for maintaining chromosome length and integrity through rounds of replication. In most normal somatic tissues telomerase is present at undetectable levels, it is however readily detectable in germ line tissues and cancers (Kim et al., 1994). In certain tumors, including those of breast and prostate tissue, a loss of differentiation occurs concurrently with re-activation of telomerase and this is considered a marker of advanced disease (Baykal et al., 2004, Tricoli et al., 2004).

One model of cancer progression proposes that the negative effect of differentiation on proliferative capacity is overcome by the acquisition or maintenance of a more stem cell like phenotype, including reactivation of telomerase (De Marzo et al., 1998). Theoretically this can be achieved in one of two ways i) de-differentiation of mature cells to a more stem cell like phenotype ii) genetic alteration of a stem or transit amplifying cell (Al-Hajj et al., 2004). Both of these situations give rise to a small population of cells (possible tumour stem cells) capable of not only forming a heterogeneous tumour but also maintaining self-renewal. In acute myeloid leukaemia (AML), breast and prostate cancer, a small fraction of cells (1:100 to 1:10000) has been isolated that is capable of colony formation (Bonnet and Dick, 1997, Al-Hajj et al., 2004). It has been suggested that mutational events in stem cell colonies create a background of genetic instability in the daughter cells that is maintained by self-renewal of the cancer stem cell. This genetic instability is proposed to act as a field for the cancerization process (Braakhuis et al., 2003). The cancer stem cell model suggests that longevity of stem cells allows them to accumulate the 5-7 mutations required for a cancer to develop (Reya et al., 2001). Once genetically altered this stem cell could go on to transmit these alterations to a population of daughter cells, creating a patch of mutant cells. This patch would in turn undergo expansion creating a field of genetically altered cells in which further mutational events may occur leading to carcinoma (Braakhuis et al., 2003). See Figure 1.5.

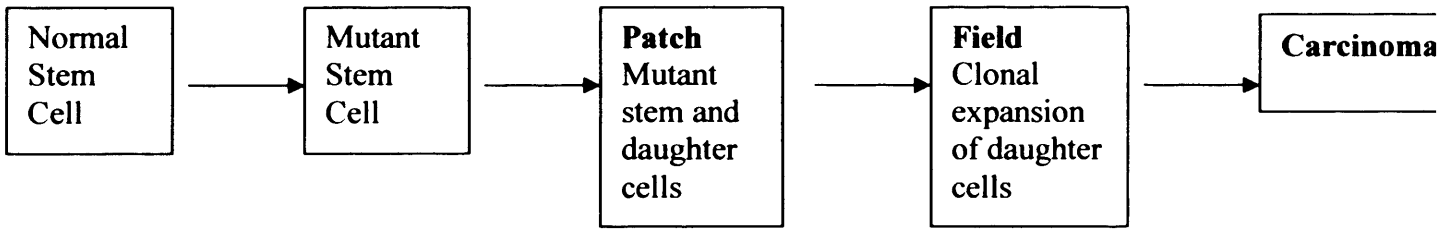



**Figure 1.5.** The cancer stem cell model of field cancerization (Braakhuis et al., 2003).

Evidence for this field cancerization model can be derived from studies in several cancers such as lung (Wiencke and Kelsey, 2002), HNSCC (van Oijen and Slootweg, 2000, Hafner et al., 2001)), bladder (57) and skin (Carlson et al., 2001), where pre-neoplastic changes are present at multiple sites before the occurrence of a carcinoma (Braakhuis et al., 2003). The above theory suggests a single genetic origin of the

neoplastic process, which indicates that the same mutation should be present throughout the lineage of cells if they are all derived from a single cancer stem cell ancestor. Confirmation of this was seen in HNSCC where patches of cells >200 cells in diameter were found to share a common genotype displaying p53 mutations (Garcia et al., 1999). Cells displaying a common clonal identity have also been noted in bladder carcinoma, even for lesions >7cm apart (Simon et al., 2001). Further evidence of a common clonal origin can be observed in the results of a number of studies comparing genetic alterations in apparently normal tissue surrounding areas of tumour resection, to those observed in the tumour specimen. In HNSCC for example, tumour associated genetic alterations in neighbouring normal mucosa were discovered in 33% of cases whose surgical margins were declared tumour free by histopathology (Tabor et al., 2002).

A stem cell model for prostate cancer, proposed originally by Isaacs et al, suggests that an increase in the total prostatic stem cell number and/or an increase in the clonal expansion of the stem cells into transit-amplifying cells may lead to neoplasia (Isaacs and Coffey, 1989). Subsequently, researchers have attempted to identify the stem cell fraction of normal and cancerous prostate tissue. In normal prostate tissue 80% of cell proliferation occurs within the basal cell layer and this layer is consequently thought to contain the stem cell fraction (Bonkhoff et al., 1994). As yet, no marker exists that identifies the prostate stem cell fraction, however through observation of the colony forming ability of epithelial cells cloned directly from areas of benign prostate hyperplasia a subset of cells with a high proliferative capacity which display pluripotency in three dimensional culture can be isolated (Hudson et al., 2000). It is postulated that these putative prostate stem cells possess decreased androgen dependence and are thus capable of the re-growth of the prostate gland following ablation of the prostate by castration. In the male rat 90% of prostate epithelial cells are lost following castration, the remaining 10%, possibly containing the stem cell fraction are androgen independent but remain androgen sensitive as restoration of androgens leads to regeneration of the prostate gland (Kyprianou and Isaacs, 1988). The androgen independent nature of these putative prostate stem cells has led to the suggestion that these cells are responsible for tumour reoccurrence and androgen insensitivity seen following hormonal treatment of prostate cancer (Bui M, 1998-99, Isaacs, 1999). This model suggests that there a subset of genetically altered (cancer)

stem cells which aberrantly mimic the normal process of tissue renewal. These genetic alterations may consist of early carcinogenic events such as mutation of tumour suppressor genes leading to genomic instability and/or mutational activation of oncogenes, leading to over proliferation of the daughter or clonally derived cells in which further mutations may lead to carcinoma. The cells, under the selective pressure of hormonal therapy, may retain the capacity for androgen independence seen in the progenitor cells. The origin of these cancer stem cells remains unclear, they may result from a genetically altered normal stem cell or a de-differentiated cell from another part of the population. Elucidation of prostate stem cell markers will assist in the clarification of this issue.

### 1.2 Prostate cancer

Prostate cancer is the second most common cause of cancer death in men in Western countries and the incidence has increased in recent years. In England and Wales, for example, death rates have trebled in the last 30 yrs (Cussenot et al., 1998). There are several possible explanations for the increased incidence of prostate cancer (PCa), the most apparent of which is the increasing age of the population. Age is the most important risk factor in PCa, with a median age at diagnosis of 72 (Cussenot et al., 1998). A heightened awareness of PCa amongst the public and the use of prostate specific antigen screening (PSA) have added to the rise in PCa diagnosis.

Globally there is wide racial variation in PCa incidence ranging from 0.1:100 000 Thailand- 30:100 000 West Indies (Gao et al., 1997). There is also variation in severity of disease with African Americans suffering more aggressive cancers, which may be due to their higher testosterone levels (Gao et al., 1997). Racial differences may be enhanced by environmental factors such as diet. A high saturated fat and low selenium, Vitamin E and D, containing diet confers the greatest risk (Chan et al., 2005). Proximity to radiation may also be a risk factor, as PCa was found to be one of the cancers inducible by exposure to atomic radiation (Beral et al., 1985). It is possible that environmental effects have a greater bearing on the incidence of prostate cancer than hereditary factors, as it has been inferred that less than 5% of PCa are inherited (Cussenot et al., 1998).

There are however, several genetic factors associated with increased prostate cancer risk. These include alteration of members of the BRCA gene family and those involved in insulin-like growth factor (IGF) signalling. BRCA mutations have been studied extensively in relation to familial female cancers such as those of the ovaries and breast where mutation of BRCA1 is associated with a significant increase in cancer risk (Robson et al., 1998). It has been alleged that prostate cancer is over-represented in familial groups with a history of BRCA associated female cancers (Sigurdsson et al., 1997). BRCA1 and BRCA2 have been studied extensively in Ashkenazi Jews due to the high frequency of three founder mutations in these genes. Studies of prostate cancer incidence in this population have found that mutations in BRCA2 confer a significantly increased risk of prostate cancer (Kirchhoff et al., 2004). The association between BRCA1 mutation and prostate cancer has been widely debated, studies have reported a lower (Brose et al., 2004) and increased risk (Ford et al., 1994) of prostate cancer in family groups with a BRCA1 mutation. BRCA1 has a putative role as a tumour suppressor involved in the maintenance of genomic integrity (Bradbury, 2002). This function, however may not be restricted to the BRCA1 gene itself, as genetic analysis of the area surrounding the BRCA1 locus (17q21) has indicated a distal genomic region that is deleted in 70% of prostate tumour cells, which may contain one or more tumour suppressor genes (Williams et al., 1996, Newman and Liu, 1998). As germ line mutations of BRCA1 and BRCA2 genes have been found at a relatively low frequency in prostate cancer cells, 0-2.3% and 0-3.2% respectively (Kirchhoff et al., 2004), it is possible that alterations in regions surrounding these genes may have a greater significance in terms of prostate cancer risk. The link between prostate cancer and BRCA mutations is the focus of an ongoing study into the identification of men with a genetic predisposition to prostate cancer and their clinical treatment (IMPACT)(Bonn, 2002), which should give further insight into the inherited aspects of prostate cancer.

Defects in the IGF-1/IGFR (Insulin-like growth factor receptor) axis are indicated as predisposing factors to prostate cancer. An increase in IGF-1 induced cellular activity, either through high circulating IGF-1 levels, increased availability of free IGF, or IGF-1R over expression has been associated with many cancers including prostate, ovarian, melanoma, colon and rhabdomyosarcoma (Hellawell et al., 2002, Playford et al., 2000, Renehan and Howell, 2005). At the cellular level IGF-1 has been implicated

in carcinogenesis through autocrine and paracrine regulation of both mitogenic and anti-apoptotic effects, in tumour progression through ligand independent activation of other receptor tyrosine kinases and enhancement of matrix-metalloprotease (MMP) expression (Papatsoris et al., 2005). Both high circulating levels of IGF-1 and over expression of IGF-1R have been associated with increased risk of prostate cancer. High levels of circulating IGF-1 are associated with prostate cancer risk most strongly in men under 59, where there is a four fold higher risk with elevated IGF-1 serum levels (Papatsoris et al., 2005). High IGF-1 levels appear to be an early finding in prostate cancer, sometimes detected up to 5 years before prostate cancer is diagnosed (Papatsoris et al., 2005). This and the recent finding that circulating IGF-1 levels are high in individuals with high grade prostate intraepithelial neoplasia (HGPIN), a possible precursor of prostate cancer, has led to the conclusion that IGF-1 has an important role in early carcinogenesis (Nam et al., 2005). In the transgenic adenocarcinoma of the mouse prostate model (TRAMP) germ line mutations which decrease the level of circulating IGF-1, delayed neoplastic changes to the prostate and improved survival, providing further evidence of a role for IGF-1 in early carcinogenesis (Majeed et al., 2005). It has been proposed that small foci of prostate cancer cells secreting high levels of IGF-1 could have a paracrine neoplastic effect on surrounding cells leading to field cancerization (Papatsoris et al., 2005). Increased levels of IGF-1 are thought to be due to a polymorphism in a cytosine:adenosine repeat (CA19) upstream of the IGF-1 gene transcription site. CA19 homozygotes have a decreased risk of prostate cancer, interestingly this genotype is found more frequently in Caucasians than those of African descent (Friedrichsen et al., 2005, Schildkraut et al., 2005).

In addition to increased secretion of IGF-1, increased bioavailability of the compound has also been associated with increased risk of prostate cancer (Hellawell et al., 2002). In serum, 90% of IGF is associated with insulin like growth factor binding protein 3 (IGFBP3) (Papatsoris et al., 2005). IGFBP3 modulates the activity of IGF and shows an inverse correlation with prostate cancer risk. IGFBP3 levels are highest in healthy individuals, intermediate in those with localised prostate cancer and lowest in individuals with metastatic disease (Kehinde et al., 2005). Differences in the ratio of IGF-1:IGFBP3 correlates with prostate cancer risk in global populations, the ratio is lowest in African Americans who have a high risk of prostate cancer (Papatsoris et al.,

2005). Low levels of both proteins are seen in Arab and Chinese populations who have a low risk of prostate cancer, further implicating the IGF-1 axis in global variation in prostate cancer incidence (Kehinde et al., 2005).

Over expression of the cellular transducer of IGF-1, IGF-1R, has been observed in primary prostate tumours when compared to benign prostate epithelium (Hellawell et al., 2002). Activation of this receptor appears to have a strong proliferative effect on prostate cells (Friedrichsen et al., 2005) which may in part be due to a transactivating effect on the androgen receptor (Papatsoris et al., 2005). This cross regulation is reciprocal, as androgens can cause a six fold increase in IGF-1R expression, an effect which is not negated by conventional anti-androgen treatment such as Casodex (Pandini et al., 2005). The cross talk between androgen and IGF pathways has been postulated as a possible explanation for androgen insensitive tumour re-growth seen in late stage prostate cancer (Papatsoris et al., 2005), (Pandini et al., 2005). However, IGF-1R expression appears to be inversely proportional to tumour stage, with expression reduced or lost in metastatic disease. This is observed in the TRAMP model, where IGF-1R expression is absent from metastasis and androgen independent disease (Kaplan et al., 1999). The significance of this finding for human prostate cancer appears somewhat limited as a recent study found that only 25% of cases over expressing IGF-1R in the primary tumour displayed reduced expression on progression to a androgen insensitive/metastatic phenotype (Hellawell et al., 2002).

### 1.2.1 Diagnosis

The majority of prostate cancers occur within the peripheral zone of the gland. Due to the anatomical position of this zone early disease is often asymptomatic, although in some cases there may be problems with urination due to overgrowth of the tissue surrounding the urethra (Mazhar and Waxman, 2002). Of presenting cases, 95% are identified as adenocarcinoma histologically based on the architecture of the tissue and the cell cytology. Prostate cancers are diagnosed using these features and graded using the Gleason scale (Mazhar and Waxman, 2002). Due to its asymptomatic and insidious onset, the need for effective screening for prostate cancer has become a research priority. At present, in the UK only 10% PCa cases are diagnosed in screening via digital examination, most are symptomatic and the diagnosis has to be

confirmed by transrectal biopsy. Digital examination screening has poor predictive value, however, as the cancers are often not detectable by palpation until an advanced stage (Mazhar and Waxman, 2002). Transrectal ultrasound has been used to identify cases of PCa this technique has proved as ineffective in determining the spread of the tumour as digital examination (Mazhar and Waxman, 2002).

Both digital examination and ultrasound screening require the cancer to be well established, for screening to be effective the cancer needs to be detectable at an early stage. Pre-malignant changes called prostatic intraepithelial neoplasia (PIN) have been discovered that occur in 70% of prostates containing high-grade cancer. Their value as a prognostic marker however remains undecided, as PIN is seen in 40% of non-cancerous prostate tissue (Mazhar and Waxman, 2002). Screening for changes such as PIN would involve invasive procedures such as biopsies, which in turn carry risks of infection. There is, therefore a need for non-invasive screening technique that has the following characteristics:

- Measurable in serum or from tissue specimens
- Useful in screening, diagnosis, prognosis, monitoring therapy, or can be followed for early indication of relapse.
- High sensitivity, specificity, reproducibility,
- Practical cost efficient test
- Organ specific
- Cancer specific

(Gao et al., 1997)

Prostate specific antigen (PSA) is a potential serum marker of PCa first described in 1971 when it was found to be elevated in 96% of patients with newly diagnosed untreated prostate cancer (Stamey et al., 1987). The PSA gene is located on chromosome 19 and the 34 kD single chain glycoprotein product is a serine protease with trypsin like activity. PSA is synthesized by epithelial cells of prostatic acini and ducts where it liquefies seminal coagulum, PSA is also found in large amounts in the cytoplasm of cancerous prostate cells (Charrier et al., 2001). The use of PSA as a prognostic marker followed the finding that the serum level increases with clinical stage and tumour volume, falls following radical prostatectomy and then rises with

tumour reoccurrence (Stamey et al., 1987). PSA held promise as a prostate cancer marker in diagnosis and prognosis; however, its clinical use is limited. PSA, despite its name, is not organ or disease specific, as it has been found in other tissues and is present at varying levels in female serum (Yu and Berkel, 1999). PSA has also been found to be elevated in 65% of patients with non-malignant prostate disease i.e. benign prostate hyperplasia and prostatitis (Charrier et al., 2001, Gao et al., 1997). Differentiation between the malignant and non-malignant state using PSA levels has been the subject of much research. It has been shown that calculating the serum ratio of free versus total PSA (free PSA plus that bound to protease inhibitor) increases the sensitivity of PSA screening and decreases the number of unnecessary biopsies by 20% (Charrier et al., 2001). Despite such improvements it appears that the marker property of PSA seems related more to changes in gland architecture than malignant status (Stenman et al., 1991, Charrier et al., 2001). As a marker PSA, therefore, is not tissue specific and does not distinguish between malignant and benign disease, it also exhibits a poor correlation with cure rates (Charrier et al., 2001). Another complication with the clinical use of this marker is that in rare cases, aggressive cancers are PSA negative (Pryor and Schellhammer, 2002). Considering these limitations it is apparent that PSA is not an ideal marker of prostate cancer, however, it is the only molecular marker in widespread clinical use for the monitoring of this disease (Gao et al., 1997). There is obviously a need for further research into markers that fulfil more of the criteria listed above and are able to diagnose cancers with a high level of confidence.

### 1.2.2 Treatment

The current treatment of prostate cancer largely depends on the stage at which the cancer is diagnosed.

Prostate cancer progression commonly involves:

1. Asymptomatic early disease, possible problems with urination
2. Invasion of seminal vesicles, ureters, bladder base and external urethral sphincter erosion often causing haematuria.
3. Lymphatic spread into the iliac chain and para-aortic lymph nodes
4. Metastasis, vascular spread to bone in 80% of cases, also to liver, lung and adrenal glands. Perineal pain, weight loss, cachexia, bone pain and

neurological complications usually related to metastases (Denmeade and Isaacs, 2002).

The treatment of clinically localized disease currently includes active surveillance for many cases especially those with other medical complications. Other more invasive management strategies are used which carry associated risk factors. Radical prostatectomy is a surgical solution for those with organ confined disease but carries a mortality risk of up to 5%, an impotence risk of 70% and incontinence risk of 5% (Mazhar and Waxman, 2002). Prostate cancer is relatively insensitive to radiotherapy due to the slow growth of the tumour; this treatment gives similar survival and incontinence rates as surgery, however, the impotence risk decreases to 10-40% (Mazhar and Waxman, 2002, Denmeade and Isaacs, 2002). A form of radiotherapy, called brachytherapy, has increased sexual potency but led to a higher risk of urinary problems (Denmeade and Isaacs, 2002). Radiotherapy is most effective as a palliative treatment in advanced prostate cancer as studies have shown that 90% of patients with metastatic disease undergoing radiotherapy were relieved of bone pain (Tong et al., 1982).

Hormonal therapy is often used in conjunction with radiotherapy or alone in advanced disease as it reduces the size of the tumour by 30-40% (Mazhar and Waxman, 2002). The treatments used in advanced disease include anti-androgens and gonadotrophin releasing factors, oestrogens have also been used but these can lead to an increased risk of cardiac disease (Denmeade and Isaacs, 2002). Hormone therapy is commonly used to alleviate the symptoms of metastatic disease, but is not curative and leads to androgen insensitivity of the tumour after a median period of 18 months at which point the treatment becomes ineffective (Mazhar and Waxman, 2002). Hormonal treatment can also be used as an adjuvant to chemotherapy in a palliative capacity.

Due to the insidious onset of the disease, prostate cancer often presents at a late stage, where there may be extra capsular invasion and metastasis to surrounding tissue. By definition in low Gleason grade tumours (more differentiated cancers) stromal invasion is present and as the tumour becomes more undifferentiated (higher Gleason grade), the risk of metastasis increases. Clinicians use Partin's tables, PSA level,

Gleason grade and tumour stage to calculate the chance of the patient having localized or locally advanced cancer. In patients with PCa which has extended outside the prostate, initially to seminal vesicles or local lymph nodes, curative treatment becomes less feasible (Stamey et al., 1999). In late stage cancers the treatment is often palliative however, there has been an emergence of new therapeutic strategies and chemotherapeutic agents. These include second-line hormonal therapy (Wilkinson and Chodak, 2004), chemotherapy (Oh et al., 2005), immunotherapy (Lee, 2002), granulocyte macrophage-colony stimulating factor (GM-CSF) therapy (Pandha et al., 2004, Small et al., 1999), dendritic cell therapy (Pandha et al., 2004), gene vaccination therapy (Gulley et al., 2002), inhibition and/or blockade of growth factor receptors or growth factor receptor pathways (Hudes, 2002) and inhibition of neo-angiogenesis (Sternberg, 2003).

Many of these treatments display anti-proliferative and anti-angiogenic properties in vitro and in mouse models and may decrease PSA levels in vivo, but none yet have produced significant prolonged survival in patients with late stage prostatic disease. This is exemplified in the case of immunotherapy with interferons. Stimulation of the body's immune system by enhancement of endogenous processes or by exogenous administration of cytokines has been a popular approach to anti-cancer therapy. Enhanced cytokine signalling by either of these means has been shown to instigate anti-proliferative (Hobeika et al., 1997, Huang et al., 2002, Tsai et al., 2002), proapoptotic (Bauer et al., 2003, Huang et al., 2002, Harris et al., 1999, Kramer et al., 2001, Selleck et al., 2003), anti-metastatic (Tsai et al., 2002, Street et al., 2001, Huang et al., 2002), immunostimulatory (Kuratsukuri et al., 2000) and anti-angiogenic (Cao et al., 2001, Bauer et al., 2003) effects. By virtue of these effects cytokines could be seen as the ideal tumor suppressors, however, it has been noted that many tumors display a lack of immunogenicity possibly due to a lack of MHC class 1 expression (Kuratsukuri et al., 2000). Prostate cancer is one such tumor, the prostate cancer cell line LNCaP is unresponsive to interferons due to epigenetic silencing of JAK1 expression, a protein required for interferon signal transduction (Dunn et al., 2005). In addition, prostate cancer metastases, from which the LNCaP cell line is derived, express little or no MHC class 1 protein (Blades et al., 1995). It has been postulated that this lack of immunogenicity is an important event in tumourigenesis as it allows the cancer cells to evade host immune responses (Blades et al., 1995, Bronte et al.,

2005). This tumor mediated immunosupression may also occur at the systemic level as elevated levels of cytokine receptors identified in cancer patient serum have been found to bind to and inhibit cytokine activity (Ambrus et al., 2003).

Several studies have examined the effects of cytokines, particularly interferons, on prostate cancer cells. These cytokines are known to have cytotoxic and immunomodulatory effects on cancer cells, not only at the primary site but also at a cellular level in disseminated cancer (Ronchese et al., 2002). Interferon alpha (IFNα) has been the most extensively studied of the interferons in relation to prostate cancer. IFNα is produced by lymphocytes, lymphoblasts and macrophages and is part of innate immunity, activating macrophages and monocytes, stimulating natural killer cells and enhancing antigen expression by MHC1 (Kuratsukuri et al., 2000). In vitro IFNα has been found to inhibit growth of the prostatic cell line DU145 by limiting progression from G1 to S phase of the cell cycle in a p53 and pRb independent manner (Hobeika et al., 1997). In xenograft mouse models of prostate cancer, IFNα has been shown not only to have an anti-proliferative effect, mediating a 37% reduction in tumour volume, but also an anti-metastatic effect in decreasing lymph node metastases. This is possibly due to an increase in the ratio of E-cadherin to matrix metalloprotease 9, as the ratio of these proteins has been suggested to be an indicator of metastatic risk (Huang et al., 2002). Clinically, IFNα is in therapeutic use for renal cell carcinoma, melanoma, transitional cell carcinoma and has undergone phase I and II trials for use in prostate cancer (Kuratsukuri et al., 2000, Kramer et al., 2001, Huang et al., 2002). In the largest of these trials IFNα treatment produced a response rate of 5% and a >50% reduction in PSA in 23% of patients (van Haelst-Pisani et al., 1992). This minimal beneficial effect was accompanied by high toxicity with patients experiencing weight loss, fatigue, malaise, central nervous system toxicity, leucopenia, nausea and vomiting. This study concluded that patients cannot tolerate the high doses of IFNα required to produce a beneficial effect in prostate cancer (Kuratsukuri et al., 2000). Subsequently, to limit the dose of IFNα required, many clinical trials have been conducted combining IFNα with various other treatments see Table 1.1 below.

**Table 1.1.** Clinical trials of IFNα combination therapy

| Treatment | No. of participants | Response rate | >50% PSA reduction | Reference |
|---|---|---|---|---|
| IFNα, IL-2 and dendritic cells primed with prostate specific membrane antigen (PSMA) | 13 | 31% | 23% | (Maffezzini et al., 1996) |
| IFNα , Retinoic acid | 14 | 15.3% | 7.7% | (Thalasila et al., 2003) |
| IFNα, TNFα | 10 | 38% | 18-87% | (Kramer et al., 2001) |
| IFNα, 5-fluorouracil | 28 | 0 | 14% | (Daliani et al., 1995) |

As Table 1.1 demonstrates, the greatest cytokine-based therapeutic effect in prostate cancer is achieved by combination of two cytokines and co-administration of primed dendritic cells able to further stimulate the host immune system. Patients within these trials however, did still experience varying levels of toxicity. Further investigation will be required to ensure the benefits of IFNα treatment outweigh the side effects.

The therapeutic potential of interferon beta and gamma (IFNβ, IFNγ) in prostate cancer has been studied far less than that of IFNα. IFNβ is produced by fibroblasts and epithelial cells and like IFNα has an anti-tumour activity (Kuratsukuri et al., 2000). In vitro, IFNβ has been found to suppress the growth of JCA-1 cells by 15-30% and when combined with the anti-tumour agent Onconase, by 42-51% (Tsai et al., 2002). In mouse prostate cancer xenograft models IFNβ suppressed tumour and metastasis formation by 80% and eradicated tumors in 20% of mice (Cao et al., 2001). This effect may be due to the anti-angiogenic properties of IFNβ in decreasing the expression of MMP's and proangiogenic factors (Huang et al., 2002), as prostate tumours from mice treated with IFNβ contained fewer microvessels and a greater degree of apoptosis than untreated tumors (Cao et al., 2001). IFNβ is in clinical use for renal cell carcinoma and has been used in clinical trials for advanced prostate cancer (Bulbul et al., 1986). However 65% of participants in this study could not complete due to disease progression and those who did continue showed no response

in terms of tumour volume, spread or PSA level, they also suffered various side effects such as shivers and fever (Bulbul et al., 1986). It remains to be seen whether the anti-tumor effects of IFNβ will be mirrored in clinical trials.

IFNγ is a type II interferon produced predominantly from CD4+ lymphocytes and natural killer cells that affects cells by signalling through the IFNγ receptor (IFNγR) and the Janus kinase-signal transducer and activators of transcription (JAK-STAT) pathway. Activation of this pathway by IFNγ leads to the transcription, via the ISGF3γ transcription complex, of interferon inducible genes containing gamma activated sequence (GAS) elements (Deb et al., 2003, Aboagye-Mathiesen et al., 1999). In humans, the IFNγ inducible genes thought to be involved in PCa progression are located on the 10q 23-26 and 17q 21 chromosomal loci, regions which are commonly deleted in PCa (Shou et al., 2002). Recent studies found this region to be deleted in 30% of 49 PCa samples and is thought to encode tumour and metastasis suppressor genes (Shou et al., 2002, Nagano et al., 2003).

The cell surface effects of IFNγ treatment in PCa cells include increased adhesion via modulation of P-cadherin, α integrins and ICAM1, increased density of cell receptors, clustering of receptors into large lipid raft structures or "signalosomes" and an increase in the display of proteins signalling the status of the cell (e.g. MHC-1, tumour associated antigens) (Bacso et al., 2002). Through modulation of these various cell surface proteins including CAM's and RTK's, cytokine treatment can alter several PCa attributes closely associated with tumour invasion and the acquisition of a metastatic phenotype. In addition, interferon γ has been found to decrease the binding affinity for bone matrix stroma in PCa cells, indicating that this cytokine may be effective in decreasing the secondary skeletal tumours common in PCa (Sokoloff et al., 1996). In the prostate cancer cell lines PC3 and LNCaP, in vitro exposure to IFNγ enhanced Fas-mediated cell death, rising from 40% to 60%. In a mouse model of prostate cancer IFNγ treatment lead to a three-fold increase in apoptosis in the primary tumor (Selleck et al., 2003). This study suggests that IFNγ acts to sensitize prostate cancer cells to the effects of Fas inhibition. In mouse models of metastatic prostate cancer, PCa xenografts showed a distinctly smaller tumour volume than those left untreated, even at the lowest dose (Shou et al., 2002) and mice lacking IFNγ progress to metastasis quicker than those expressing the cytokine. These IFNγ

negative mice are as susceptible to tumour metastasis as mice lacking NK cells (Street et al., 2001). IFNγ may be of therapeutic use for prostate cancer where it could be beneficial in late stage and hormone refractory disease by retarding invasion and metastasis. The finding that mice lacking the IFNγ receptor develop spontaneous tumours more rapidly than those with functional receptors, indicates that IFNγ is involved in both immune surveillance and retardation of invasion and metastasis, making this cytokine a true candidate for tumour/metastasis suppressor status (Shou et al., 2002, Nagano et al., 2003).

### 1.3 The proteome

The term proteome was coined in 1994 to describe the linguistic analogue of the genome, being the entire protein complement expressed by a genome or a cell/tissue type (Naaby-Hansen et al., 2003, Cai et al., 2004). Proteomics can be defined as the study of all proteins at the subcellular, cellular, tissue or even organism level (Cai et al., 2004). Proteomics is the large scale study of proteins, ideally proteomic studies seek to characterise and quantitate all proteins within a sample at a particular time under a particular set of environmental conditions (Fey and Larsen, 2001). However, proteomics is not solely used to obtain a snapshot of proteins at a given time, it can also be used to identify protein interactions with other proteins, DNA, or RNA, allowing analysis of complex biochemical pathways (Emili and Cagney, 2000). Studies of protein expression and interaction are not mutually exclusive, as one gives functional significance to the other. In proteomics, however, these studies are split into two disciplines "Expression proteomics" and "Functional or Cell map proteomics" (Blackstock and Weir, 1999). The difference between the two disciplines lies mainly in the techniques used and the number of proteins studied.

The term "Expression proteomics" is associated with the description of the total proteome of the subject, the differential expression of proteins between subjects and under different treatment conditions. This is sometimes denoted "discovery proteomics" as the researcher is seeking to identify both known and unknown proteins (Cai et al., 2004). Global analyses such as these require the separation of thousands of proteins from the original sample. The proteome unlike the genome is in a constant state of dynamic flux producing a mixture of heterogeneous proteins with differing

biochemical properties and expression levels. Considering the above, analysis of the prokaryote proteome with its genome complement of less than 3000 genes appears daunting and analysis of eukaryote proteome with greater than 10,000 proteins and the added complication of mRNA processing and complex post translational modification, impossible (Zuo and Speicher, 2000). There is however, a technique commonly used in the global expression analysis of complex genomes which is both sensitive enough to give single protein resolution and discrimination of post-translational modifications (PTM). Proteomic methods such as 2D electrophoresis are the only techniques available that allow the analysis of splice variants and post translational modifications, as well as more conventional expression level quantification (Naaby-Hansen et al., 2001). Such modifications may be functionally critical and determine a disease, i.e. phosphorylation, glycosylation, as is the case of the prion diseases CJD and Alzheimer's respectively (Banks et al., 2000). Novel post translational modifications, mostly phosphorylation, of several oncogene products and cell cycle components (including p53) have been identified in transformed liver cells (Yan et al., 2004). In addition to this aberrant glycosylation of several cancer associated proteins has also been reported, proteomics based approaches are ideally placed to characterise such aberrations (Banks et al., 2000).

2DGE is a gel-based assay in which proteins are separated in two dimensions, firstly by their isoelectric charge and secondly by molecular weight, this method has therefore been termed two-dimensional gel electrophoresis (2DGE). 2DGE is a technique developed in the early 1970's which due to its ability to separate thousands of proteins at a time has become synonymous with the term proteomics (Cai et al., 2004). Although this technique has been available to researchers for 35 years its application to analysis of cellular proteomes has been limited by the detection sensitivity of mass spectroscopy (MS). Recent technological advances such as electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI) have, however, improved the detection limit of MS allowing 2DGE when coupled to MS to characterise thousand of proteins from a single sample (Cai et al., 2004).

Ideally 2DGE results in proteins separated into discrete spots, with each spot representing one protein, a typical gel is able to resolve 2,000 protein spots whereas the best gel systems presently available can resolve up to 11,000 protein spots

(Dalton, 1999, Zuo and Speicher, 2000). This level of resolution appears appropriate for the quantitative analysis of the eukaryote proteome, however due to PTM and mRNA splicing each protein species may have a number of isoforms, which may on a well-resolved gel all separate out as distinct spots. This complication means that each of the 10,000 protein species of the eukaryote genome may be present as multiple isoforms, leading some to suggest that 2DGE does not have the dynamic range suitable for the global expression analysis of eukaryote proteomes (Zuo and Speicher, 2000). 2DGE is also limited in its sample load capacity, as the amount of sample applied increases the resolution of 2DGE decreases, leading to spots containing more than one protein and at high sample loads streaking may occur (Zuo and Speicher, 2000, Fey and Larsen, 2001). In addition, there has traditionally been a poor representation of certain protein species on 2D gels including proteins with high molecular weight, low abundance, basic and hydrophobic properties (Fey and Larsen, 2001, Naaby-Hansen, 2004).

It is thought that proteins with low abundance may be masked by other "housekeeping proteins" present at high levels within the cell, this can be rectified by selective removal of abundant proteins. This as with all pre-fractionation techniques has the associated risk of protein loss (Zuo and Speicher, 2000). Basic and hydrophobic proteins are thought to be underrepresented due to differing solubility or interactions with support materials in the first dimension. Many pre-fractionation techniques have been employed to increase the sensitivity of 2DGE including sequential extraction with different solvents (Thoren et al., 2002), subcellular fractionation (Lehr et al., 2005), chromatography (Nagano et al., 2003), removal of abundant proteins (Lollo et al., 1999) and preparative isoelectric focussing (PIEF) (Zuo and Speicher, 2000). The latter technique aims to reduce the diversity and complexity of the protein mixture so that subsets of proteins can be resolved over complementary narrow pH range second dimension gels, termed the "zoom" technique (Naaby-Hansen, 2004). PIEF allows a higher sample volume application, better solubility and better representation of low abundance proteins, with a resolution limit of proteins present at 1000 copies per cell. The zoom technique using on average 5 gels covering an overlapping pH range, allows the detection of 2000-3000 spots per gel giving a total of 10-15000 protein spots for MS analysis (Zuo and Speicher, 2000). Despite the increased level of resolution pre-fractionation does have certain disadvantages. Firstly due to increased

sample handling and risk of proteolysis pre-fractionation can lead to protein loss of up to 20% and as such a larger sample volume is required (Dalton, 1999, Fey and Larsen, 2001). Secondly IEF resolution in the basic range (> pH 7) is not as good as in the acidic range. As it is thought that one third to a half of cellular proteins have an isoelectric point above pH 7, this could lead to a under-representation of basic proteins and therefore false interpretation of expression levels (Fey and Larsen, 2001). Finally, all 2DGE methods have the complication of poor reproducibility, this is because 2D gels require a high level of technical skill to run, have no standard running conditions and are time and resource intense and therefore not suited to high throughput applications. Fluorescence labelling techniques such as difference gel electrophoresis (DIGE) have attempted to increase the quantitative range of 2DGE. This technique allows two or three samples labelled with separate fluorescent dyes to be run on the same 2D gel allowing direct comparison of different samples and avoidance of problems due to poor gel reproducibility (Naaby-Hansen, 2004). This technique however is disadvantaged not only by limited sample volume application, but also by fluorophore labelling bias.

The high variability of 2DGE makes comparison of these data within the scientific community difficult, the Human Proteome Organisation (HUPO) has however released an initiative to standardise the representation of data from human studies involving techniques such as 2DGE in an effort to consolidate and improve dissemination of data pertaining to the human proteome (Hanash, 2004). Despite its disadvantages, 2DGE remains the only appropriate method for quantifying changes in the protein profiles of cells, tissues and organisms. There are however several emerging techniques which seek not only to match the level of protein separation obtained using 2DGE but also to improve the representation of proteins poorly studied due to the limitations of this technique. Several of these new techniques utilise technological advances in MS to obtain protein identification without the complications of a gel-based step. An example of this can be seen in Liquid chromatography followed by MS, or so-called "shotgun" proteomics. This technique isolates proteins based on their biochemical properties, sometimes using a two dimensional approach, followed by MALDI or ESI MS protein identification. The advantages of this technique are that speed, sensitivity and scope of protein characterisation is increased, making the technique more dynamically flexible than

2DGE. The main disadvantage with this technique however is its lack of quantativity, although this may be overcome with the advent of isotope-coded affinity tagging (ICAT) (Cai et al., 2004).

Surface enhanced laser desorbption and ionisation (SELDI), is also becoming increasingly used in global high throughput analyses, where samples are applied to capture chips (capture is based on varied binding surfaces such as metal affinity or ion exchange). MS is then performed through ionisation of the proteins directly from the chip (Stevens et al., 2004). The advantages of this technique over 2DGE are that sensitivity is increased so that only 500-1000 cells are required for protein identification, there is better representation of high molecular weight and basic proteins and that reproducibility is enhanced (Cai et al., 2004). This technology, in conjunction with bioinformatics, has been used in the profiling of tumour biomarkers in ovarian cancer, where signature protein expression patterns were able to predict the presence of the disease with 99-100% specificity (Petricoin et al., 2002). Despite the potential use of this system in high throughput applications such as cancer diagnostics, there remain complications in that the signature patterns used to identify disease differ between research groups focussing on the same disease. This may be due to differences in technique, the age of the equipment, or may truly represent a number of signature patterns all with potential as diagnostic criteria, further research is required before such a clinical application can be made.

Expression proteomics is a commonly used technique in cancer research as it enables the comparison of protein expression in normal and cancerous cells from the same tissue and of cells at various stages of tumourigenesis. In a recent differential expression analysis of proteins derived from oesophageal squamous cell carcinoma (ESCC) tissue 15 proteins, including manganese-containing super oxide dismutase (MnSOD), were found to be up regulated and the expression of five proteins including squamous cell carcinoma antigen 1 (SCCA1), down-regulated in cancer tissues when compared to normal epithelium (Qi et al., 2005). Studies such as this have not only provided vital information regarding protein expression in carcinogenesis and tumourigenesis, but have also provided a wealth of biomarkers with a potential use in the diagnosis, prognosis or treatment of cancer.

Functional proteomics is a system-based approach, which directly analyses a specific subset of proteins that may be related in terms of function, sequence or structure. The central aim of this research approach is to characterize biological functions of proteins through their interactions with other proteins, DNA and RNA (Cai et al., 2004). These function-based studies have different protein separation requirements to those based on global analyses. Due to the nature of these studies, being the analysis of small sub-groups of proteins, there is no requirement for the resolution of large numbers of proteins and therefore 2DGE is seldom used apart from in the analysis of post-translational modifications (PTM). Functional studies often require the protein to be in its natural functional conformation, a condition difficult to maintain in gel based systems.

Peptide or protein arrays are best suited to studies of this nature, these can represent all the variants of a bioactive peptide, protein family or species, an array of antibodies, or in fact the whole proteome of a cell/tissue or organism. These arrays coupled to MS can then be used to answer specific questions concerning protein or nucleic acid interactions, or to examine differing protein expression following various treatments (Fey and Larsen, 2001). These 'non-living' arrays can provide detailed information on protein interactions in detailed pathways such as those involved in cell signalling or enzymatic processes, there are however problems concerning quantitation. The main limitation of a protein array is one shared with 2DGE, the purification and stabilisation of a heterogeneous protein mixture (Emili and Cagney, 2000), which may cause misrepresentation of protein species in the array. An additional limitation with regard to protein arrays is that proteins often function as part of a complex with other proteins and may be falsely represented as inert proteins or peptides on an array. A solution to this would be the formation of living arrays of cells. These would be able to address functional questions regarding thousands of proteins in complex pathways such as cell signalling, in which there are many multi-component complexes (Emili and Cagney, 2000). At present, this technique is only useful in simple genomes such as yeast due to their relatively low protein complexity. Creating yeast living arrays, however has allowed large yeast two-hybrid screens to identify the protein-protein interactions of 1004 proteins, some of which were previously unclassified (Uetz et al., 2000).

### 1.3.1 The plasma membrane proteome

The plasma membrane proteome can be defined as the entire complement of proteins present in the plasma membrane at a specific time. The process of carcinogenesis leads to changes in the array of proteins present in the plasma membrane proteome. Changes may be seen for example, in the number or activity of growth factor receptors heightening growth factor responsiveness, in the number or type of cell adhesion molecules expressed e.g. integrins, or in the number/activity of proteases present on the cell surface. Analysis of differential expression of such proteins in cancer is extremely important, as due to their position on the cell surface they have potential for use as diagnostic and/or prognostic markers and therapeutic targets. In the latter capacity, it may be possible to tailor treatments more specifically to the cancer cells and reduce the harmful side effects of anti-cancer treatments. The plasma membrane proteome, therefore, could not only be an indicator of the malignant status of the cell but also a resource for targeted cancer therapy. Research has lead to the identification of a number of cancer associated plasma membrane proteins, which currently account for 70% of all known pharmaceutical drug targets, some of which are in clinical use (See Table 1.2 below) (Wu and Yates, 2003).

**Table 1.2.** Disease specific anti-cancer therapeutics and their plasma membrane targets, adapted from (Adam et al., 2003)

| **Product Name** | **Drug Target** | **Cancer Treated** |
|---|---|---|
| Gleevec | abl-kinase | Chronic Myeloid Leukaemia, Gastrointestinal stromal |
| Herceptin | her2neu | Breast |
| Panorex | Ep-CAM | Colon |
| IRESSA | EGF receptor | Non small cell carcinoma |

A common feature to all of the acquired capabilities leading to the cell acquiring a cancer phenotype is the involvement of the plasma membrane. This is unsurprising as the plasma membrane (PM) is the interface between the cell and its immediate environment acting both as a barrier and allowing the exchange of information

between the segregated environments. The basic structure of the plasma membrane is a lipid bi-layer, studded with integrally and peripherally attached proteins. These membrane-associated proteins confer the majority of the plasma membrane's unique functions. Within the plasma membrane, further complexity is present in the form of subdomains called lipid rafts, enriched in cholesterol, glycosphingolipids and signalling molecules. These structures appear small in size but can represent a relatively large proportion of the PM and are thought to be involved in the regulation of signal transduction (Pike, 2003). It has been suggested that these sub-domains represent areas of increased order, with a corresponding decrease in fluidity, likely to be due to the lipid/cholesterol composition and abundance of transmembrane proteins (Pike, 2003). Further plasma membrane complexity is conferred by the aggregation of these lipid rafts into membrane invaginations or calveolae. This process clusters proteins into a plasma membrane microenvironment conducive to the initialisation of signalling cascades (Simons and Toomre, 2000).

In addition to its therapeutic target potential, the potential of the cell membrane proteome as a resource of prognostic and/or diagnostic indicators is also under extensive research. In PCa, a number of proteins have been discovered, the aberrant expression of which in can be exploited to determine the nature and progression of the disease. Such markers may detect early malignancy, especially in diseases with insidious onset such as prostate cancer. The protein PSA held promise as a diagnostic/prognostic marker; however the shortcomings of this protein as an indicator of prostate cancer have been illustrated in that it is neither organ nor disease specific (Charrier et al., 2001, Gao et al., 1997). Other potential markers of this disease have been identified, i.e. prostate specific membrane antigen (PMSA), chromogranin A, glutathione S-transferase p1 and prostate stem cell antigen. A multiple marker approach may be required to overcome the shortcomings of each of these markers (Tricoli et al., 2004, Gao et al., 1997).

Protein based approaches have been used in the discovery of tumour markers for many years, from the discovery of Bence Jones proteins in the 1800's, to the generation of tumour specific antibodies in epithelial cancer (Banks et al., 2000). Recent technological advances in the field of proteomics have led to research into a comprehensive definition of cancer cell membrane associated proteins. This approach

may reveal those proteins involved in cancer progression that could be used as new biomarkers or therapeutic targets. The application of proteomics to cancer research has already proved to be as successful as genomics in generating quantitative expression data in the study of tumours of the liver (Chignard and Beretta, 2004), breast (Zhang et al., 2005), oesophagus (Qi et al., 2005) and prostate (Meehan et al., 2002). In addition to this proteomic mapping of the vasculature of normal and cancerous tissue have led to the identification of drug delivery targets on the endothelium (Jain, 2000).

In oncology, proteomics is fast becoming the most widespread clinical application of research, making the technique translatable from bench to bedside. Genetic markers are also entering clinical practice, however some important carcinogenic changes are multi-factorial and unlikely to stem solely from a genetic lesion. A successful application of proteomics to oncology can be observed in the example of squamous cell carcinoma of the bladder, where several proteins unique to metastatic lesions were discovered. From this study, psoriacin was highlighted as a potential marker of the primary bladder carcinoma. An ELISA assay is currently being developed for psoriacin allowing its potential as a diagnostic marker to be evaluated. These researchers have now set up a comprehensive proteomic database for bladder carcinoma including profiles for both transitional and squamous cell tumours (Chambers et al., 2000, Celis et al., 1999)

Despite the benefits of proteomic technologies, they have yet to fulfil their potential in the analysis of the plasma membrane proteome. Although 30% of the human genome is alleged to code for membrane proteins (though not all are expressed in every cell) these proteins are commonly under-represented in proteomic data sets and only 30 membrane proteins have been fully structurally characterised (Wu and Yates, 2003, Werten et al., 2002). This deficit of characterised membrane proteins is thought to be due to their biochemical nature. Integral membrane proteins are amphipathic containing both hydrophobic and hydrophilic regions allowing them to be retained in both phases of the membrane. This amphiphillic nature makes membrane proteins notoriously difficult to study with proteomic technologies. The main issue with such proteins is their solubility, especially from highly water resistant tissues such as hair and skin (Wu and Yates, 2003). To conduct 2D electrophoresis the proteins must first

be solubilised in a non-ionic sample buffer, as in the first dimension proteins are separated on their relative isoelectric charge. Membrane proteins do not solubilise well in such buffers and have a tendency to precipitate at their isoelectric point (Naaby-Hansen et al., 2001). There are additional problems in the analysis of membrane proteins caused by their relative rarity. It has been suggested that these proteins make up only 5-8% of the proteome and as such, their expression may be masked by more abundant soluble proteins (Naaby-Hansen et al., 2001). Due to the problems with using conventional 2D gel electrophoresis techniques to analyse the PM proteome, many investigators have eliminated the isoelectric focussing step and returned to "the ageless workhorse" the one-dimensional gel (Wu and Yates, 2003), or favoured other non gel-based methods.

### 1.3.2 Proteomics and the study of invasion

The plasma membrane proteome contains a wealth of potential therapeutic targets, diagnostic and prognostic indicators. In relation to the present management of prostate cancer, there is a need for biomarkers at either end of the treatment spectrum, as early indicators of disease and as therapeutic targets in advanced disease. Standard proteomic strategies have been applied in the search for early indicators of PCa and as mentioned previously there are several promising targets under investigation. In contrast, there has been little application of proteomics to the study of advanced disease, for example invasion and metastatic processes in PCa. Previous research has included protein-based analysis of the cancer cell membrane to discover changes, which could contribute to an invasive phenotype. Studies of this nature have yielded several classes of proteins that are implicated in cancer cell invasion. The most studied of these are cell adhesion molecules (CAMs), endopeptidases and receptor tyrosine kinases (RTK's) (Hanahan and Weinberg, 2000).

In prostate cancer, the effects of local invasion are not fatal but may cause unpleasant side effects such as urinary retention. Prostate cancer becomes lethal when metastasis occurs (Quaranta and Giannelli, 2003). This stage can only be reached through invasion and intravasation, as metastasis cannot occur without invasion. In PCa invasion often involves spread to the seminal vesicles, followed by spread to local lymph nodes. Prostate cancers are prone to metastasis, especially to bone, in that 80%

of patients with advanced disease have bone metastasis (Stewart et al., 2004). There is no effective long-term cure for such disease and the survival ranges between 9 months to a year (Mazhar and Waxman, 2002). Palliative hormonal treatments can decrease the size of primary and secondary tumour masses, but as reported previously the tumour cells become increasingly androgen refractory (Mazhar and Waxman, 2002). It has been suggested that androgen refractory cancers are actually more invasive, this is possibly due to the up regulation of MMP's (MMP2) and the conversion of integrins subtypes to those favouring motility (Baldi et al., 2003). Various CAM proteins are implicated in PCa progression, primarily the integrins and cadherins. Integrins are heterodimeric cell adhesion receptors, with as many varied combinational subtypes. Increased expression of integrins increases their adhesion to the ECM and therefore limits movement. Certain integrin subtypes, however, favour motility and cells switching expression to such proteins can be come highly invasive. For example in prostate cancer, cells with increased expression of $\alpha 6$ $\beta 1$ integrin are found to invade the seminal vesicles (Stewart et al., 2004). There also appears to be a shift in integrin expression in more advanced PCa, as metastatic cell lines express integrins that interact with the bone matrix (Stewart et al., 2004).

Cadherins are classed as cell surface adhesion glycoproteins that contain highly conserved sections that interact with cytosolic proteins, loss of either of these functional partners leads to lack of adhesion and increase the potential for invasion. E-cadherin is an epithelial specific calcium binding protein, loss of which has been implicated in the invasive process of a number of cancers. This cadherin is lost with increasing grade and stage in PCa (Stewart et al., 2004). Conversely, N-Cadherin, which facilitates cell-cell contacts, is up regulated in the advanced stages of PCa. N-cadherin is alleged to facilitate tissue invasion by allowing the cell to interact with the stroma (Mareel and Leroy, 2003). This cadherin switch is not unique to PCa or to cancer states, as it occurs during gastrulation in normal embryonic development (Mareel and Leroy, 2003).

The endopeptidase proteins implicated in invasive progression include MMP's, serine, aspartic, threonine and cysteine proteases (Hanahan and Weinberg, 2000). Local proteolysis is facilitated by proteases outside the cell and thus these proteins are either localised to the membrane in an integral fashion or peripherally attached. Some

proteases are produced in precursor form, secreted and cleaved on the cell surface, a process which often requires a binding site on the extra cellular face of the plasma membrane. These protease-binding sites are often aberrantly expressed in carcinomas, as increasing the binding platforms for such proteins increases the cells invasive potential. Annexin II is an example of such a protease binding platform implicated in metastatic progression (Mai et al., 2000c).

### 1.4 Annexins

The annexins constitute a subgroup of structurally similar calcium dependent phospholipid binding proteins (Gerke and Moss, 2002, Huo and Zhang, 2005, Turnay et al., 2005). Over fifty annexins have been described in 65 different species, thirteen of which occur in vertebrates, including humans and are denoted A1-13 (Turnay et al., 2005, Gerke and Moss, 2002). The structure of A5 was the first to be solved using crystallography in 1990, this revealed five alpha-helical repeats wound into a right-handed super helix, which formed a membrane-binding domain. All annexins possess at least four of these 70 amino acid residue repeats (Huber et al., 1990). In contrast to this conserved core region, annexins possess a highly variable amino terminus which in certain members of the family serves as a site for phosphorylation and protein-protein interaction (Rety et al., 2005). Several human annexins (A1, 2 and 12) interact with members of a second calcium binding protein family, the S100 proteins. Interactions with the S100 proteins have been associated with the function and localization of annexins in both cellular and extracellular environments. In the case of A2 (also known as AII) for example, suppression of its heterotetrameric binding partner S100A10 via small interfering RNA (siRNA) significantly reduces the cell surface expression of AII (Deora et al., 2004).

Although much is known regarding the structural attributes of annexins in their ability to bind negatively charged phospholipids in a calcium dependent fashion, their functional roles within the cell remain largely undocumented. GFP localization studies have indicated that annexins appear to be mostly intra-cellular proteins with possible roles in membrane trafficking and organization (Radke et al., 2004) as they have the ability to shuttle between membrane and cytoplasmic compartments

regulated by intracellular calcium concentrations (Deora et al., 2004, Gerke and Moss, 2002).

Annexin 1 (A1) is the most functionally characterized member of the annexin family. Studies have discovered A1 involvement in diverse cellular mechanisms such as cell signalling (Croxtall et al., 2000), regulation of cell growth and differentiation (Elbtaouri et al., 1994) and apoptosis (Huo and Zhang, 2005). These mechanisms are often deregulated in cancer cells, thus A1 has been implicated in carcinogenesis. Suppression of A1 by RNA interference was recently found to block differentiation of erythroid cells, whilst over expression of A1 increased differentiation of these cells in vitro (Huo and Zhang, 2005). In vivo an increase in A1 expression correlates with differentiation in embryonic development in both mouse (Chepenik et al., 1995) and human cell lines (Fava et al., 1993). A direct link between A1 and differentiation in cancer cells has been observed in the lung adenocarcinoma cell line A549, where induction of differentiation of these cells with phorbol 12-myristate 13-acetate (PMA) treatment leads to an up regulation of A1 expression (Solito et al., 1998). In clinical specimens A1 mRNA and protein expression is reduced in 79% of poorly differentiated oesophageal squamous cell carcinomas (Hu et al., 2004) and shows a 5 fold reduction in breast cancer samples when compared to normal tissue (Shen et al., 2005). The latter study did however only include a small sample size, a total of 2 normal and 7 cancer samples, therefore their findings require confirmation in a larger sample. A1 over expression has also been associated with carcinogenesis in hepatocellular carcinoma, possibly through interactions with the EGFR-MAPK-ERK signalling pathway (Radke et al., 2004, Huo and Zhang, 2005). In addition to its role in differentiation, A1 has also been found to serve a function in the protective process of the inflammatory response. In normal mice treated with sub-lethal doses of lipopolysaccharide (LPS), A1 is up regulated in epithelial cells, neutrophils and macrophages that are all known to be involved in protection from endotoxemia. In the same study when A1 knockout mice were administered a sub-lethal dose of LPS, a toxic response, characterised by organ injury and lethality, occurred within 48 hrs. This could be rescued by administration of exogenous A1 (Damazo et al., 2005).

Annexin II (also known as A2 and AII) is a 36kD calcium dependent phospholipid binding protein that has been shown to be a co-receptor for several proteins including

endopeptidases. The most characterised role of annexin II is in the generation of the serine protease plasmin, a key protein in fibrinolysis (Falcone et al., 2001). In this capacity AII acts as a binding site for the precursor plasminogen and the activator, tissue plasminogen activator (tPA) (Fogg et al., 2002). Annexin II is over expressed in leucocytes of a subset of patients having the hemorrhagic form of acute promyelocytic leukaemia (APL) (Rand, 2000, Deora et al., 2004). Increased surface production of the fibrinolytic protein plasmin by annexin II up regulation may thus be associated with the haemorrhagic complications in these patients (Menell et al., 1999). Annexin II mediated plasmin generation has also been shown to facilitate matrix invasion and degradation by macrophages (Falcone et al., 2001) and neurite development in differentiating PC-12 cells (Jacovina et al., 2001). AII knock out mice display inefficient clearance of injury induced thrombi due to the lack of plasmin generation, these AII null mice also display reduced activation of MMP 9 and 13 (Ling et al., 2004). This finding further implicates AII in cell matrix invasion through the activation or stabilisation of certain MMP's. AII is predominantly found in a heterotetrameric complex with the S100A10 (p11) protein and is located, in vivo, on endosomal and plasma membranes (Zobiack et al., 2002). The protein, however has an independent ability to bind phospholipids and can do so in monomeric and dimeric conformations. See Figure 1.6.

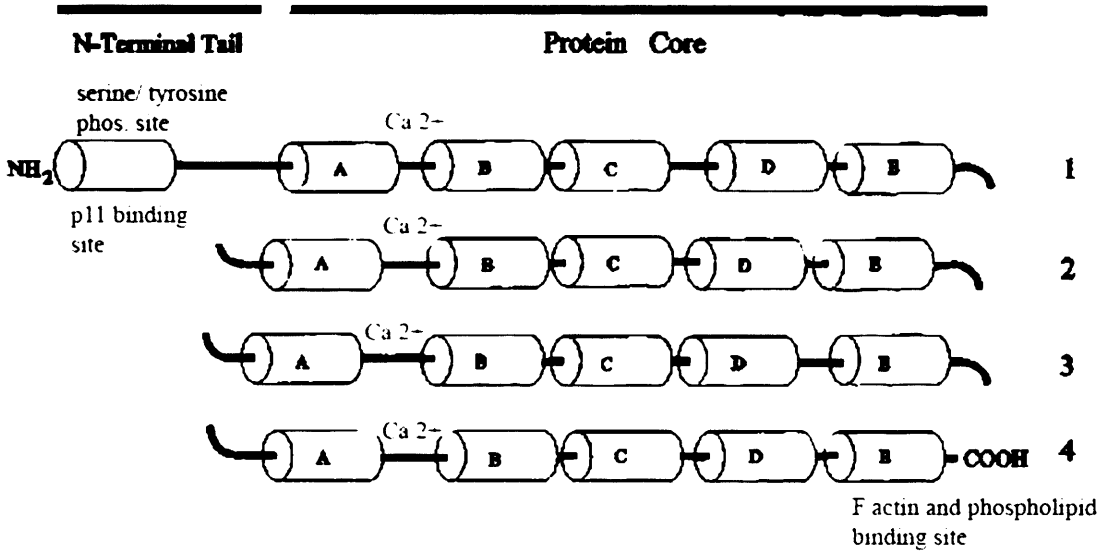



**Figure 1.6.** Schematic representation of the structure of AII, indicating binding sites for various factors. Adapted from (Hajjar et al., 1996).

The AII heterotetrameric complex is formed of two AII and two p11 subunits, which are connected by disulphide bonds making the complex highly stable (Mai et al., 2000c). p11 is an 11kD member of the S100 calcium dependent binding family of proteins and has no other known function other than to bind AII (MacLeod et al., 2003). This AIIp11 complex, when located on the plasma membrane, has several alternative functions in addition to its role in generating plasmin. AII is also a binding platform for the precursor of the cysteine protease Cathepsin B (Mai et al., 2000b). Annexin II is found on the surface of many cell types including neuronal cells (Jacovina et al., 2001), leucocytes (Menell et al., 1999), monocytes, macrophages (Falcone et al., 2001), endothelial cells (Chung and Erickson, 1994, Hajjar et al., 1994, Ma et al., 2000). AII is over expressed in many tumour types and also shows a change in localisation becoming a cell surface protease in tumour cells (Mai et al., 2000c). In addition to stimulating the activity and providing a structural support for endopeptidases cell surface AII is thought to play a role in cell-cell and cell-matrix adhesion through interaction with collagen and high affinity binding of β2-Glycoprotein I to endothelial cells, immunoglobulin transport, endo and exocytosis and Tenascin C signal transduction (Ma et al., 2000). Tenascin C is expressed during normal development and at sites of tissue regeneration and invasion, namely tumours (Ma et al., 2000). Activation of Tenascin C by AII leads to a loss of focal adhesions, enhanced cell division and increased invasive capacity. AII has also been characterised as a binding platform for various pathogens, such as cytomegalovirus and E.coli, indicating that in addition to its role in facilitating extracellular degradation it also acts as a receptor for the transduction of signals to the intra-cellular phase (Depla, 2000).

AII in acting as a binding platform for proteases and ECM components (collagen and Tenascin C), facilitates the remodelling of the basement membrane (Ma et al., 2000). AII present on the outer leaflet of the cellular membrane may also contribute to the cells invasive potential by the plasmin-mediated activation of other metallo-proteases (Kim and Hajjar, 2002). The presence of annexins on the outer leaflet has historically been a contentious issue; however, following a number of localisation studies this is no longer in dispute (Falcone et al., 2001, Chung and Erickson, 1994, Mai et al., 2000a, Brownstein et al., 2003, Kim and Hajjar, 2002, Mai et al., 2000c).

In relation to metastatic progression, AII over expression on the surface of cells has been reported in a number of cancers including acute promyelocytic leukaemia (APL) (Olwill et al., 2005), pancreas (Paciucci et al., 1998), breast (Schwartz-Albiez et al., 1993), lung (Cole et al., 1992, Brichory et al., 2001b), brain (Nygaard et al., 1998), HNSCC (Wu et al., 2002) and colorectal carcinoma (Emoto et al., 2001). In such neoplasms, surface annexin II over expression has been linked to a more aggressive tumour phenotype and concurrent poor prognosis (Emoto et al., 2001, Wu et al., 2002). The potential role of AII in basement membrane remodelling and its prevalence in aggressive tumours suggests that it is an important protein in the invasive potential of tumour cells. The link between AII over expression and an aggressive phenotype was recently explored in macrophages where it was found to be correlated with an invasive and degradative phenotype (Falcone et al., 2001). AII expression has been explored in a number of cancers where it has also been found to promote metastatic growth via the evasion of host-derived and chemo-therapeutic agents (Falcone et al., 2001). This research indicates that AII secreted at high levels in sera from patients with renal carcinoma, may have an immunosuppressive effect (Mai et al., 2000c). This immunosuppressive effect may be necessary for the maintenance of surface AII and therefore invasive potential, as auto antibodies to surface exposed AII are frequently observed in patients with lung cancer (Brichory et al., 2001a). In addition, it has been shown that AII and Activated Leukocyte Cell Adhesion Molecules (ALCAM) are involved in the metastatic progression of tumour cells following chemotherapy with adriamycin (Choi et al., 2000). AII has a potential role in several of the stages in the multi-step process of metastatic progression, see Figure 1.4, as due to its role in plasmin production it facilitates angiogenesis and in addition potentiates the invasive capabilities of this and other endopeptidases in remodelling of the basement membrane. The activation of Tenascin C by AII aids the separation of the cell from the primary tumour and the presence of AII in sera may assist in the evasion of host responses (Murphy-Ullrich, 2001). It appears that AII has a remarkable effect on invasive capacity, this protein therefore, has a potential therapeutic use as a target for the restriction of invasion and therefore the treatment of advanced cancer.

### 1.4.1 Annexin II in prostate cancer

AII is over expressed in cancer cell lines and in vivo where it is commonly localized to areas of basement membrane invasion and metastatic foci (Liu et al., 2003a). In contrast, research suggests that in prostate cancer AII expression is lost during the progression to a cancerous phenotype. The loss of AII expression in prostate cancer cell lines has been widely documented (Smitherman et al., 2004, Banerjee et al., 2003, Kirshner et al., 2003, Liu et al., 2003a, Liu et al., 2003b, Fakih et al., 2002, Kumi-Diaka, 2002, Tekur and Ho, 2002, Chetcuti et al., 2001b). A comprehensive study of five normal prostate cell lines and seven cancer cell lines found that AII was expressed strongly in the cytoplasm and nucleus of normal cells, with cancer cells exhibiting a reduced and altered pattern of expression. The same study found that a reintroduction of AII to LNCaP cells, known to lack AII, inhibited the migratory capacity of these cells (Liu et al., 2003b). This result is contrary to many studies indicating that over expression of AII in cancer cells increases their invasive potential (Podgorski and Sloane, 2003, Mai et al., 2000b, Diaz et al., 2004). The expression pattern of AII in prostate cancer cell lines is unclear, as several studies have suggested that reduced expression of AII is observed in PC3 and DU145 in comparison to normal prostate cell lines (Liu et al., 2003b). However, there is evidence to suggest that these two cell lines do express high levels of AII (Banerjee et al., 2003). The only cell line consistently reported as lacking AII expression is LNCaP. A recent study employing subtractive hybridization and Northern blot analysis of LNCaP cells suggests that Annexin II heavy and light chains are frequently lost in prostate cancer and in its precursor, prostatic intraepithelial neoplasia, due to hyper-methylation of the *p36* gene (Chetcuti et al., 2001a). Further research however has indicated that hyper-methylation is not the only cause of AII suppression as treatment with 5-aza-deoxycytidine failed to promote re-expression of AII (Banerjee et al., 2003).

There have been several reports of loss of AII expression in clinical tissue from primary prostate carcinoma. A reduction in AII expression was observed in a panel of prostate tumour samples with Gleason grades ranging from 6-10. However, 25% of the samples with a Gleason score of >8 displayed focal AII expression on the cell

surface (Banerjee et al., 2003). In this study, it was alleged that AII is lost in early stages of PCa and re-expressed at the later undifferentiated cancer stage (Banerjee et al., 2003). However, a subsequent study into AII expression in various stages of PCa found that AII expression decreased by 71% from BPH to androgen stimulated prostate cancer (AS-CaP) and declined a further 31% from AS-CaP to recurrent (androgen insensitive) prostate cancer (R-CaP). No evidence of AII re-expression in advanced cancer was observed in this study (Smitherman et al., 2004). Consideration of the literature indicates that further research is required to explain the deregulation of AII expression and its importance in prostate carcinogenesis and progression.

### 1.5 Rationale

This PhD project originated from a 2D gel-based proteomic analysis of a matched pair of isogenic prostate cell lines. This study consisted of global protein analysis of the cell lines which resulted in the identification of 22 proteins differentially expressed between the normal and cancer cell lines. Nine proteins were found to be down regulated in the cancer cell line including HSP70, Bip and MxA. Thirteen proteins were up regulated in the cancer cell line including Cathepsin D, gp96, HnRNP-K and PSMA5 (Nagano et al., 2003). This PhD project extended the characterisation of the isogenic cell lines through comparative analysis of the plasma membrane proteome.

The central aim of this PhD project was to analyze proteins differentially expressed on the surface of normal and neoplastic prostate epithelial cells using a proteomic approach, with a view to identifying proteins with potential value as diagnostic or therapeutic protein markers. The hypothesis to be tested was that there are differences in the plasma membrane proteome between normal and cancer cells that confer a determinative phenotype and are instrumental in the carcinogenic process.

The initial objective was to develop a reproducible and efficient method for extracting and analyzing cell surface proteins. This was required due to the poor representation of plasma membrane proteins commonly encountered with standard proteomic methods which include hydrophobic, high molecular weight and basic proteins (Naaby-Hansen, 2004, Fey and Larsen, 2001). The optimized cell surface analysis technique was then applied to the comparative analysis of the cell surface proteome of

1542 NPTX and CP3TX cells. The 1542 NPTX and CP3TX cell lines were the chosen model for this analysis because due to their isogenic nature and their commonality in transformation, any differences in their protein expression profile are likely to be a result of the carcinogenic process (Bright et al., 1997). The cell surface proteome of these isogenic cell lines was chosen for comparative analysis because it not only contains factors and receptors involved in many of the signal transduction pathways altered in cancer cells, but also due to its accessibility in terms of providing diagnostic, prognostic or therapeutic targets for the management of the disease.

In addition to global analysis of protein expression in the isogenic prostate cell lines, the earlier work investigated the effect of stimulation with interferon α and γ on protein expression in the isogenic cell lines. Stimulation with IFNγ caused a decrease in protein synthesis which was 2-fold greater in the cancer cells (Nagano et al., 2003). Several proteins displayed differential expression in normal and cancer cell lines when stimulated with IFNγ, these included MxA, Nmi and HnRNP-K. Approximately one third of the proteins in this study showing differential regulation between normal and cancer cells in response to IFNγ had some role in endoplasmic protein processing and many represented the products of interferon inducible genes. This indicated that IFN-induced signalling pathways were possibly altered in the prostate cancer cell line 1542CPTX (Nagano et al., 2003).

As an extension of this work, IFN signalling was studied through application of the optimized cell surface analysis technique to 1542 NPTX and CP3TX cells in the presence or absence of stimulation with IFNγ. IFNγ induced signalling was chosen for analysis due to the previous findings that this cytokine induced differential protein expression in cancer cells and in light of its reported potential use as a tumor and metastasis suppressor, in the retardation of tumourigenesis and metastatic progression (Shou et al., 2002, Nagano et al., 2003, Selleck et al., 2003).

The analysis of IFNγ signalling identified several cell surface proteins with patterns of expression modulated by IFNγ. One of these proteins, identified as Annexin II, was chosen for further characterization due to its reported role in ECM invasion and basement membrane remodelling, processes integral to the spread of prostate cancer to surrounding tissues (Falcone et al., 2001, Ma et al., 2000).

In summary the aims of this PhD project were;

- To optimize a biochemical method for specifically extracting and separating membrane proteins.

- To identify the differences in expression of membrane associated proteins between normal and cancerous prostate cells.

- To identify any differences in the expression of cell surface proteins and those in the IFN signalling pathway between normal and cancer prostate cells, that may contribute to the difference in IFN-induced membrane protein expression.

- To investigate the functional role of key identified proteins and confirm differential expression using several cell lines.

Completion of these objectives should result in greater knowledge of differences in the cell surface proteome of these isogenic cell lines and as they share a single genomic origin and indicate the cell surface proteins involved in carcinogenesis or metastatic progression. This study should also increase the knowledge of IFNγ signalling in these cell lines and indicate whether this cytokine could be of potential therapeutic use in prostate cancer.

# Chapter 2

# Materials and Method

## 2.1 Materials

The following section details the materials used within this study and their source. The cell lines, solutions, antibodies and buffers used are also outlined.

### 2.1.1 Cell lines

The cell lines used in this study were primarily adherent epithelial cells derived from normal prostate tissue, prostate tumour or metastasis, see table 2.1.

**Table 2.1.** Human prostate cell lines used in this study, their origin and growth media.

| CELL LINE | ORIGIN | MEDIA |
|---|---|---|
| **1542 CP3TX** | **Primary adenocarcinoma (Bright et al., 1997)** | Keratinocyte serum free (KSFM), 5% foetal bovine serum (FBS), Bovine pituitary extract (BPE) 1.5ml, 0.2 ng/ml Epidermal Growth Factor (EGF), 100 U/ml penicillin, 100 µg/ml streptomycin (PS). |
| **1542 NPX** | **Benign Prostate epithelium (Bright et al., 1997)** | KSFM, 10% FBS, BPE, EGF, PS |
| **1532 CPT** | **Primary adenocarcinoma (Bright et al., 1997)** | KSFM, 10% FBS, BPE, EGF, PS |
| **1532 NP** | **Benign Prostate epithelium (Bright et al., 1997)** | KSFM, 10% FBS, BPE, EGF, PS |
| **1535 CPT** | **Primary adenocarcinoma (Bright et al., 1997)** | KSFM, 10% FBS, BPE, EGF, PS |
| **1535 NP** | **Benign Prostate epithelium (Bright et al., 1997)** | KSFM, 10% FBS, BPE, EGF, PS |
| **PC3** | **Prostate cancer bone metastasis (Kaighn et al., 1979)** | RPMI 1640, 20% FBS, PS, L-Glutamine |
| **Pre 2.8** | **Normal prostate epithelium (Alam et al., 2004)** | RPMI 1640, 20% FBS, PS, L-Glutamine PrEGM at 33°C |
| **LNCaP** | **Prostatic cancer lymph node metastasis (Horoszewicz et al., 1980)** | RPMI 1640, 20% FBS, PS, L-Glutamine |
| **DU145** | **Prostate cancer brain metastasis (Mickey et al., 1980)** | RPMI 1640, 20% FBS, PS, L-Glutamine |

The cell lines used most extensively within this study were 1542 NPX and CP3TX, a pair of isogenic cell lines derived from a radical prostatectomy specimen. These cell lines were immortalized by transduction at the third passage with a recombinant retrovirus encoding the E6 and E7 transforming proteins of the human papilloma virus (See Figure 2.1) (Bright et al., 1997). The epithelial and prostatic origin of these cell lines was confirmed by immunocytochemical analysis of cytokeratin and prostate specific marker (PSA, PAP) expression. Due to the heterogeneous nature of prostate cancer, primary cultures derived from fresh tumours often contain a mixture of benign and malignant cells. To confirm the identity of the 1542 CP3TX cell line a panel of eight microsatellite markers were examined for loss of heterozygosity (LOH) by PCR. These markers are located on chromosome 8p, a chromosomal region displaying a high degree of allelic loss in primary prostate tumours and thus postulated to be the sites of putative tumour suppressor genes (Bergerheim et al., 1991, Vocke et al., 1996). This panel of microsatellite markers has been previously shown to detect LOH in microdissected prostate cancer specimens with a high degree of reliability (Vocke et al., 1996). 1542 CP3TX displayed LOH at four of the loci examined whilst in 1542 NPX LOH was not detected. No LOH was detected in seminal vesicles or fibroblasts from the same patient indicating that the LOH was specific to the prostate cancer cells. 1542 CP3TX was subsequently characterised as representing a prostate cancer cell line (Bright et al., 1997). These 1542 cell lines therefore represent a unique opportunity to study the origin of cancer from a single genome perspective, where differences between the cell lines may be solely carcinogenic.

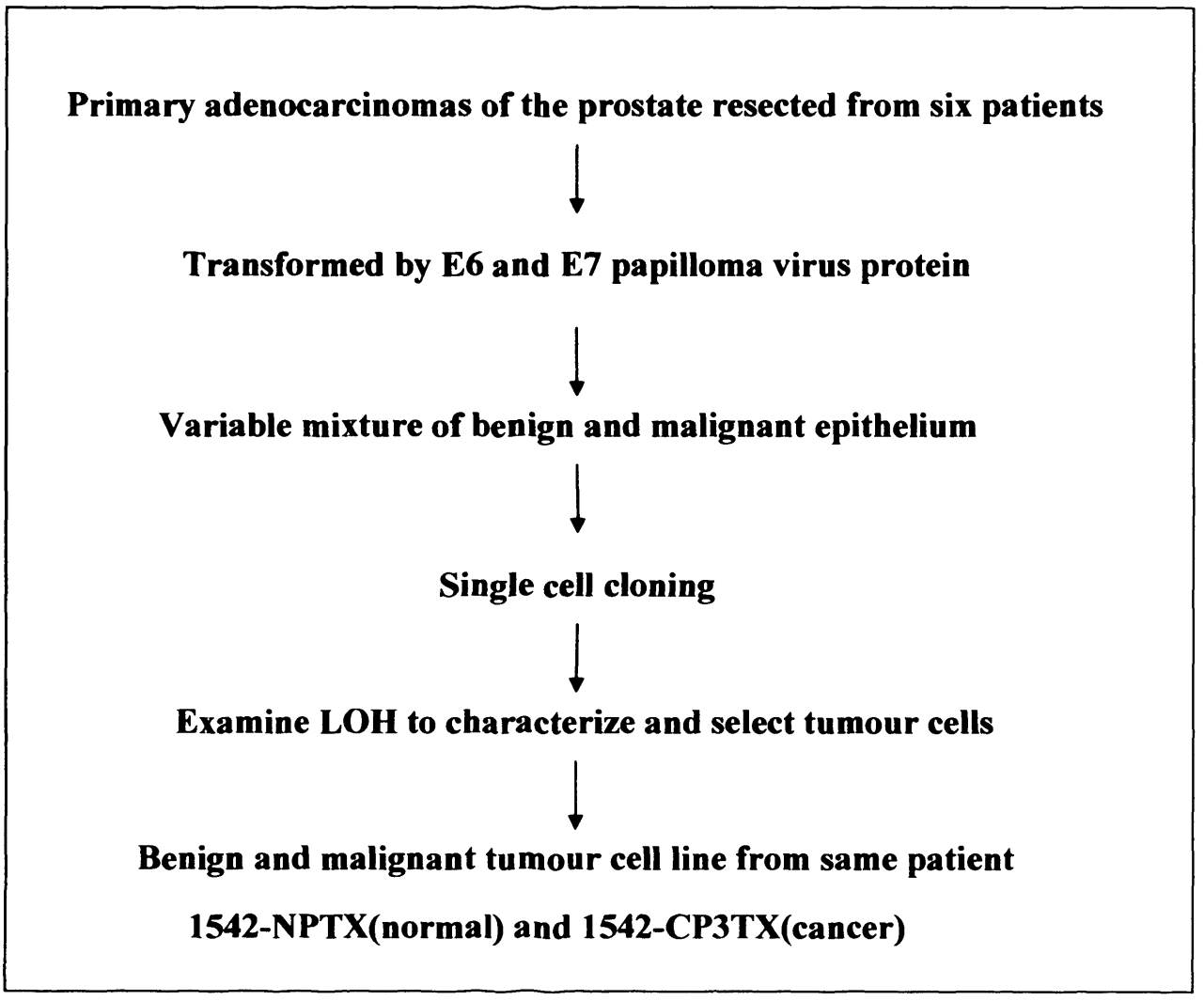



**Figure 2.1** A schematic representation of the formation of 1542 NPTX(normal) and 1542 CP3TX (cancer) cell lines, adapted from (Bright et al., 1997).

The media in which the various cell lines were cultured were obtained from Invitrogen; all additives were available from central services in the Ludwig Institute for Cancer Research (LICR) except for those needed to complete KSFM, which were also purchased from Invitrogen. In certain experiments, it was necessary to exclude one or more components of the media and/or to add IFNγ at a concentration of 500 units/ml (Sigma). All cell lines were obtained from Prof. J Masters at the Prostate Cancer Research Centre, UCL.

### 2.1.2 Source companies

This section includes the source companies for the materials and reagents used in this study, see table 2.2.

**Table 2.2.** The source companies for the materials used in this study and their location.

| | |
|---|---|
| Amersham Biosciences | Little Chalfont, Buckinghamshire, UK. |
| BD biosciences | Oxford, UK. |
| Bio-Rad | Hemel Hempstead, Hertfordshire, UK. |
| Calbiochem | Nottingham, UK. |
| Cambrex | Nottingham, UK. |
| GRI | Braintree, Essex, UK. |
| Invitrogen | Paisley, UK. |
| Merck Biosciences, Calbiochem, Novagen | Nottingham, UK. |
| Molecular probes | Paisley, UK. |
| New England Biolabs | Hitchin, Hertfordshire, UK. |
| Novus Biologicals | Littleton, USA. |
| Nunc | Hereford, UK. |
| Perbio | Tattenhall, Cheshire, UK. |
| Qiagen | Crawley, West Sussex, UK. |
| Santa Cruz Biotechnology | Heidelberg, Germany. |
| Sigma-Aldrich Ltd | Poole, Dorset, UK. |
| Upstate | Milton Keynes, UK. |
| VWR | Poole, Dorset, UK. |
| Watson Marlow | Falmouth, Cornwall, UK. |
| Zymed | Cambridge, UK. |

## 2.1.3 Antibodies and fluorophore conjugates

This section includes the antibodies and fluorophore conjugates used in this study and details their source and optimised concentrations for use in various immunoassays, see Table 2.3.

**Table 2.3.** Detailing the concentrations of antibodies and fluorescent conjugates used for various experimental techniques in this study including immunodetection on Western blot (WB), immunofluorescence (IF) and immunoprecipitation (IP).

| **Antibody/ fluorophore conjugate** | **Company** | **Dilution used in WB** | **Dilution used in IF** | **Dilution used in IP** |
|---|---|---|---|---|
| **Anti-ABCA1 rabbit polyclonal** | Novus Biologicals | 1:100 | - | - |
| **Anti-annexin II** | Zymed | - | - | 1:50 |
| **Anti-GRP 94 (gp96).** | Santa Cruz Biotechnology | 1:3000 | 1:100 | - |
| **Anti-pro-cathepsin B** | Upstate | 1:1000 | - | - |
| **Anti-plasminogen** | Abcam | 1:1000 | - | - |
| **Anti-β actin** | Sigma | 1:1000 | - | - |
| **Anti-annexin II** | BD Biosciences | 1:5000 | 1:100 | - |
| **Anti-annexin II light chain (p11).** | BD Biosciences | 1:200 | 1:100 | - |
| **Anti-Rabbit IgG** | Amersham Biosciences | 1:3000 | - | - |
| **Calpain II (C-19). antibody** | Santa Cruz Biotechnology | 1:100 | - | - |
| **Cholera toxin β subunit** | Molecular probes | - | 1:100 | - |
| **Fluorocein goat anti-mouse IgG** | Molecular probes | - | 1:100 | - |
| **Goat anti-rabbit IgG Texas Red** | Molecular probes | - | 1:100 | - |
| **Mouse IgG** | Amersham Biosciences | - | - | - |
| **Mouse IgG, HRP-Linked Whole Ab** | Amersham Biosciences | 1:5000 | - | - |
| **Phalloidin** | Molecular probes | - | 1:100 | - |
| **Phospho-Threonine-Proline Monoclonal Ab** | New England Biolabs | 1: 100 | - | - |

2.1.4 Buffers

This section details the recipes for the various buffers used in this study and the source company of the reagents used.

**Lysis Buffers**

1% Octylglucopyranoside (OGP)

1% OGP (Sigma) in 1 X PBS (Invitrogen)

1 complete protease inhibitor tablet (Amersham) per 50ml

2D Lysis buffer

4% Chaps (Sigma)

8M Urea (Sigma)

2% NP40 (Sigma)

Complete protease inhibitor tablet, as above

Phosphatase inhibitors (Amersham)

Leupeptin 1μg/ml

Aprotinin 1μg/ml

Pepstatin 1μg/ml

**Labelling Buffers**

Blocking buffer

50mM NHCl (Sigma) in 1x PBS

Biotin

5mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Perbio) in 1 x PBS

5mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Perbio) in 1 x PBS

**Sample Buffer**

62.5mM Tris-HCl, pH 6.8 (Sigma)

20% Glycerol (Sigma)

2% SDS (Sigma)

5% β- Mercaptoethanol (Sigma)

**Running Buffer (1x)**

25mM Tris

192mM Glycine (Sigma)

0.1% SDS

pH 8.3

**Live cell immunofluorescence buffer**

9.9g Hanks BSS (Cambrex)

5ml 10% sodium azide (Sigma)

50ml heat inactivated donor calf serum (Invitrogen)

4.76g Hepes free acid (Sigma)

5.2g Hepes sodium salt (Sigma)

Double distilled water ($ddH_2O$) to 1l

**Western Transfer Buffer**

20mM Tris

192mM Glycine

0.1% SDS

20% MeOH (VWR)

**Equilibration buffer**

6M Urea

2 % SDS

1% DTT (Sigma)

30% Glycerol

50mM Tris pH 6.8

**Agarose**

0.5% Agarose (Sigma)

In 1x running buffer with 0.03 % Bromophenol Blue (Sigma). added

**Storage buffer**

0.2% sodium azide in 1 x PBS

**Affinity chromatography buffers**

Binding

1% OGP lysis buffer

Elution

100mM DTT

8M Guanine HCl (Sigma)

Regeneration

8M Guanine HCl

Wash buffers

1% OGP

1% NP40

**TBST**

25mM Tris-HCl, pH 8.0

125mM NaCl

0.1% Tween 20 (Sigma)

**Coomassie staining solution**

0.2% R250 (Sigma)

40% MeOH

10% Acetic acid (VWR)

**Coomassie destain**

10%MeOH

5% Acetic acid

## 2.2 Methods

The practices and procedures used throughout this thesis are described in the following methods section.

### 2.2.1 Cell Culture

All cell lines were obtained from Prof. Masters in either T-80 or T-25 cell culture flasks (Nunc) and detached using 0.25% trypsin (Invitrogen). The cells were split from one into three new flasks containing the appropriate media (see table 2.1 above). These cells were then incubated at 37 °C, in an atmosphere of 5% $CO_2$ in air and fed twice weekly until reaching 80% confluence at which point they were either split again or used for experimental study. Cells were kept in continuous culture for no longer than 3 months or 10 passages, whichever was the sooner, to avoid potential phenotypic or genotypic changes associated with long-term culture. Cells were observed for signs of infection, overcrowding or starvation with the aid of an inverted microscope. All cell handling was performed in a cell culture hood with a laminar airflow system and aseptic technique was used to prevent IFNection. This protocol was adapted from (Hudson et al., 2000)

### 2.2.2 Counting cells

When using cells in experimental procedures it became necessary to accurately measure the number of cells present within a given solution. The general protocol for this procedure is as follows, although time to detachment differed within each cell line.

Spent medium was removed and trypsin added at a volume of 1ml per $25cm^2$. The trypsin was distributed over the growing surface and the excess was removed. Cells were then incubated for approximately 5 minutes at 37°C and observed for signs of detachment. When all cells were floating in the trypsin solution 10ml of medium was used to wash the cells from the growing surface, this solution was then transferred to a universal container (Nunc). The cell solution was mixed thoroughly and a 200µl aliquot was removed to an eppendorf tube (VWR). This aliquot was then diluted with 200µl Trypan Blue (Sigma) and mixed thoroughly. A small drop of the diluted cell solution was added to both edges of the cover slip on a haemocytometer and the cells

counted with an inverted microscope at x10 magnification. At least 100 cells were counted for an accurate representation of the cell population; dead cells (stained blue with trypan blue) were not counted. The number of cells present within the original solution was estimated using the following calculation.

Volume of a square = $0.1mm^3 = 1x10^{-4}ml$
Therefore, multiply by 10000 to get number of cells per ml

The sample was originally diluted with an equal volume of Trypan blue so to calculate the cells per ml the above figure was multiplied by 2, which equals $28 \times 10^4$

$28 \times 10^4$ = 280,000 cells per ml

The original solution was 10ml hence;

280,000 x 10 = 2,800,000 cells in the original solution (10ml).

This protocol was adapted from (Caprette, 2000).

2.2.3 Freezing cells

To maintain long term cellular integrity it was necessary to transfer the cells to long-term frozen storage (except the isogenic cell lines 1542, 1535 and 1532, frozen stocks of which were retained by Prof. Masters). The protocol for this was as follows.

The cells were cultured and counted as above, then spun down in a universal container and re-suspended in freezing medium consisting of RPMI + 30% FBS + 10% DMSO. 1ml aliquots of this solution containing the cells were put into 1ml cryotubes (VWR) and placed in a box containing isopropanol. This box was then placed immediately into a -80°C freezer for 24 hrs. The final step was to transfer the cryotubes containing the cells into liquid nitrogen, noting the position of the aliquots.

It was necessary, when frozen cells were required, to defrost them quickly in a water bath to avoid formation of ice crystals that may affect the integrity and viability of the

cells. Once defrosted the cells were transferred to a centrifuge tube, spun down, re-suspended in the appropriate medium and transferred to a tissue culture flask. The cells were then cultured following the standard protocol (see section 2.2.1). Protocol adapted from (Warnasuriya, 2001).

### 2.2.4 Cell Lysis

Although several lysis buffers were used in this study, depending on the different requirements of the experimental procedure, the basic protocol for lysis was identical. The cells were observed for their degree of confluence and providing they were at least 70% confluent, were selected for lysis and put on ice. The old medium was removed and the cells were washed three times with cold 1x PBS. Following the final wash, all traces of PBS were removed by aspiration and a volume of lysis buffer was added. The amount of lysis buffer added was dependent on the area of the growing surface and the requirement for sample concentration. The lysis buffer was run over the growing surface several times within 1 minute, following which the cells were removed from the growing surface into the buffer using a rubber scraper (VWR). Subsequently the lysis buffer containing the cells was aliquotted into an eppendorf and spun down for 1 min at 13000rpm. The supernatant was transferred to a clean eppendorf, labelled and then stored frozen or used immediately. The pellet, containing nuclear components and cell debris was also retained. This protocol was adapted from (Nagano et al., 2003).

### 2.2.5 Protein concentration assay

Following cell lysis, it was necessary to measure the amount of protein within the sample. The protein concentration assay varied according to the lysis buffer. Commonly a Bradford protein concentration assay (Perbio) was used. However, lysis buffers such as 1% OGP interfere with this assay and in this case, a Lowry assay was used (Bio-Rad). The protocols for these assays are described in Appendix 2.

### 2.2.6 Biotin Labelling

To selectively enrich the cell surface protein fraction of the sample it was necessary to label surface proteins so they could be purified by affinity chromatography. The label

chosen was a biotin construct that binds to proteins at their lysine residues. Two different types of biotin tag were used in this study, a long chain Sulfo-NHS ester, EZ-Link Sulfo-NHS-LC-Biotin and a reducible form with a cleavable disulphide bond in the spacer arm, EZ-Link Sulfo-NHS-SS-Biotin (Perbio). The cell labelling protocol detailed below was used for both forms.

The required solutions, biotin, blocking buffer, 1% OGP and 1x PBS, were chilled in advance. The cells were washed 3 times in PBS to minimise contamination from media and cell debris during labelling, during the final wash the cells were observed for any loss of adhesion. The wash solution was removed, 5ml of biotin solution added and incubated for 5 mins on ice. Excess biotin solution was removed, the quench solution added and incubated for 10 mins on ice, the cells were again checked for signs of detachment. Following this procedure, the cells were washed again in PBS and left in the final wash solution. The PBS was carefully removed to prevent dilution of the lysis buffer and the cells lysed using 500µl 1% OGP and the protein concentration assayed using the Lowry method. This protocol was adapted from (Naaby-Hansen et al., 1997).

### 2.2.7 Affinity Chromatography

Samples for affinity chromatography were prepared using three T-80 cell culture flasks of 1542 NPX and CP3TX each containing approximately 750,000 cells. These cells were labelled and pooled into 500µl 1% OGP lysis buffer to increase the protein concentration and assayed as above using the Lowry method (see Appendix 2). A control sample of 1542 NPX cell lysate was prepared following the same method, with the exception that the cells were unlabelled. Several methods of affinity chromatography were compared during this study including the use of various avidin columns. The protocol that follows is the optimised form of affinity chromatography used in the majority of experiments. The elution step varies depending on the type of biotin used. The two alternatives used are described.

All solutions (see above under affinity chromatography solutions), were allowed to equilibrate at room temperature. Meanwhile immobilised Neutravidin beads (Perbio) were packed into three 1ml columns following the protocol described in Appendix 3.

These columns were then clamped upright and attached to a peristaltic pump (Watson Marlow) at a flow rate of 0.5ml/min and equilibrated by the addition of 10ml binding buffer. The sample was then applied to the column and the effluent collected. The sample was left to incubate in the column for 30mins. Following this the effluent was re-applied and run-through the column. This process was repeated and the final effluent collected. The column was then washed with 10ml binding buffer from which two 5ml aliquots of the wash buffer were retained. The sample was eluted from the column in one of two ways, described as follows:

1. For SS biotin labelled cells, 1ml of DTT elution buffer was added and left to incubate on the column for 15mins. This buffer was drawn off, reserved and a further 1ml of elution buffer applied. This process was repeated once more and finally 1ml of regeneration buffer was added and the flow through retained. The fractions were pooled giving a final elution fraction of 4ml. The column was regenerated with a further 9mls of regeneration buffer and then stored with the addition of 5mls of storage buffer, at 4°C.
2. For the LC biotin labelled samples, the elution protocol was the same as above except using 5mls of Guanine HCl as the elution buffer.

The protein concentration in the elution fractions was found to be extremely dilute and it was necessary therefore to dialyse the fractions using 10,000 molecular weight dialysis cassettes (Perbio) followed by vacuum concentration overnight at a low heat setting. The fractions were then stored as a frozen pellet, or if used immediately reconstituted in $ddH_20$ and subjected to a Bradford assay (as in Appendix 2).

2.2.8 Calcium Dependent Release

For the study of cell surface AII and its response to IFNγ, it was necessary to devise a simple way of enriching the cell surface fraction of this protein in the sample. Enrichment was achieved using two different approaches. The first method used $CaCl_2$ solution at 0, 0.5, 1 and 1.5mM, to compete with the calcium binding of AII to the cell surface. Following three washes with 1x PBS, 5ml of the solution was applied to the cells and left to incubate for 5 mins. The solution was then removed, vacuum concentrated and the pellet treated as described for the eluate above.

The EDTA based method of extraction followed the same protocol as above, but used EDTA concentrations of 0, 0.5, 1, 5 and 50mM. This experiment demonstrated that 0.5 to 1mM EDTA provides the highest yield of cell surface AII. Therefore, the optimised protocol for subsequent experiments was as follows. 750,000 cells were required for each condition or cell line under study, which were maintained on ice throughout the procedure. The growing surface of these flasks was washed with ice-cold 1x PBS three times, aspirated thoroughly and 1ml 0.5mM EDTA added. The EDTA solution was run over the growing surface several times for 1min. The solution was then removed, vacuum dried or subjected to a Bradford protein assay (see Appendix 2).

### 2.2.9 1D SDS PAGE

To resolve, characterise and identify the proteins present within the various samples it was necessary to separate them on a resolving gel. 1D SDS PAGE was the method of choice because of its suitability for membrane protein analysis. In this study, a range of acrylamide gels were used. The formulation of these gels is shown in Table 2.4. All gels were either cast using the Bio-Rad (commonly) or Hoeffer (longer gels for greater resolution) casting systems (Amersham). Large format gels were used most often because of their increased resolution. However, in some cases where only one protein required identification the mini gel protean system was used (Bio-Rad). The amounts below apply only to gels cast on 16x20 cm Bio-Rad plates. Other systems required modifications to the amount, but not proportion of the constituents listed below.

**Table 2.4.** **A** Recipes for 1D SDS PAGE running gels of varying acrylamide concentrations. All values are in ml. **B** Recipe for 4.5% stacking gel, all values in ml.

**A**

| | 10% | 12.5% | 15% |
|---|---|---|---|
| 30% Bis-Acrylamide (Bio-Rad). | 10 | 12.5 | 15 |
| 1.5M Tris HCl pH 8.8 (Sigma). | 7.5 | 7.5 | 7.5 |
| 10% SDS (Sigma). | 0.3 | 0.3 | 0.3 |
| $ddH_20$ | 12.15 | 9.5 | 9 |
| 10% APS (Bio-Rad). | 0.15 | 0.15 | 0.15 |
| Temed (Sigma). | 0.02 | 0.02 | 0.02 |

**B**

| | |
|---|---|
| 30% Bis-Acrylamide | 1.35 |
| 0.5M Tris HCl pH 6.8 | 2.25 |
| 10% SDS | 0.09 |
| $ddH_20$ | 5.35 |
| 10% APS | 0.038 |
| Temed | 0.011 |

The gel mixture was prepared using the proportions given above, leaving addition of Temed until last to prevent premature setting and mixing well at each step. The mixture was then added to clamped plates of the appropriate size, pre-cleaned with $ddH_20$, set in the casting system. The gel was then overlaid with enough $ddH_20$ to cover the exposed top of the gel and create a straight edge. The gel was then left to set for 1 hour. Each resolving gel required a stacking gel, the recipe for which is shown in Table 2.4. Again, these amounts apply only to the larger gel size quoted above. The water was removed from the top of the set resolving gel and the stacking gel applied. A sample comb of sufficient well number and size was then added and the gel left to set for 30mins.

Meanwhile the samples were prepared. Accurate determination of the protein concentrations allowed equal protein aliquots of each sample to be prepared. These aliquots were then diluted with a third of the volume of 3x sample buffer. The choice of sample buffer, with or without reductant, depended on whether the sample contained reducible biotin in which case the non-reducible form was used. The

samples were then mixed and heated to 95°C for 1min. In some cases where there was a necessity to resolve large proteins the heating step was omitted. For ease of explanation, the following applies to the Bio-Rad protean II system and was modified for other electrophoresis systems. Once set the sample comb was removed, the gel was attached to the cooling unit of the electrophoresis system. The gel was then placed in the electrophoresis tank and 1x tank buffer added so that both exposed ends of the gel were covered and the top reservoir full. The samples were then applied to the gel, taking care not to cause any overspill to neighbouring wells. A volume of broad range rainbow marker (Amersham) appropriate to the size of the gel was also loaded. The electrophoresis conditions were dependent on the size of gel and the system used. Generally, the Bio-Rad gels were run for 1hr at 40mA per gel with cooling and then overnight at 7mA. Some gels were run quickly at 50mA for 4 hrs with cooling.

The preparation of samples, casting and running of gels for mass spectroscopy was done solely in a clean room environment to reduce the risk of contamination. This protocol was adapted from (Amersham, 2005b).

2.2.10 Western Blotting

To immobilise the resolved proteins in the gel onto a medium more suited to antibody detection, the proteins in the gels were transferred to nitrocellulose paper using the wet transfer western blotting technique. For this the Bio-Rad protean II system was used. The nitrocellulose and filter paper were cut to appropriate size (to cover the gel surface) and marked in one corner for orientation of the gel. All equipment, tray, sponges, frames and tank, were washed in dd$H_2O$. The sponges and nitrocellulose were then soaked in a volume of transfer buffer large enough to submerge the frames and sponges in a tray or container. The frames were then submerged in transfer buffer. The gel was removed from the electrophoresis tank and from the plates and the stacking gel portion (or agarose and IEF strip in the case of 2D gel electrophoresis) was carefully removed. The western transfer materials were then compiled, submerged in transfer buffer in layers of the following:

- Frame
- Foam

- Filter paper
- Gel
- Nitrocellulose (orientating the mark on the paper with a feature of the gel).
- Filter paper
- Foam
- Frame

Once the layers were assembled, a clamp was applied and the frame was taken out of the buffer and placed in the western transfer tank, ensuring that the gel side faced the negative electrode. The tank was then filled to the top with 1 x transfer buffer and run either for 3 hours with cooling at 300mA, or overnight at 60mA. Once transferred the gel was discarded, the membrane washed in TBS-T and stored in dry, protective conditions. This protocol was adapted from (Amersham, 2005d).

### 2.2.11 Coomassie staining

To visualise the proteins directly whilst still in the gel it was necessary to apply a general protein stain. Coomassie blue was chosen for its ease of use, there follows a description of the two main coomassie staining methods used in this study.

**Standard Coomassie staining**

The gel was removed from the electrophoresis apparatus and glass plates and the stacking gel removed. The gel was then put into a clean plastic tray, covered with 100ml of filtered coomassie blue solution and left to incubate overnight on a rocking platform at slow speed. Following this the coomassie solution was carefully removed and the gel was washed in de-stain solution until the background appeared reasonably clear. The gel was then ready for scanning, quantitation of bands, or cutting of bands for mass spectroscopy. The latter required all preparation, running and staining of gels to be done in a clean room environment. This protocol was adapted from (Amersham, 2005c).

**Biosafe Coomassie staining**

This method was used when more sensitive detection of proteins was required. It also has the added benefit of requiring less incubation time. The manufacturer's protocol used in this study is described in Appendix 34.

2.2.12 Silver Staining

When extremely sensitive protein detection was required, silver staining was performed. As the usual method is not compatible with mass spectroscopy, a modified form of staining was used. This protocol was based on the Shevchenko technique (Shevchenko et al., 1996) and is described in Appendix 5.

2.2.13 2 Dimensional Gel Electrophoresis (2DGE)

To analyse certain proteins more thoroughly, especially with regard to their post translational modifications, it was necessary to perform 2DGE to separate proteins depending on their isoelectric point and mass. The protocol for this is described below.

**First dimension/ Isoelectric focussing (IEF)**

The first step of 2DGE was performed on Immobiline dry strip gels (Amersham, Bio-Rad) of varying pH range. Those most frequently used in this study were pH 3-10 or 3-6. The gels were thawed, labelled and left on a glass plate, while the other reagents, including mineral oil, IPG buffer (Amersham) or Ampholyte mix (Bio-Rad), 2D lysis buffer and bromophenol blue were brought to room temperature. The strips were re-hydrated before use in a solution containing:

IPG buffer/ampholyte mix at 2% of the final volume
Sample at a concentration of 150 µg/µl
2D lysis buffer* up to a final volume of 300-350µl (depending on strip size)

* = 2D lysis buffer with bromophenol blue added at a concentration of 2µl per 5ml

This solution was prepared, mixed and loaded into the sample groove of a re-swelling tray (Bio-Rad). The corresponding labelled strip was then removed from its protective covering and carefully placed gel side down in the re-swelling tray groove. Approximately 2mls mineral oil was then added on top of the strip to exclude all air. The strips were then allowed to re-hydrate overnight undisturbed.

Once re-hydrated the strips were ready for IEF using the Bio-Rad Multiphor system. Preparation for IEF involved cleaning all equipment with $ddH_2O$ and cutting filter paper wicks (Bio-Rad) for each end of the strip and moistening them with $ddH_2O$. The electrophoresis platform was then made ready by the addition of 15ml mineral oil (Amersham) and positioning of the glass electrophoresis plate to exclude as much air as possible. Oil was added to the inside of the plate and the strip divider was applied, again excluding as much air as possible. The re-hydrated strips were then placed in the correct orientation gel side up in the grooves of the strip divider and the wicks added extending 3mm onto the surface of the gel. Electrodes were then attached on top of the wicks 2mm from the start of the gel. Mineral oil was added so that the wicks were submerged and IEF was performed as below using a gradient current increase:

| | | | |
|---|---|---|---|
| 300V | 5mA | 10 W | 1 hr |
| 300V | 5mA | 10 W | 2 hrs |
| 3500V | 5mA | 10 W | 3 hrs |
| 3500V | 5mA | 10 W | 19 hrs |
| 300V | 5mA | 10 W | 2 hrs |

**Second Dimension**

The second dimension was similar to 1D SDS PAGE with the exception that gradient gels were used and the sample was applied in the form of an IPG strip. The gels for the second dimension were cast using a gradient former and multiple casting tank (Bio-Rad). The recipe for casting the 18x20cm gradient gels used in this study is shown in Table 2.5 below.

**Table 2.5**. Recipe for 9-16% gradient gel, values are shown in ml.

| | 9% Chamber | 16% chamber |
|---|---|---|
| 30% Bis-Acrylamide | 8.9 | 15.8 |
| 1.5mM Tris HCl pH 8.8 | 7.4 | 7.4 |
| $ddH_2O$ | 13.3 | 6.4 |
| 10% SDS | 0.3 | 0.3 |
| Temed | 0.0118 | 0.0118 |
| 10% APS | 118.2 | 118.2 |

The glass plates were placed into the multiple casting chamber separated by plastic films to allow ease of separation. The top of the chamber was added and attached to the gradient former. The gradient former was then placed on a stirring platform 5cm higher than the casting chamber, with the connecting tube clamped. The 9% and 16% solutions were prepared (excluding APS) and were added to separate chambers and whilst stirring the APS was added. Subsequently the communicating valve between the two solutions was opened and the connecting tube unclamped. The plates were filled to 1 inch below the top of the plate and overlaid with $ddH_2O$ as for 1D SDS PAGE gels. These plates were labelled and left covered overnight in a cold room, to allow full polymerisation of the acrylamide. Before application to the gel, the IPG strips required treatment with equilibration buffer. The strips within the strip divider were placed in a plastic container and covered in 100ml equilibration buffer. Filter paper wicks were added to each end of the strip and clamped and the strips were then left on a rocker for 30 mins with a buffer change halfway through.

The gels for the second dimension were prepared by removal of the $ddH_20$ overlay and addition of 0.5% agarose solution to the top of the plate. The strips were removed from equilibration buffer and blotted to remove excess fluid. Each strip was then added to a gel using forceps, noting the orientation, pushing the strip to the bottom of the agarose so that it sat on the top of the gradient gel. The agarose was then left to set and the Bio-Rad Protean II system was prepared as for 1D SDS PAGE. The gels were then applied to the electrophoresis apparatus and run using the same settings as mentioned previously for SDS PAGE. Following the completion of the second dimension, the gels were subjected to western transfer. This protocol was adapted from (Amersham, 2005a).

### 2.2.14 Immunodetection of proteins bound to nitrocellulose membrane

There follows the general scheme of immunoblotting used in this study. Primary antibodies were each used at their optimal dilutions (see table 2.3) and secondary antibodies were used at a dilution of 1:5000.

The nitrocellulose membrane was re-hydrated in TBST for 15 mins and then put into blocking buffer for 1 hour at room temperature or overnight at 4°C. The choice of blocking buffer was dependent on the primary antibody used, antibodies to phospho-

proteins for example required blocking in BSA. Following blocking, the membrane was transferred to a solution containing the correct dilution of primary antibody, in TBST and left for 1 hr at room temperature or overnight at 4°C. Some antibodies required the addition of 2% blocking protein to the primary antibody to limit background interference. The membrane was then washed three times in TBST for 5-10mins each wash. After washing, the secondary antibody was diluted in TBST, added to the membrane and incubated for 1 hr at 20°C. The membrane was washed as before and the antibody signal detected using enhanced chemiluminesence (ECL) detection reagent kit (Amersham). The membrane was incubated in ECL for 1 min following which excess fluid was removed and the membrane placed face down in a plastic folder excluding all air. The membrane was then placed right side facing into an x-ray film cassette and taken to a dark room where blue Fuji film (GRI) was exposed to the blot for 1, 5, 20 and 60mins. These exposure times were adjusted depending on the strength of signal. The films were processed automatically in the majority of cases.

Following immunodetection the membranes were either washed in TBST and stored, or placed in stripping buffer for 2 x 30mins to remove the antibodies, rejuvenating the membrane for subsequent immunodetection. This protocol was adapted from (Amersham, 2005d).

2.2.15 Immunofluorescence

In this study two main immunofluorescence techniques were performed, the choice of which depended on the protein localisation requirements of the experiment.

**Paraformaldehyde fixation**

The first technique used fixation of cells grown to 60% confluence on cover slips in a 24 well plate, with 4% paraformaldehyde. These cells were initially washed three times in 1x PBS and then fixed for 10mins. The cover slips were then washed three more times, put into 1ml of blocking buffer and incubated for 30mins at room temperature. The primary antibodies were prepared in 1x PBS at the appropriate dilution (see table 2.3) and applied to the cells for 1 hr at 20°c. Subsequently the cells were washed three times, fluorophore-conjugated secondary antibody added and

incubated for 1hr at 20°C. The cover slips were then given a final three washes and mounted on a microscope slide with Mowiol mountant (Sigma). The slides were stored at in the dark at 4°C for no longer than 3 days before viewing. This protocol was adapted from (Wojciak-Stothard et al., 1998).

**Live cell immunofluorescence**

Live cell immunofluorescence was used when it was necessary to observe the cell surface localisation of AII and p11 and their interaction with lipid rafts and the actin cortex. The cells were cultured on glass cover slips in a 24 well plate. After 24 hrs, IFNγ was added to half the wells and the cells left to incubate for a further 24hrs. The cells were then washed three times with ice cold 1 x PBS and incubated with the correct dilution of primary antibody for 1 hr at 20°C. The cells were washed again three times in 1xPBS and incubated at 4°C with FITC-conjugated cholera toxin B (Molecular probes) for 30mins. The wash procedure was repeated and the cells fixed with 4% paraformaldehyde (see above). A further 3 washes were performed and the cells were permeabilised with 0.2% TX100 (Sigma) for 6 mins. A further wash step was performed in triplicate after which blocking solution was added to the cells for 30mins. Following this, the correct fluorophore-conjugated secondary antibody and phalloidin were added to the cells at the appropriate concentrations and incubated for 1 hr at room temperature. A final wash step was performed, after which the cover slips containing the cells were mounted cell side down onto microscope slides using Mowiol mountant. The slides were stored in the dark at 4°C for no longer than 3 days before viewing. This protocol was adapted from (Groves, 1992).

2.2.16 Cell invasion assays

In this study a technique was required that would assay the invasive potential of cultured cells, for this the Biocoat matrigel invasion chamber system (BD biosciences) was used. 750,000 cells at 70% confluence in a T-80 tissue culture flask were incubated with or without IFNγ for 24 hrs. The invasion chambers were thawed and a coating of fibronectin (50µl of fibronectin at a concentration of 0.01mg/ml in 1% PBS) (Invitrogen) was applied to the base of the chamber and left to dry for 1 hr. The matrigel inside the chamber was then re-hydrated with the appropriate medium lacking serum, but including 0.1% BSA, with or without IFNγ, for 1.5 hrs. The cells

were detached from their growing surface by addition of 3ml of cell dissociation fluid (Sigma) and counted using the above protocol. Aliquots of 2 x $10^5$ cells in 250µl were then prepared in the appropriate serum free medium plus or minus IFNγ. Excess medium from the re-hydration of the matrigel was removed and the transwell chambers were placed into a 24 well plate. In this plate 500µl of the medium plus or minus IFNγ, was added to the wells, underneath the chamber. The 250µl cell aliquot was then added to the top of the assay chamber, following which the plates were covered and incubated for 24 hrs at 37°C in 10% $CO_2$ in air. After this time period, the membranes at the bottom of the chambers were washed three times in media plus 0.2% BSA. The cells remaining in the upper chamber were removed with a cotton swab. The membranes were fixed for 10mins in 4% paraformaldehyde and stained in haematoxylin solution for an additional 10mins. After rinsing in 1x PBS the membranes were then either placed in storage buffer and kept at 4°C, or counted immediately. Quantitation of migrant cells was performed by counting 6 random fields on each membrane at x20 magnification. This protocol was adapted from (Falcone et al., 2001).

The invasion assay protocol was adapted for both an assay of invasive capacity with exposure to an anti-AII antibody and transfection of LNCaP with a GFP AII construct. In the first case the alterations to the protocol included re-hydrating the matrigel with media plus or minus 25µg/ml Anti-AII antibody (Zymed) incubated at 4°C. This medium was also used in the bottom of the wells, to incubate cells for 15mins before detachment and in the suspension of cell aliquots. The control for this experiment involved the addition of anti-mouse IgG at 25µg/ml (Amersham) in place of the AII antibody used above. In the GFP transfection study, the same protocol was used, with the exception that the cells were obtained from a 24 well plate, were not stained with haematoxylin and the migrant cells were counted using a fluorescence microscope. This protocol was adapted from (Falcone et al., 2001).

2.2.17 Transfection of LNCaP cells with a green fluorescent protein AII (GFPAII) construct

To examine the effect of AII expression in LNCaP cells, the cells were transfected with an AII GFP construct or with the GFP vector alone as a control. The cells were then observed for GFP expression and subjected to an invasion assay.

**Bacterial transformation**

A small aliquot of the wild type AII construct was obtained from Dr S Moss (Merrifield et al., 2001). The GFP construct was based on the pEGFP-C1 plasmid vector, a diagram of which can be seen in Figure 2.2. This plasmid encodes a variant of wild type GFP and resistance to kanamycin and neomycin. The plasmid DNA was amplified by transformation into competent E. Coli JS4 cells, in the following manner. The bacteria were removed from storage at -80°C and defrosted on ice, 1µl of GFPAII DNA was then added to a 100µl aliquot of bacteria, left to incubate for 30 mins on ice and the bacteria were transformed using a heat shock of 45 secs at 42°C. The samples were then left for 2 mins on ice, following which 500µl Luria Broth (LB) (Qiagen) was added. This mixture was incubated for 1hr at 37°C, whilst pre-prepared bacterial plates, containing agar, LB and 30µg/ml Kanamycin, were warmed in a 37°C oven. Following incubation a 150µl aliquot of transformed bacteria was spread onto the bacterial plate using a glass spreader. The plates were incubated inverted for 12-16 hrs. Colonies were then selected, placed in 2ml of LB medium and left to incubate overnight. Plasmid DNA extraction was performed by means of a maxi prep kit (Qiagen), the protocol for which is included in Appendix 6. DNA concentration and purity were quantified by reading absorbance at 260/280nm using UV spectrotrometry. The entire DNA used for transfection had a purity reading of over 98%. This protocol was adapted from (Qiagen, 2005a).

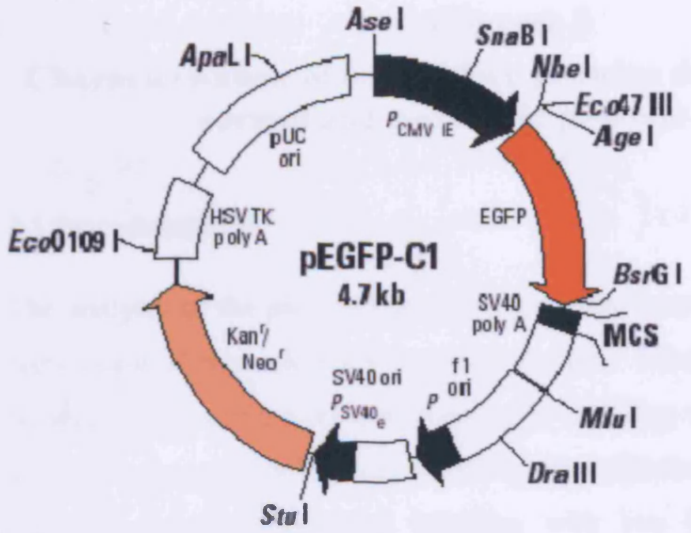



**Figure 2.2.** A schematic diagram of the pEGFP-C1 vector (BD_Biosciences, 2005).

**LNCaP transfection**

The transfection of LNCaP cells with the GFPAII construct and vector only (BD biosciences) followed the same protocol. The LNCaP cells were plated into a 24 well plate containing cover slips at a density of 71,250 cells per well, appropriate medium was then added and the cells were allowed to adhere overnight. The transfection was performed using FuGENE 6 reagent (Roche) the protocol for which is included in Appendix 7. A ratio of 9:1 FuGENE reagent to DNA was used, adding 1.8µl FuGENE to 0.4 µg DNA. The cells were observed 18 hrs following transfection and then either fixed in paraformaldehyde (see section 2.2.15), or re-transfected with either GFPAII or empty vector following the same protocol and subject to an invasion assay, (see notes at end of section 2.2.16). This protocol was adapted from (Roche, 2005a).

# Chapter 3
# Characterisation of cell surface proteins differentially expressed by normal and neoplastic prostate epithelial cells

## 3.1 Introduction

The analysis of the plasma membrane proteome requires strategies optimised for the enrichment of rare, relatively insoluble proteins. Many strategies have been employed to analyse cell surface proteins from radio-labelling with $^{125}I$ or $^{3}H$ (Naaby-Hansen et al., 2003), to complex three-dimensional crystallisation analyses (Werten et al., 2002). Recently however, vectorial labelling with less hazardous compounds, such as fluorophores and biotin, followed by affinity chromatography has been employed in the enrichment of this protein fraction (Naaby-Hansen et al., 2003).

A long chain sulphonamide ester of biotin, called Sulfo-NHS-LC-Biotin, has been successfully used to study spermatozoa cell surface proteins. In this study, 98 sperm cell surface proteins were identified, 22 of which were phosphorylated, including several novel protein identifications (Naaby-Hansen et al., 1997). Tagging with biotin derivatives, as opposed to His, FLAG or HA tagging, is advantageous, as the addition of the sulpho group makes the biotin impermeable to the cell, thus limiting unwanted intracellular labelling. In one of the first studies to employ biotin labelling, it was found that this labelling technique minimised cytoplasmic contamination (Busch et al., 1989). The study of membrane proteins is commonly hampered by their insoluble nature, biotin labelling also increases the solubility of the compounds it is associated with, a quality which is a distinct advantage as success in purification of membrane proteins depends on solubility (Viard et al., 2002).

The use of the biotin tags such as Sulfo-NHS-LC-Biotin is however, of limited use in proteomics as the tag itself can lead to the generation of multiple isoforms due to the negative charge it carries. This can be avoided by the use of a reducible biotin derivative, such as Sulfo-NHS-SS-Biotin, which can be removed before protein separation (Werten et al., 2002, Naaby-Hansen et al., 2003). This reducible form of vectorial labelling has been successfully used to analyse cell surface protein

composition, one of the first applications being the analysis of cell surface proteins on rat hepatocytes (Busch et al., 1989).

Biotin is a small co-enzyme synthesised by bacteria, plants and some fungi (Cronan, 1990). Biotinylated proteins are rare in nature, it is estimated that there are only 4 mammalian proteins with this modification (Cronan, 1990). These are acetyl coA carboxylase (Chandler and Ballard, 1988), pyruvate carboxlyase, methyl crotanyl-CoA carboxylase and propianyl CoA carboxylase (Robinson et al., 1983). The rarity of endogenous biotinylated proteins indicates that biotin labelling provides a specific way of tagging surface proteins with little to no background from endogenously biotinylated molecules (Cronan, 1990). Avidin has an extremely high affinity for biotin, therefore avidin-biotin affinity chromatography is an efficient, high yield method of cell surface protein purification (Werten et al., 2002).

Pre-fractionation steps, such as affinity chromatography, are often used in proteomics to decrease the diversity and complexity of a protein mixture, giving a sample enriched in the number and concentration of a desired subset of proteins (Naaby-Hansen et al., 2001). Biotin-avidin affinity chromatography has been improved by the engineering of monomeric avidin columns with a decreased affinity for biotin, allowing competitive elution with free biotin. These columns however, are prone to degradation by protease and reducing agents. Therefore a streptavidin tetrameric column has been developed which has superior stability (Cronan, 1990).

The aim of this study was to characterise the differential protein expression of an isogenic pair of prostate cell lines (1542 NPX and CP3TX), using biotin labelling and affinity chromatography. Each step including lysis, labelling and affinity chromatography, was optimised to find the most effective method of obtaining a sample enriched in cell surface proteins. These proteins were then characterised by mass spectroscopy.

## 3.2 Methods and Results

### 3.2.1 Identification of surface proteins

Cell surface proteins differentially expressed between 1542 NPX and CP3TX cell lines were analysed by the use of biotin-avidin affinity chromatography. This was achieved through optimisation of the manufacturers affinity chromatography protocol (Perbio) to give an enriched cell surface protein yield in the sample. This enrichment was necessary due to the inefficient resolution of 1D SDS PAGE as with this technique many protein species may be present in a single band. 1 dimensional SDS PAGE was the chosen technique for protein separation, as the increased resolution of 2DGE systems is outweighed by the difficulty of resolving hydrophobic proteins (Thoren et al., 2002). Due to the protein complexity in 1D SDS PAGE gel bands, to achieve a protein concentration appropriate for mass spectroscopy identification it was necessary to increase the biotinylated protein yield from pre-fractionation to a concentration that was easily observable with coomassie staining. Once this goal was achieved, bands were cut and sent for analysis by mass spectroscopy.

### 3.2.2 Biotin labelling

Two biotin derivatives were considered for use in this study, namely Sulfo-NHS-LC-Biotin (LC) and Sulfo-NHS-SS-Biotin (SS). These two labelling compounds were compared for efficiency and yield of putative cell surface proteins. Three T-80 cell culture flasks each containing approximately 750,000 cells for both 1542 NPX and CP3TX cell lines were labelled with either LC biotin or SS biotin and three flasks of each cell line were left unlabelled as a control. The cells were lysed in 1% OGP lysis buffer and the lysates fractionated over neutravidin columns, according to their elution conditions (see section 2.2.7). The samples were resolved on 12.5% gels, run in duplicate for coomassie blue staining or western transfer. This experiment was repeated in triplicate. Representative gel images are shown in Figure 3.1. The neutravidin columns were recycled for use in all three replicates.

The results of these studies indicate that in the gels containing SS biotin samples (Figure 3.1 A.), there are a large number of bands within the control lanes, which

appear to increase in number and density in each replicate. These bands may represent unlabelled proteins binding to the neutravidin beads or support. There are however, bands present within the sample lanes which are absent in the control, which may represent cell surface proteins. The LC gels did not contain enough protein to be observed by coomassie staining. The biotin labelled proteins in these gels could only be visualised on immunoblotting with avidin. On the LC biotin labelled gels there were also bands within the control lanes, although far more bands were present in the labelled sample lanes (see Fig.3.1 B).

Labelling with SS biotin gave a yield of putative cell surface proteins high enough for detection by coomassie staining and this therefore was the labelling method of choice. Further optimisation was required, however, before this technique could be applied to the characterisation of cell surface proteins. Background contamination in the control samples made it extremely difficult to observe differentially expressed proteins between the normal and cancer samples. In the affinity chromatography protocol the wash step is required to remove any contaminating non-labelled material. To optimise this step of the protocol, the final elution product following application of several different wash solutions were compared. Eight T-80 cell culture flasks each containing 750,000 unlabelled 1542 NPX cells were lysed with 1% OGP and split into three aliquots. These samples were then run over three newly formed neutravidin columns, washed with each of the following made up in 1x PBS;

8M urea+ SDS+ 1% OGP
0.5% SDS
0.1% OGP

The proteins present within the elution fraction following affinity chromatography of the unlabelled samples were resolved on a 12.5 % gel and visualised by coomassie staining. This experiment was repeated in duplicate and a representative gel is shown in Figure 3.2.

Unexpectedly, the wash buffer 1% OGP used in the original cell labelling experiments gave the elution fraction with the least background contamination. The most stringent wash containing 8M urea gave the most contaminated sample. It was

therefore concluded that in the initial labelling experiments, recycling of the neutravidin columns led to a build up of contaminating proteins observed as bands in the control unlabelled sample. The results of the wash buffer study suggested that using a fresh column and washing with 1% OGP gave the cleanest elution fraction. The optimised features of SS labelling and 1% OGP wash buffer were therefore added to further improve the affinity chromatography protocol.

### 3.2.3 Lysis conditions

The cell lysis conditions were optimised using 150,000 cells from each of the 1542 NPX and CP3TX cell lines at 70% confluence labelled as per protocol with LC biotin (see section 2.2.6). The yield of biotinylated protein was examined using two different lysis buffers, 1% NP40 and 1% OGP. Both of these buffers provide a gentler method of membrane solubilisation than the more frequently used detergent SDS (Roche, 2005b). Following labelling, the cells were lysed with 500µl of either detergent and the protein concentration analysed according to the Lowry protocol (see Appendix 2). Protein concentrations were higher in NP40 lysed cells at 2.0mg/ml, compared to 0.8 mg/ml in OGP treated cells. The samples were then resolved on a 12.5% gel subject to SDS PAGE. The proteins were transferred from the gel to a nitrocellulose membrane by Western transfer. The membrane was then probed using HRP-streptavidin and visualised with ECL. This experiment was repeated in triplicate. Figure 3.3 shows a representative result.

Although it appeared from the Lowry assay that NP40 gave a better protein yield, the avidin blotting showed that 1% OGP solubilised a greater number and range of biotinylated cell surface proteins. The NP40 lysis appeared to yield biotinylated proteins in the range of 75 to 160kD, whereas OGP lysis allowed resolution of biotinylated proteins from 15 to 160kD. Differences in the surface composition between 1542 NPX and CP3TX cells lysed with 1% OGP were also apparent on the avidin blot featured in Figure 3.3A. The greater yield in range and quantity of biotinylated proteins using OGP lysis buffer is probably attributable to its ability to solubilise membrane proteins sensitive to hydrophobic environments. This detergent was therefore chosen as the lysis buffer for solubilizing proteins for affinity chromatography.

The results of the above experiments were used to create an optimised protocol (see sections 2.2.6 and 7) that could be employed for the pre-fractionation and cell surface enrichment of samples for 1D SDS PAGE analysis.

### 3.2.4 Surface protein identification

Once the labelling and affinity chromatography protocol had been optimised it was possible to apply this protocol to the pre-fractionation of 1542 NPX and CP3TX samples and run long Hoeffer 12.5% gels to obtain maximum resolution of the surface protein enriched sample. Samples from three T-80 tissue culture flasks each containing 750,000 1542 NPX or CP3TX cells were labelled with SS biotin and lysed with 1% OGP. 50μg of each 1542 NPX and CP3TX sample were run against the same quantity of unlabelled sample (lysate from 2,250,000 NP cells) as a control and duplicate gels were run for coomassie blue or silver staining. All running and staining of these gels was performed as per protocol, (see sections 2.2.9 and 2.2.11) with the exception that all preparation, running and staining was performed in a clean room environment. This location was necessary to minimise the protein contamination of bands cut from the gels so that any samples being sent for mass spectroscopy analysis would be as free from contaminating protein as possible. Representative gel images can be seen in Figure 3.4.

The stained gels were screened for bands absent from the control and showing apparent differential expression between 1542 NPX and CP3TX. Sample bands were cut from several gels representing replicates of the above experiment and the proteins were identified by Nano-HPLC-ESI-MS/MS by the Mass spectroscopy group at Ludwig Institute for Cancer Research (LICR). The results of the protein identification are shown in Tables 3.1 and 3.2.

**Table 3.1.** Cell surface associated proteins identified via Nano-HPLC-ESI-MS/MS from 1542 NPX and CP3TX cell surface protein enriched lysates.

| Protein | MW (kD). | Postion in Figure 3.4 | Location | Function |
|---|---|---|---|---|
| **4F2 heavy chain antigen** | 58 | 4-7 | Transmembrane | Amino acid transport |
| **A tyrosine kinase** | 110 | | Transmembrane | Cell signalling |
| **Annexin III** | 36 | 11 | Peripheral | Signal transduction |
| **Aminopeptidase** | 80-90 | 6 | Peripheral | ECM modulation, Signalling |
| **BiP** | 70 | | Membrane | Molecular chaperone |
| **β-Galactosidase** | | 4,6,7 | Peripheral | Lysosomal hydrolase |
| **Calnexin** | 90 | 6 | Plasma membrane, assoc lipid rafts | Molecular chaperone |
| **CD9** | 26 | 14 | Transmembrane | Cell adhesion |
| **CD 109** | 160 | 2 | GPI anchored | Cell-cell interactions |
| **CD44** | 44 – larger splice variants | 2-4 | Cell surface glycoprotein, lipid rafts | Cell adhesion |
| **CTL2** | 80 | 6 | Transmembrane | Cell signalling |
| **E-Cadherin** | 95 | 4 | Transmembrane | Cell adhesion |
| **EGF receptor** | 138 | 1-2 | Transmembrane | Growth signalling |
| **Emmprin** | 29 | 9 | Peripheral | Induction of MMP's |
| **Ephrin type A** | 110 | 4 | Transmembrane | Cell signalling |
| **F11 receptor** | 33 | 10 | Integral | Cell signalling, adhesion |
| **gp96** | 92 | 6 | Peripheral | Molecular chaperone |
| **Glucosidase II** | 60 | 6 | Transmembrane | Chaperone, glucose trimming |
| **HSP 70** | 71 | 8 | Peripheral | Molecular chaperone |
| **HSP27** | 27 | | Plasma membrane | Cell adhesion |
| **HSP60** | 60 | 9 | Mostly mitochondrial, observed on plasma membrane | Expressed on cell surface in infection, autoimmune disease and in tissue culture |
| **Integrin alpha 2** | 130 | 3 | Transmembrane | Cell adhesion |
| **Integrin alpha 3** | 130 | 4 | Transmembrane | Adhesion, Migration, Signal |
| **Integrin alpha 5** | 110 | 4 | Transmembrane | Cell adhesion |
| **Integrin β1** | 91 | 1-5 | Transmembrane | Cell adhesion |
| **Integrin β4** | 200 | 1 | Transmembrane | Cell adhesion |
| **Integrin β6** | 89 | 4 | Transmembrane | Cell adhesion |
| **LAR receptor** | 211 | 7 | Transmembrane | Signal Transduction |
| **MEMD** | 65-100 | 4-5 | Transmembrane | Cell adhesion |
| **Neuropilin like protein** | 85 | 4 | Transmembrane | Cell signalling |
| **Porin 1** | 30 | 11 | Transmembrane | Voltage gated ion channel |
| **Porin 2** | 33 | 11 | Transmembrane | Voltage gated ion channel |
| **Prostagladin F2 receptor** | 99 | 4 | Transmembrane | Cell signalling |
| **Scavenger receptor B1** | 62 | 7 | Transmembrane | Lipid and lipoprotein receptor |
| **SCYLP** | 21 | 14 | Peripheral | Protein folding |
| **Thioredoxin** | | 13 | Peripheral | Formation of S-S bridges |
| **Transglutaminase 2** | 80 | 7 | Plasma membrane localizes with HSP27 in keratinocytes. | Cell adhesion, spreading, cell-matrix interactions |
| **VLA-3 alpha** | 115 | 4 | Transmembrane | Cell adhesion |

The cell surface analysis of 1542 NPX and CP3TX cells resulted in the identification of 69 proteins by Nano-HPLC-ESI-MS/MS, of which 35% were integral membrane proteins and 21% peripheral membrane proteins (see Table 3.1). However, MS analysis revealed a high level of complexity in several of the protein bands excised, with non-membrane proteins also present (see Table 3.2). These non-membrane proteins may represent proteins associated with membrane proteins, or artefacts due to sample contamination. The presence of various keratins indicates that the latter may be the main causative factor. Although the bands excised were selected on the basis that they appeared to show differential expression between the two cell lines, the complexity of these bands negates any inference regarding relative expression levels of individual proteins from the normal and cancer cells. To quantitate the relative expression of individual proteins, sensitive immunotechniques are required. These techniques are however, limited by the availability of discriminatory primary antibodies. Due to this limitation and time constraints, further analysis could only be conducted for two of the identified proteins.

Proteins identified from the cancer cell line included EGF-R, several integrins and molecular chaperones. The latter include proteins with the endoplasmic reticulum (ER) retention KDEL sequence, indicating as has been previously noted (Altmeyer et al., 1996), that the localisation of ER associated proteins is deregulated in the 1542 CP3TX cell line. An 11kD band that appeared to contain more protein in the 1542 CP3TX sample shown in Figure 3.4 C, was identified as containing three proteins including a fragment of the CD 9 antigen. The abundant expression of this band in the cancer cell line indicated that the proteins therein may have potential as tumour markers and therefore CD9 was selected for further analysis. Lysates obtained from affinity chromatography enriched samples of three T-80 cell culture flasks each containing 750,000 1542 NPX or CP3TX cells were subject to electrophoresis on 15% gels and transferred as per protocol (see section 2.2.10). These membranes were then probed with the anti-CD9 antibody. This experiment was repeated on several occasions, however the only commercially available CD 9 antibody did not give a signal in the 1542 cell extracts even when used at a concentration of 1:50. The antibody did however, detect a protein at the correct molecular weight for CD9 in a control extract produced by the antibody manufacturer.

Calnexin, a 90 kD molecular chaperone was also isolated from a protein band showing greater intensity of staining in the 1542 CP3TX cell line. As this band contained other proteins, further analysis of the relative expression level of calnexin was required. To explore the expression of this protein in several isogenic cell lines, the following study was performed. Three T-80 cell culture flasks containing 750,000 cells, were set up for each of the 1532 NP/1542 CP3TX, 1535 NP/1535 CP3TX and 1542 NP/1542 CP3TX cell lines. The cells were washed and lysed in 500μl 1% OGP. The samples were loaded onto a 12.5% gel at a concentration of 50μg per well and subject to electrophoresis and western transfer as per protocol. The membranes were then probed with anti-calnexin antibody; this was repeated in triplicate. Representative results are shown in Figure 3.5. The results from these triplicate experiments were analysed by densitometry. These readings were then normalised to those for β actin expression to compensate for any differences in loading. The normalised data were then used to calculate the fold difference in calnexin expression between the normal and cancer isogenic cell line pairs. The fold differences were as follows; 1532 CPT 1.4 fold higher expression than 1532 NP, 1542 CPTX 2.25 fold higher expression than 1542 NP and 1535 CPT 1.9 fold higher than 1535 NP. These data were also used in a student's t-test to infer the significance of the fold differences. All fold differences in calnexin expression were found to be significant with P values of 0.04, 0.002 and 0.0005 for the 1532, 1542 and 1535 isogenic pairs respectively, indicating that it is highly unlikely that the differences in calnexin expression observed are due to random fluctuations in these data. These results indicate that calnexin expression is significantly up regulated in the 1542 CP3TX cell line and that the same pattern of up regulation is observed in the other cancer cell lines when compared to their normal counterparts. This pattern of over expression is most apparent in the 1535 and 1542 isogenic cell lines.

**Table 3.2.** The non-membrane associated proteins identified from the gels in Figure 3.4. Fourteen additional proteins were isolated but were unidentifiable by database searches and de novo sequencing.

| Protein | MW (kD). | Postion in Figure 3.4. |
|---|---|---|
| Pyruvate kinase | 53 | 12 |
| Phospholipase alpha | 50.5 | 12 |
| Alpha tubulin | 45 | 9 |
| Beta 5 tubulin | 44 | 9 |
| Beta actin | 37.5 | 11 |
| Keratin 10 | 58 | 9 |
| Myosin heavy chain | 196 | 3 |
| Keratin 9 | 62 | 9 |
| T-complex protein 1 | 55 | 9 |
| Elongation factor 2 | 51 | 11 |
| Enhancer protein | 19 | 14 |
| Pyruvate kinase (muscle isoform) | 53 | 9 |
| Keratin 17 | 43 | 12 |
| Keratin 18 | 43 | 12 |
| Keratin 6A | 56 | 12 |

## 3.3 Discussion

The aim of this study was to develop an optimised strategy for membrane enrichment, so that the cell surface proteome of prostate epithelial cells could be compared. The rationale for this was that proteins exhibiting differential expression could possibly be involved in the progression to a cancerous phenotype. Proteins exhibiting differential expression patterns between normal and cancerous cells also have the potential to be exploited as markers of malignant disease or as targets for treatment of such disease. The advantage of using isogenic cell lines in a study of this nature is that any inter-individual variation is removed and therefore any differences observed are more likely to be representative of the carcinogenic process. The cell surface protein analysis was performed using biotin vectorial labelling and avidin affinity chromatography to pre-fractionate the sample into a membrane enriched protein

extract. Initially two biotin derivatives were compared for their efficiency in labelling cell surface proteins. This comparison was achieved through the observation of the range and quantity of proteins biotin labelled with each of the biotin derivatives. Irreducible forms of biotin such as the LC derivative require either competitive elution or elution under extremely acid conditions. Both of these elution methods require severance of the biotin-avidin association and consequently produce lower yields than the reducible biotin label. This elution method also interferes with the resolution of samples, as they must be dialysed prior to running. Sulfo-NHS-SS-Biotin gave the higher yield of cell surface proteins. The efficiency of this label probably lies in its ability to be dissociated from the protein of interest. The SS tag is cleavable by the use of a reductant, such as DTT or β mercaptoethanol, allowing elution of the target protein without interference with the biotin-avidin interaction. The interaction between these proteins is extremely strong, having an affinity constant of $>10^{15}$ $m^{-1}$ and therefore even when using the modified streptavidin compound the energy required to break the interaction is considerable.

In addition to the choice of protein label, the selection of wash buffer for use in affinity chromatography proved critical for obtaining a resolvable cell surface enriched protein fraction. The purpose of the washing stage of the pre-fractionation process is to remove any contaminating unlabelled cellular protein, which may be loosely bound to the avidin or support. In an attempt to preserve interactions between the intracellular domains of the biotinylated integral membrane proteins and their cytosolic binding partners during the isolation procedure, high-salt, alkaline buffers recently demonstrated to efficiently remove non-biotinylated cytoskeletal protein from Streptavidin beads (Zhao et al., 2004), were omitted from the washing procedure in this study. As a consequence five of the nine protein bands examined by mass spectrometry were found to contain contaminating cellular proteins such as, α-actinin 4, β 5 tubulin, elongation factor 2 (EF2) and pyruvate kinase in addition to one or more surface proteins. Integrins, ALCAM and CD44 are strongly anchored to the cytoskeleton, which could explain the detection of actinin and tubulin molecules in the affinity enriched fractions. However, neither EF2 nor pyruvate kinase interact directly or indirectly with membrane proteins, suggesting that they represent procedural contaminants, rather than proteins co-purified in complex with a transmembrane partner.

As biotinylated proteins are rare (Cronan, 1990), very few contaminating bands should be seen in the control lanes. The results shown in Figure 3.2 appear to suggest that use of a more stringent wash buffer results in more contaminating unlabelled protein bands following affinity chromatography. The highest level of contamination is seen with the use of 8M Urea in the wash buffer. This contamination may be due to the stringent nature of this chemical and its ability to degrade the avidin and column support. Once the column has been degraded there could be a variety of binding sites available for unlabelled cellular proteins, perhaps explaining the multitude of proteins observed in the elution fraction when the 8M Urea wash buffer was used. The results indicated that 1% OGP wash buffer gave the least background contamination when used with a fresh neutravidin column. The problems with protein build up on these columns when recycled may also be attributable to degradation as the regeneration solution contains low pH guanidine HCl, an agent that also may damage the columns.

The choice of lysis buffer was also crucial for obtaining a cell surface protein enriched sample. Two lysis buffers were compared in triplicate for their ability to solubilise a large range and quantity of labelled surface proteins. Avidin immunoblotting on whole cell lysates showed that 1% NP40 detergent gave higher protein concentrations, but solubilised a small range of labelled high molecular proteins. Despite appearing to yield less protein, 1% OGP lysis buffer solubilised a larger molecular weight range of proteins. The ability of the OGP lysis buffer to solubilise labelled cell surface proteins lies in its properties as a detergent. OGP is a non-ionic detergent capable of solubilizing membrane proteins in a gentler manner, often allowing retention of native structure (Roche, 2005b). Recently this detergent has been used to improve the yield of proteins in MS, where it was found to facilitate the recovery of hydrophobic peptides and increased the total number of peptides detected to 85% (van Montfort et al., 2002). A 1% OGP solution was therefore deemed the most appropriate lysis buffer for the cell surface analyses conducted in this study.

The experiments described above resulted in the formulation of an optimised protocol for cell surface protein analysis, which provided a high yield of cell surface proteins with minimal contamination from unlabelled proteins and containing a wide range of

molecular weight proteins. The efficacy of this protocol was then tested by its application to the cell surface analysis of 1542 NPX and CP3TX cells.

The pre-fractionation, analysis and identification of labelled cell surface proteins from the two isogenic cell lines was conducted and these data pooled. This resulted in the mass spectroscopy identification of 69 proteins in total, 35% of which were integral and 21% peripheral membrane proteins. There were however, 16 known non-membrane proteins identified and a further 14 proteins of unknown identity. Despite the potential association of such proteins with true membrane proteins, the identification of non-membrane associated proteins is an indication that there is contamination at some level. This suggests that further improvements are required to optimise the membrane protein enrichment procedure to achieve a less contaminated sample for MS. This experiment would benefit from being conducted in a solely clean room environment to minimise the contact with exogenous proteins. Improvements could also be made in the labelling and affinity chromatography protocols to minimise endogenous and non-specific protein elution.

The results from this study indicate that the cell labelling/affinity chromatography protocol was capable of producing a cell surface protein enriched sample. It is assumed that 5-8% of cellular proteins are membrane proteins, compared to 56% of the protein mixture using this cell surface analysis technique. This represents a greater than 10 fold enrichment of cell surface proteins. This level of enrichment is comparable to several other recent studies employing various cell surface proteomic techniques. In the analysis of neural cells from the human brain a 60% enrichment of membrane proteins was observed using stepwise depletion of non-membrane proteins, density gradient fractionation and liquid chromatography followed by MS (LCMS)(Nielsen et al., 2005). A similar study examining cell surface protein expression in human keratinocytes via 2 dimensional LCMS achieved a 57.3% enrichment of such proteins (Blonder et al., 2004). Recently a 67.3% enrichment of membrane proteins from a lung cancer cell line was achieved using an approach similar to that used in this study (Zhao et al., 2004). The increased level of cell surface protein enrichment in this lung cancer study may be due to their utilisation of HPLC as an additional step following avidin chromatography. This strategy may be

beneficial, although any further fractionation steps within the protocol do increase the risk of sample loss and therefore interfere with quantitation.

The bands excised were selected based on apparent differential expression between the 1542 NPX and CP3TX cell lines. However, on MS analysis several bands were found to be extremely complex. This degree of complexity is to be expected as 1D SDS PAGE gels do not give the single protein degree of resolution of techniques such as 2DGE. The advantage of the 1D SDS PAGE technique is the large range of hydrophobic proteins that can be resolved without problems with crystallization or interaction with gel supports. Due the band complexity it is impossible to define which proteins may be over expressed in the cancer cells without further analysis. It is interesting to note however that a large proportion of the proteins identified are involved in cell adhesion. These proteins include E-cadherin, CD44 and 6 integrin subtypes, cell adhesion molecules that have been implicated in malignant and metastatic progression (Stewart et al., 2004). E-cadherin, for example, has been shown to exhibit an anti-proliferative effect, the loss of which promotes cancerous growth and increases invasive potential. Integrins, like cadherins, are implicated in malignant and metastatic progression. Several integrins were identified in the 1542 CP3TX cell line. Over expression of integrins is thought to limit movement of the cells therefore decreasing their invasive capacity. However, up regulation of several integrin subtypes favours motility and can lead to a more invasive phenotype. The integrin subtype α6 β1, is most commonly implicated in the invasion of prostate cancer cells into seminal vesicles (Stewart et al., 2004). β1 integrin was identified in a band that from the cancer cell line. Further quantitative analysis by immunotechniques will be required to infer the relative expression of these cell adhesion molecules, before any assumptions can be made regarding their role in carcinogenesis.

The expression of two proteins identified from the cancer cell line, CD9 and calnexin, was examined in detail through immunodetection of proteins separated via 1D SDS PAGE. The band found to contain the CD 9 fragment at approximately 8 kD was one of the least complex bands analysed, containing only two other proteins. CD9 was consistently detected in avidin purified biotinylated membrane fractions from the cancer cells. A ten amino acid peptide corresponding to amino acids 21-30 was

sequenced by ESI-MS/MS, indicating that the fragment originates from the N-terminus of CD9. A band containing the 8kD CD9 form was strongly stained in Sulfo-NHS-LC-biotin labelled cancer cells, but not detected in the biotinylated fraction of normal prostate cell proteins. Both extraction and enrichment were performed in the presence of EDTA and under a broad umbrella of protease inhibitors, suggesting that the biotinylated CD9 fragment is generated prior to biotinylation and extraction. CD9 is the most well-characterised member of the Transmembrane 4 subfamily and may be implicated in cell migration, proliferation and tumour cell metastasis (Mhawech et al., 2003). Although the full length CD9 molecule is thought to inhibit motility in a range of cancers (Wang et al., 2002a), expression of the CD9 antigen fragment in acute lymphoma correlates with poor prognosis (Komada and Sakurai, 1994). Loss of the full length protein has been found to have prognostic value in several tumours including adenocarcinoma of the lung, colon, breast and pancreas (Wang et al., 2002a, Komada and Sakurai, 1994). It appears that loss of the full-length CD9 molecule promotes an invasive phenotype and leads to a poor prognosis. In the 1542 CP3TX cells the presence of the CD9 antigenic fragment, may indicate that the CD9 molecule is being degraded in the cancer cells thus giving them a selective advantage in tissue invasion. Immunoblotting studies could not confirm whether there is an up regulation of the CD9 fragment in cancer cells as the only commercial antibody available did not produce a recognisable band even at high concentrations despite its ability to detect CD9 in a positive control. This result may indicate that the CD9 fragment expressed in the 1542 cell lines is undetectable by an antibody raised against the full-length protein. To address this situation further study using an antibody specifically designed to recognise the CD9 fragment peptide sequence will be required. In addition to this quantitative PCR could be performed to examine expression of the CD9 mRNA transcript in the normal and cancer cells. Confirmation of the over expression of this motility associated protein fragment in the cancer cell line could possibly indicate its utility as a diagnostic marker of prostate cancer, with the added advantage that it may be present in the extracellular milieu (Komada and Sakurai, 1994).

Calnexin a type I transmembrane protein, was also identified from fractionated 1542 CP3TX cell lysate. Due to its role as a molecular chaperone, this protein has an endoplasmic reticulum retention sequence however, despite the possession of such a sequence calnexin has been shown to be expressed on the cell surface associated with

glycoproteins (Li et al., 2001). This protein has been implicated in cell adhesion via interactions with other CAM's such as integrins β1 and 6 and expression of calnexin has been found to increase in cancer cells on initial contact with substratum (Ruoslahti and Reed, 1994, Yeates and Powis, 1997). In a recent study of a small sample of breast tumour specimens, calnexin was found to be over expressed in 75% of samples compared to adjacent normal tissue. A correlation was also seen between over expression and increasing breast tumour grade (Li et al., 2001). This correlation between calnexin over expression and increasing tumour grade suggests that the over expression of calnexin, perhaps by its interaction with CAM's, may play a role in metastatic progression. The expression of calnexin in several isogenic cell lines was analysed by immunoblotting. In all samples the cancer line was shown to express significantly higher levels than its normal isogenic pair. This over expression in cancer cells, suggests that calnexin plays some role in malignant progression, further studies are required to assess whether this role includes progression towards an invasive phenotype.

In summary the results of this study have lead to the optimisation of a protocol for cell surface protein analysis, which has been applied to the analysis of surface protein composition in 1542 NPX and CP3TX cells. This analysis has lead to the identification of cell surface proteins involved in cell adhesion, signalling and growth and that are already implicated in malignant progression. The cancer specific over expression of one of these proteins, calnexin, was confirmed in 1542 NPX, CP3TX and two other prostate isogenic cell lines. This protein with further study, may have potential as a diagnostic marker in prostate cancer.

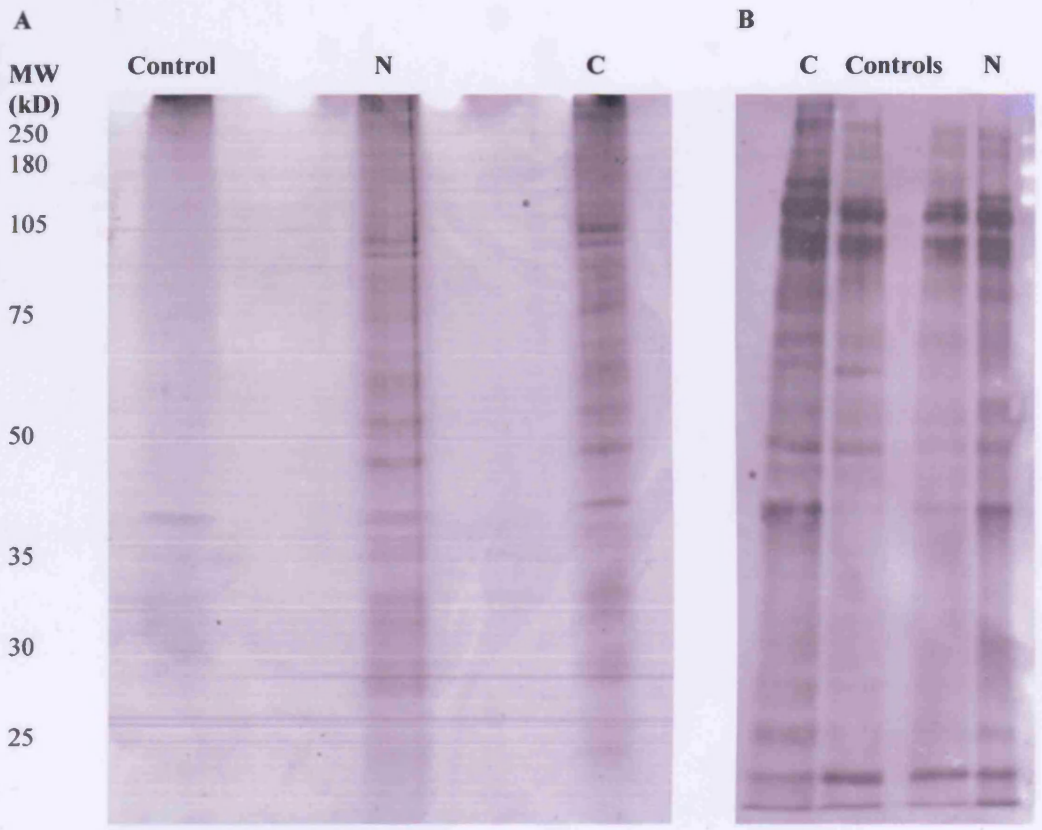



**Figure 3.1.** Cell surface fractionated NP and CPT cell lysate labelled with **A** SS biotin and **B** LC biotin. The SS biotin gave a labelled protein yield visible on staining with coomassie blue, whereas the LC required avidin blotting to visualise labelled proteins.

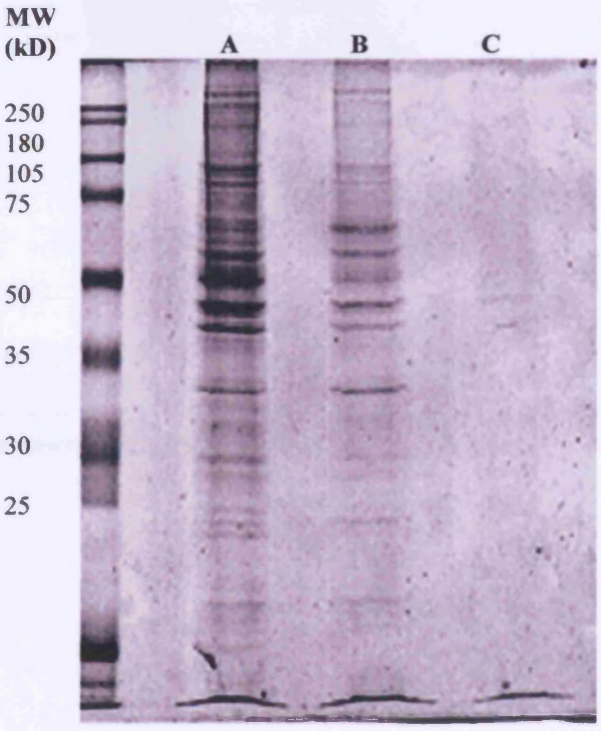



**Figure 3.2.** Elution fractions from unlabelled lysate subject to affinity chromatography using different wash buffers. **A** 8M Urea, 0.5% SDS in 1% OGP **B** 0.5% SDS C 0.1% OGP.

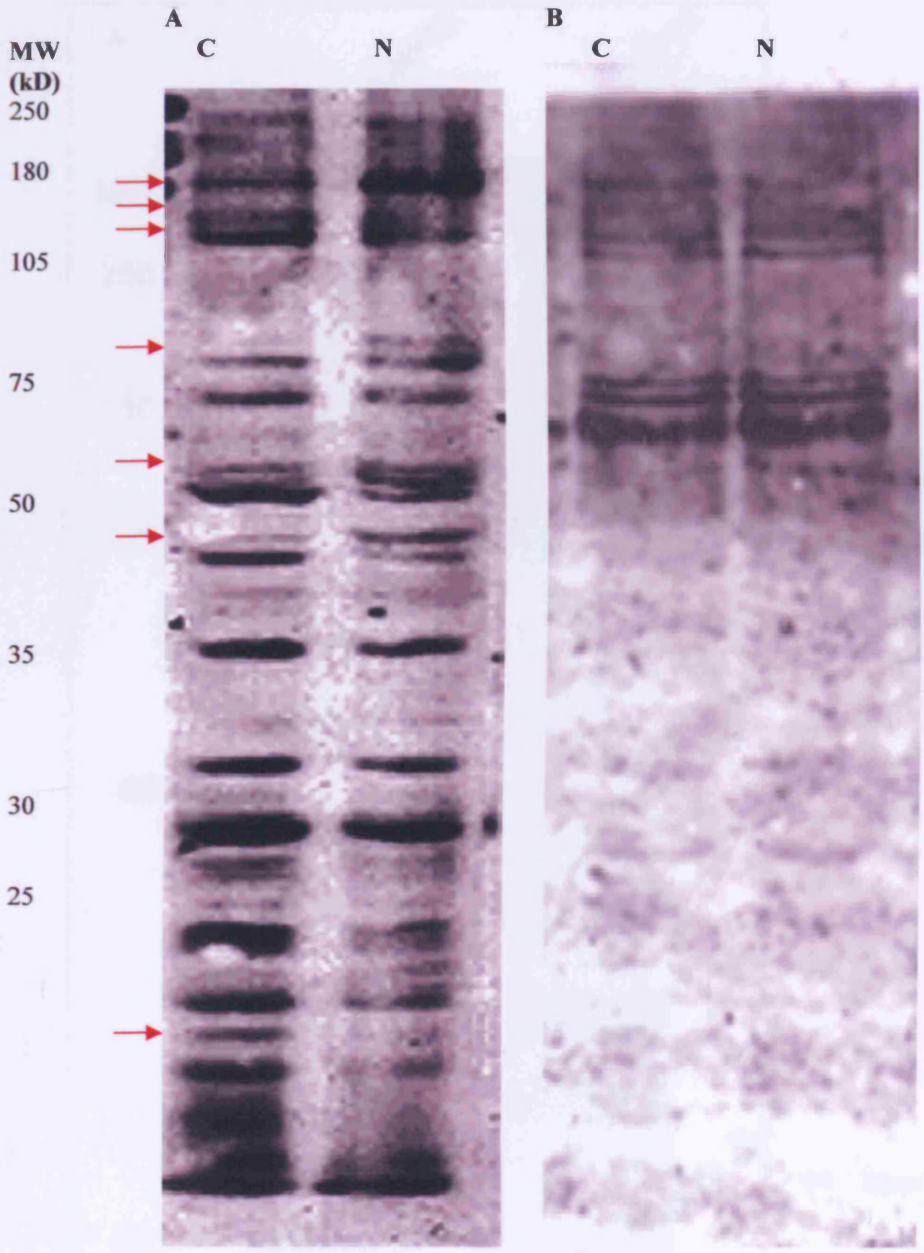



**Figure 3.3** 1D SDS PAGE avidin immunoblot of 1542 NPX (N) and CP3TX (C) biotin labelled proteins lysed in **A** 1% OGP, **B** 1% NP40. Lysis with 1% OGP appears to give a better yield of labelled proteins in both quantity and molecular weight range. Differences between 1542 NPX and CP3TX can be observed (See red arrows).

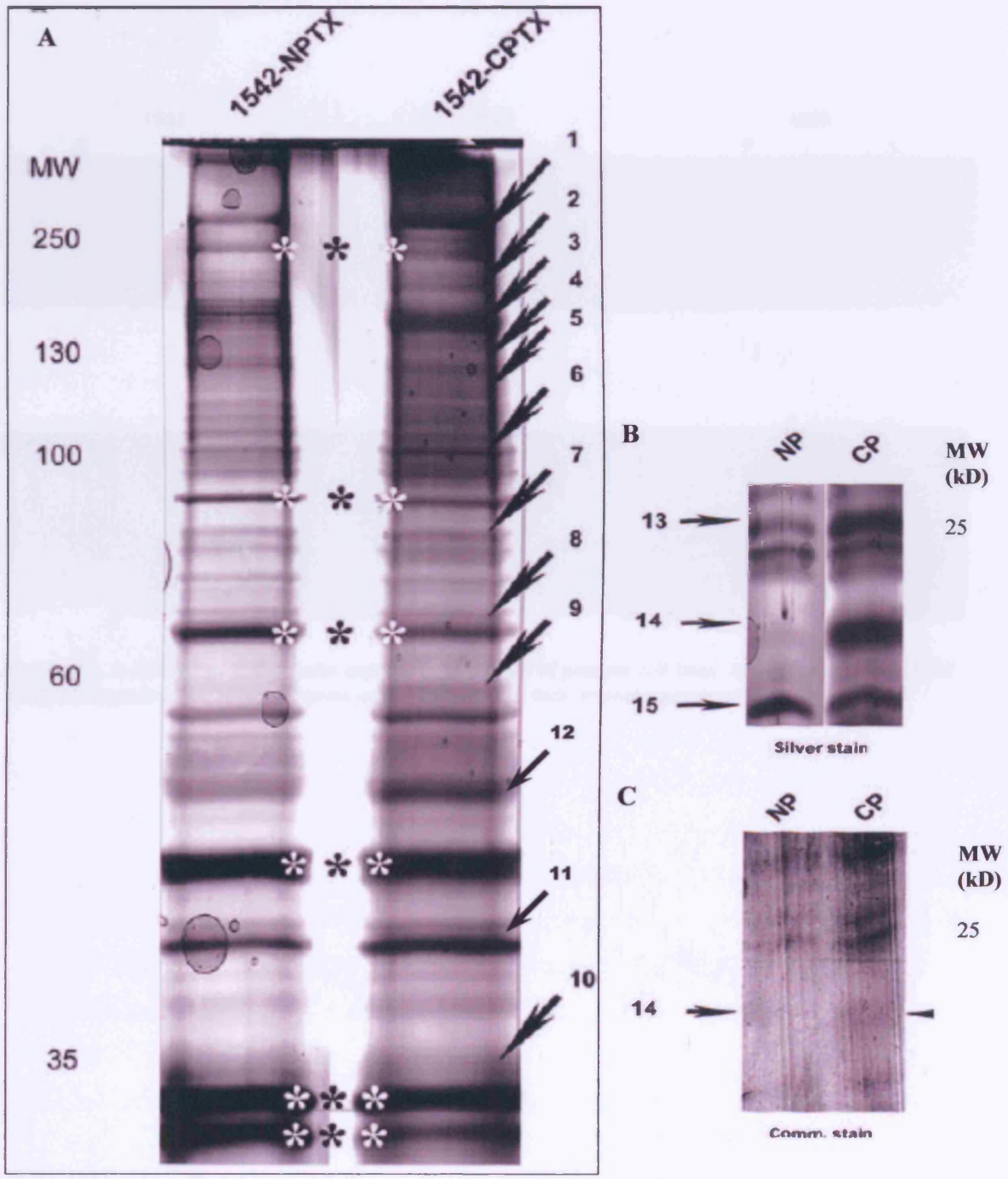



**Figure 3.4**. Affinity purification and identification of biotinylated surface proteins. **A** NHS-SS-sulpho-biotin labelled surface proteins were solubilised with 1% Octyl-b-D-glucopyranoside in the presence of protease and phosphatase inhibitors and purified by avidin affinity chromatography. The excised protein bands are indicated by arrows and numbered to the right. ESI-LC-MS/MS analysis identified 24 transmembrane proteins and 11 peripherally attached surface proteins in 15 excised protein bands. (**B** and **C**) A band containing the 8kD fragment of the 24kD tetraspanin protein CD9 was consistently detected in enriched surface fractions from the cancer cells (band 14, left images in b and c).

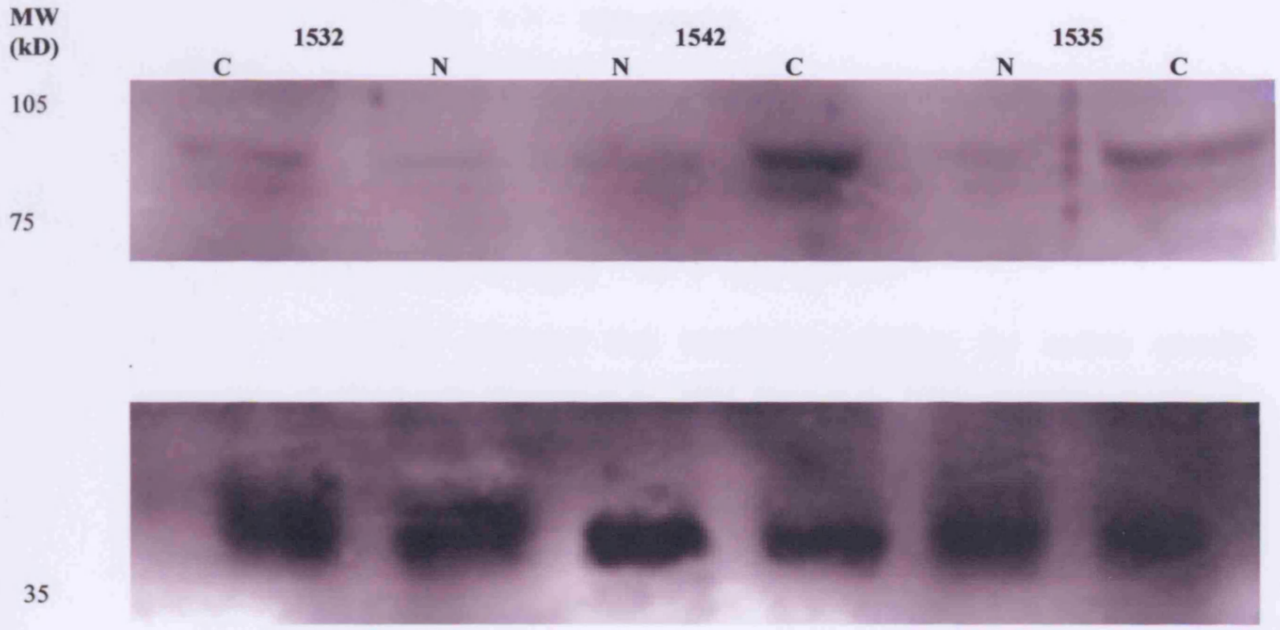



**Figure 3.5. A** Calnexin and **B** β Actin expression in a panel of prostate cell lines. All cancer cell lines from the three isogenic cell line pairs express more calnexin than their normal counterparts

# Chapter 4

# Comparative analysis of IFN signalling and cell surface protein expression in 1542 NPTX and CP3TX cells

## 4.1 Introduction

Previous studies have suggested that interferons modulate the surface protein composition of cancer cells (Nagano et al., 2003, Shou et al., 2002), including changes in the expression of a number of cell adhesion molecules and cell surface receptors (Bacso et al., 2002). Cytokines cause a number of cellular effects including cell cycle arrest, growth suppression and inhibition of metastatic potential in prostate cancer (Nagano et al., 2003, Shou et al., 2002, Sokoloff et al., 1996). In addition, IFNs play an indirect role in immunosurveillance by upregulating antigen presenting molecules such as MHC 1 (Shou et al., 2002). Interferon signalling is initiated by binding to receptors unique for each IFN (See Fig 4.1). The signalling pathways for both type I and II IFNs converge, however, at the level of signal transduction from these receptors. On stimulation by IFNγ, Janus kinases (JAK) 1 and 2 activate the cytosolic domains of the IFNγ receptor leading to the recruitment of STAT1 proteins. These STAT proteins consist of two subunits α and β, which are activated by phosphorylation and move into the cytoplasm. IFNγ signalling leads to a homodimerisation of STAT1 proteins which travel to the nucleus and initiate the transcription of genes containing specific IFNγ response elements (Sehgal et al., 2002). IFNα signalling also leads to the activation of STAT1 and STAT2 that form a transcription complex with p48 called the IFNα-induced transcriptional activator (ISGF). This complex is responsible for the transcription of genes with IFN stimulated response elements (ISRE) (Clifford et al., 2002).

STAT1 in addition to its integral role in the cross talk between the type I and II IFN signalling pathways, can also be activated by mitogenic factors such as EGF and PDGF (Marra et al., 1996). IFNs and growth factors share a common signalling pathway, crosstalk between which amplifies the mitogenic effects of EGF and PDGF. Treatment of cells with IFNγ potentiates the mitogenic activity of EGF. However, cells exposed to

both agents or to IFNγ alone exhibit an anti-mitogenic response (Marra et al., 1996). It is of note that regulation of one pathway can lead to two opposing outcomes.

Recent research demonstrated that IFN signalling is abnormally regulated in 1542 CP3TX cells when compared to the 1542 NPTX cell line (Nagano et al., 2003). In prostate cancer cell lines, many interferon inducible genes are down regulated. In addition a key member of the transcription complex, p48, displays aberrant expression in comparison to normal prostate epithelial cells (Shou et al., 2002, Nagano et al., 2003). p48 has a critical role in IFN signalling and the action of cytokines on tumour cells, in that altered expression leads to IFN insensitivity. p48 was found to be down regulated in melanomas and it has been suggested that this down regulation is an early event in the development of melanoma, conferring a selective advantage through insensitivity to IFN signalling (Clifford et al., 2002).

The finding that IFN signalling is abnormal in cancer cells indicates that a diminished response to both type I and type II interferons may lead to avoidance of immune surveillance and confer a survival advantage to the tumour cell. This insensitivity to cytokines has been proposed to be the subversion of a natural process, in that altered p48 expression can be observed in placental trophoblasts and is thought to promote feto-placental survival (Cross et al., 1999). The expression of p48, STAT 1 and of the IFNγ receptor (IFNγR) in 1542 NPTX and CP3TX cell lines is examined in the present study to observe the effect of IFN stimulation on these cells and any differences in signalling which may be responsible for modulation of IFN inducible proteins, especially those on the cell surface. Following on from the work of Nagano et al., 2003, this study focuses on IFNγ signalling in 1542 NPTX and CP3TX cell lines, including a comparative analysis of the affect of IFNγ on the expression of a cell surface protein gp96, a molecular chaperone identified in the previous chapter as expressed on the surface of 1542 NPTX and CP3TX cells. This protein was chosen for further analysis because previous studies have found gp96 to be down regulated by IFNγ and has been implicated in the immune response through delivery of peptides to MHC I molecules (Nagano et al., 2003, Dai et al., 2003). gp96 is a 96kD glycoprotein normally resident in the endoplasmic reticulum (ER) having

both a signal peptide and the tetrapeptide, Lys-Asp-Glu-Leu (KDEL), retention sequence for this location (Robert et al., 1999). gp96 has, however, also been localized to the cell surface of tumour cells (Yedavelli et al., 1999a, Manjili et al., 2002, Caudill and Li, 2001, Haverty et al., 1997a). The cell surface location of gp96 was confirmed through a series of immunofluorescence studies and found to be surface associated by biotin labelling in further research using methods similar to those used in the present study (Altmeyer et al., 1996). The over expression of gp96 has been reported in oesophageal carcinoma and is thought to confer a selective advantage to cancer cells by inhibiting apoptosis (Chen et al., 2002). In addition, studies have shown that gp96 harvested from tumours can be re-administered to the patient as an anti-tumour vaccine, due to its ability to elicit both a non-specific cytokine and a specific cytotoxic T cell response (Robert et al., 1999, Manjili et al., 2002). Cytokines have been reported to have a number of effects on gp96, however a recent study disclosed that not only was gp96 over expressed in the 1542 CP3TX cell line, but that expression could be decreased by 24 hr exposure to IFNγ (Nagano et al., 2003).

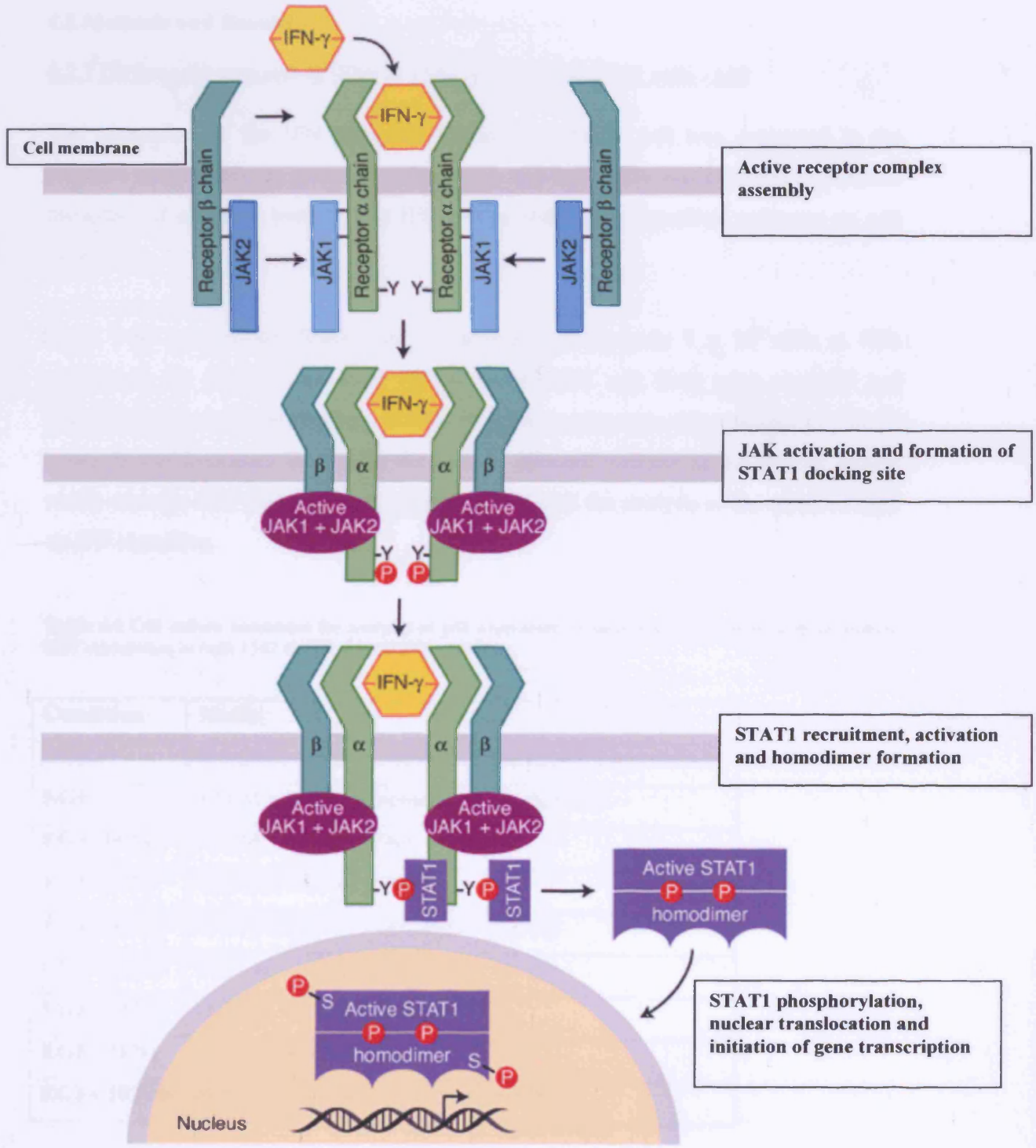



**Figure 4.1.** The IFNγ signalling pathway. Taken from (Newport, 2003).

## 4.2 Methods and Results

### 4.2.1 Differential response to IFNγ in 1542 NPTX and CP3TX cells - p48

The expression of the IFN inducible transcription factor p48 was compared in the presence and absence of cytokines IFNα and γ and EGF. EGF was included to examine the effect of crosstalk between the IFN and growth factor signalling pathways on p48 expression.

Eight T-80 cell culture flasks each containing approximately 7 x $10^6$ cells at 70% confluence for each of the 1542 NPTX and CP3TX cell lines were prepared and transferred into minimal medium (KSFM without supplements or serum added) for 24 hours. It was necessary to transfer the cells to minimal medium as the normal growth media contains EGF and therefore would interfere with the analysis of the effect of EGF on IFN signalling.

**Table 4.1** Cell culture conditions for analysis of p48 expression in response to cytokines, with or without EGF stimulation in both 1542 NPTX and CP3TX cell lines.

| Condition | Media |
|---|---|
| **EGF +** | Complete KSFM (cKSFM) (containing 0.2 ng/ml EGF) |
| **EGF-** | KSFM w/o supplements or serum (KSFM) |
| **EGF- IFNα** | KSFM, 500U/ml IFNα |
| **EGF- IFNγ** | KSFM, 500U/ml IFNγ |
| **EGF- IFNαγ** | KSFM 500U/ml IFNα, 500U/ml IFNγ |
| **EGF+ IFNα** | cKSFM, 500U/ml IFNα |
| **EGF+ IFNα** | cKSFM, 500U/ml IFNα |
| **EGF+ IFNγ** | cKSFM 500U/ml IFNγ |
| **EGF+ IFNαγ** | cKSFM, 500U/ml IFNα, 500U/ml IFNγ |

The stimulation conditions applied to both 1542 NPTX and CP3TX cell lines can be observed above in Table 4.1. As p48 is an IFN inducible transcription factor, both IFNα and γ were added alone or in conjunction. The influence of the mitogen EGF on p48

expression in response to these cytokines was analysed by repeating the cytokine stimulation with or without EGF present in the media. Each of the conditions indicated above were applied for 24hrs. The cells were then labelled with LC biotin as per protocol (see section 2.2.6), lysed in 500µl 1% OGP lysis buffer and quantitated using the Lowry protein concentration assay. The samples were resolved on a 12.5% gel at a concentration of 30µg of protein per well and subject to western transfer. This experiment was repeated in triplicate. The membranes were then probed with avidin, anti-p48, anti-STAT 1 and β-actin antibodies, representative results of which can be seen in Figure 4.2.

Avidin blotting of the resolved cell lysates revealed a number of proteins, with differing patterns of expression in response to the cytokines IFNγ and α in 1542 NPTX and CP3TX cell lines (See arrows Figure 4.2 A). These may represent cell surface proteins or a background signal from other non-labelled cellular proteins detected by the avidin probe. To examine the differences in IFN signalling further, the pattern of p48 expression was analysed, see 4.1.B. The bands from three replicate gels were analyzed as digitized images by using a GS-700 Imaging Densitometer (Bio-Rad). The mean optical density (OD) of band images is expressed as volume (OD × area) and adjusted for background to give arbitrary units of adjusted volume, these values were then normalised to β-actin to adjust for loading differences. Densitometry analysis of the results of this study indicated the following; firstly that the expression of p48 in the absence of EGF is barely detectable in 1542 NPTX cells, with higher expression in 1542 CP3TX (8.6 ± 0.9 in NPX versus 15 ± 1.3 in CP3TX). The difference in p48 expression became more marked when EGF was present in the medium, 1542 NPTX cells maintain a barely detectable level of this protein, whereas in 1542 CP3TX cells there is an approximate 2 fold increase in p48 expression (rising from 15 ± 1.3 to 33 ± 1.5).

The responses of 1542 NPTX and CP3TX cells to IFNγ and α under normal conditions and in the absence of EGF, revealed differing expression patterns of p48. In the normal cell line, in the absence of EGF, stimulation with IFNα increases expression three fold (rising from 8.6 ± 0.9 to 27 ± 1.4), whereas in the cancer cell line there is a lesser increase in expression (rising from 15 ± 1.3 to 33 ± 1.1). However, with IFNγ the

converse is the case with 1542 CP3TX displaying a greater increase in expression in the absence of EGF (rising from 8.6 ± 0.9 to 27 ± 1.3 in 1542 NPTX cells and from 15 ± 1.3 to 63 ± 1.8 in 1542 CP3TX cells). When both cytokines were added together to EGF starved cells the synergistic effect is the same as with the single cytokine showing highest expression, i.e. IFNα in 1542 NPTX cells, IFNγ in 1542 CP3TX cells. When the cytokines were applied to cells in the presence of EGF, an enhanced expression of p48 was observed that followed the same pattern as in the absence of EGF described above.

Statistical analysis using one-way analysis of variance (ANOVA) indicated that cell line has no significant effect on p48 expression, whereas the presence of EGF in the growth medium and stimulation by IFNα did elicit a significant effect on p48 expression in these cell lines ($P=0.024$ and $P<0.001$ for EGF and IFNα stimulation respectively). To examine interaction between factors a two-way ANOVA was applied to these data, which indicated that there was a significant interaction between the factors EGF and IFNα stimulation in terms of variation in p48 expression ($P=0.003$) and also between the factors cell line and IFNα stimulation ($P<0.001$). This indicates that although no significant difference was observed in p48 expression between the cell lines in the absence of other factors, when IFNα is introduced a significant difference in p48 expression occurs. The p48 expression data for IFNγ stimulation produced no significant effects in either ANOVA analysis. The two cytokines were found to have no interactive effect, in agreement with the observations made above.

The membranes from the p48 expression analysis were re-probed with an antibody to STAT1, a transcription factor involved in IFN signalling. This revealed two bands detected by the STAT1 antibody, corresponding to the correct molecular weights of the STAT1 α and β subunit. In the normal unstimulated state, the predominant subunit expressed was the higher molecular weight STAT1 α form in both cell lines (See Figure 4.2 C). The expression of the α subunit remained at a constant level in all the culture conditions in both cell lines. The STAT1 β subunit appeared to be expressed at similar low levels in 1542 NPTX and CP3TX cells in both the normal unstimulated state and in the absence of EGF. On addition of either IFN, the STAT1 β sub-unit expression

appeared to increase, indicating that the expression of this transcription factor subunit is induced by IFNs. In the 1542 NPTX cell line in the absence of EGF, expression of STAT1 β showed an apparent similar increase following the addition of either or both IFNs. When EGF was present, the effect of the two cytokines on STAT1 β expression was potentiated. In the 1542 CP3TX cell line under EGF starvation conditions, both IFNs increased STAT1 β expression. The greatest response however, followed treatment with IFNγ. The combined effect of both cytokines resulted in a STAT1 β level comparable to that with IFNγ alone. In the presence of EGF, this pattern of expression was enhanced but unchanged in the cancer cell line.

4.2.2 IFNγR

The IFNγR is up regulated by IFNγ (Bacso et al., 2002). In this study, IFNγR expression was analysed in several prostate cell lines in the presence and absence of IFNγ. Two T-80 cell culture flasks each containing approximately 7 x$10^6$ cells at 70% confluence for each cell line (1542NPX, 1542 CP3TX, DU145, LNCaP, PC3, Pre 2.8) were prepared and IFNγ was added to one of each pair of flasks for 24 hrs. The cells were then washed x 3 in 1x PBS and lysed in 1% OGP. The protein content was measured using the Lowry protein concentration assay, following which 50μg of each sample was loaded on a 10% gel and subject to electrophoresis and subsequent western transfer. The membrane was then probed using an antibody to the IFNγR. This experiment was repeated in duplicate representative results of which can be observed in Figure 4.3, the results were normalised to β actin and analysed via densitometry.

The IFNγR antibody appears to detect several bands at the approximate molecular weight for IFNγR. This observation could be due to lack of specificity of this antibody or indicate that several isoforms of the protein are present in the lysate. In the unstimulated state, the band corresponding to the correct weight for the IFNγR protein appeared to be most highly expressed in the Pre 2.8, 1542 NPTX and CP3TX cell lines, with the other cancer cell lines showing a low level of expression. On stimulation, the expression of IFNγR in 1542 NPTX cells showed an apparent increase of approximately 3 fold (rising from 11 ± 1.8 to 35 ± 3). However, in the other normal cell line, Pre 2.8, expression did

not appear to increase. PC3 and 1542 CP3TX cells displayed an increase in expression comparable to that of 1542 NPTX cells, whereas DU145 appeared to show only a slight increase in expression on stimulation with IFNγ. In contrast, LNCaP demonstrated an apparent marked decrease in expression of the IFNγR when exposed to IFNγ. Analysis of these results was hampered by the lack of specificity of the IFNγR antibody and any result observed could be entirely attributable to non-specific interactions.

4.2.3 gp96, a cell surface glycoprotein differentially regulated by IFNγ

gp96 is a glycoprotein found on the surface of certain cells where it may play a role in triggering an immune response (Robert et al., 1999, Manjili et al., 2002). Recent proteomic studies found this protein to be over expressed in the 1542 CP3TX cell line in comparison to 1542 NPTX and that this expression can be modulated by IFNγ (Nagano et al., 2003). In the present study, the expression of gp96 was explored using samples from a panel of prostate cell lines. Two T-80 cell culture flasks each containing approximately 7 x$10^6$ cells at 70% confluence for each cell line (1542 NPTX, 1542 CP3TX , DU145, LNCaP, PC3, Pre 2.8) were prepared and one of each pair of flasks stimulated for 24 hrs with IFNγ. The cells were then washed, lysed and quantitated as above. The samples were resolved on a 10% gel at a concentration of 30μg of protein per well and then subject to western transfer. The membrane was then probed using an antibody to gp96. This experiment was repeated in triplicate, a representative result of which can be observed in Figure 4.4.

The results of an anti-gp96 immunoblot on whole cell lysate from various prostate cell lines can be observed in Figure 4.4A. The results of this analysis on the panel of prostate cell lines in the presence and absence of IFNγ suggests that in the unstimulated state, the cancer cell lines expressed more gp96 than the normal cell lines. However, after normalisation to β actin any differences observed are completely attributable to differences in loading. On stimulation with IFNγ, there was also no difference in expression when the densitometry readings were normalised to β-actin.

The expression of gp96 on the surface of the 1542 NPTX and CP3TX cell lines in the presence and absence of IFNγ, was examined using the cell surface analysis protocol optimised in chapter 3 (See section 2.2.6-7). Six T-80 cell culture flasks each containing approximately 7 $x10^6$ cells at 70% confluence were prepared for both 1542 NPTX and CP3TX cell lines. Three flasks of cells were exposed to IFNγ for 24 hours. The cells were then labelled with SS biotin, lysed in 1% OGP and subject to affinity chromatography as per protocol (see section 2.27). Following pre-fractionation, the samples were dialysed, vacuum concentrated and then quantified using a Bradford protein concentration assay. 30μg of each sample was loaded onto a 10% gel, subject to electrophoresis and western transfer. This experiment was repeated in triplicate. An immunoblot of a representative result using anti-gp96 antibody is shown in Figure 4.4B. These results were analysed via densitometry, normalisation to actin was not possible as the proteins present were derived solely from the cell membrane.

These results show that in the unstimulated state an approximately 2 fold higher level of gp96 cell surface expression can be observed in 1542 CP3TX when compared to 1542 NPTX cells (17 ± 1.2 in NPTX cells compared to 34 ± 0.6 in CP3TX cells) a student's t-test shows this is a significant result, with a probability value of $P<0.001$. On stimulation with IFNγ the expression of gp96 on the cell surface of 1542 CP3TX cells shows a significant decrease ($P<0.001$ using a student's t-test) to almost undetectable levels, confirming previous findings (Nagano et al., 2003). The converse is true of the 1542 NPTX cells, which on IFNγ stimulation show a 2 fold increase in cell surface expression of gp96 (rising from 17 ± 1.2 to 37 ± 1.9, $P<0.001$). Surface gp96 expression in normal and cancer cell lines, in response to IFNγ was confirmed as significantly different by the students t test, $P<0.001$.

The gp96 expression patterns observed in 1542 NPTX and CP3TX cell lines were analyzed further by two dimensional gel electrophoresis (2DGE). Six T-80 cell culture flasks containing approximately $7x10^6$ cells were prepared for both 1542 NPTX and CP3TX cell lines. Three flasks of each cell line were stimulated for 24 hrs with IFNγ, after which the flasks were lysed and pooled into 500μl 2DGE lysis buffer (see section

2.1.4). The protein concentration of the samples was quantified by a Bradford protein assay and 300μg of protein applied to an Immobiline pH 3-6 IPG strip for each sample as per protocol (see section 2.2.13). The first and second dimensions of 2DGE were performed according to this protocol and the gels transferred to nitrocellulose. These membranes were then probed with anti-gp96 antibody.

Differences were observed between the cell lines in not only the level of expression, but also the number of isoforms induced on stimulation with IFNγ (See Figure 4.5). The 2D analysis confirms that gp96 is expressed more highly in the 1542 CP3TX cell line than in its normal counterpart and that this expression decreases on stimulation with IFNγ. In the 1542 NPTX cell line, stimulation with IFNγ causes an increase in the number of gp96 isoforms present, whereas in the 1542 CP3TX line only one isoform continues to be expressed after 24 hrs in the presence of IFNγ.

The effect of IFNγ on cell surface expression of gp96 was also analysed using immunofluorescence. One 24 well plate containing cover slips was prepared for the cell lines DU145, PC3, LNCaP, Pre 2.8 and seeded with 20,000 cells per well. The cells were cultured for 72 hours and then half the wells in each plate were stimulated with IFNγ for 24 hrs. The cells were washed and fixed in paraformaldehyde as per protocol (see section 2.2.15). Immunofluorescence was performed using an anti-gp96 antibody (See Figure 4.6). The amount of primary antibody required to achieve immunolocalisation of the gp96 protein was assessed through optimisation of the procedure using various concentrations of primary antibody. Dilutions of 1 in 50, 1 in 100, 1 in 200 and 1 in 500 were used, although no improvement in signal was seen past 1 in 100. An additional attempt to improve the signal from immunofluorescence was made in the form of adding 2% blocking buffer (BSA or Non-fat dried milk) to the primary antibody solution. This modification however appeared to weaken the signal and was therefore omitted from later experiments. Subsets of cells from each cell line, stained with secondary antibody only, were used as a control to assess the level of non-specific background staining. These controls lacked any perceivable immunofluorescent signal either with the naked eye or by cooled CCD camera detection, in all cell lines.

The localization of gp96 in unstimulated cells appeared to be predominantly nuclear and cytosolic, although cell borders can be seen, suggesting membrane localisation of the protein. In the normal cell line Pre 2.8 the expression of gp96 increased, especially in the nuclear region. In the three other cancer cell lines there was an apparent reduction in gp96 expression, with staining becoming diffusely cytosolic. These results indicate that an increase in gp96 expression is seen in the normal cells and the converse is observed in the cancer cell lines on stimulation with IFNγ.

4.2.4 Affinity chromatography of IFNγ stimulated 1542 NPX and CP3TX cells

To further examine the effect of IFNγ on the surface composition of 1542 NPX and CP3TX cells, vectorial labelling with Sulfo-NHS–SS-biotin of 2.25 x $10^4$ cells was conducted for each cell line in the presence or absence of 24 hr stimulation with IFNγ. This was followed by avidin affinity chromatography, to pre-fractionate cell surface proteins before electrophoresis and mass spectrometry identification. 50μg of each sample was loaded onto two 12.5% gels, in duplicate and subject to standard electrophoresis conditions. One set of gels was then subject to western blotting whilst the other was stained in coomassie blue. This process was repeated in triplicate, a representative coomassie stained gel can be observed in Fig.4.7A. The bands from three replicate gels were analyzed as digitized images by using a GS-700 Imaging Densitometer (Bio-Rad). The mean optical density (OD) of band images expressed as volume (OD × area) and adjusted for background to give arbitrary units of adjusted volume.

Several proteins were identified by densitometry that showed apparent differential expression on treatment with IFNγ, two of these proteins appeared to be up regulated by the cytokine, but a 36 kD surface protein found in high density was significantly down regulated in response to IFNγ stimulation (Fig 4.7A red asterisk). The three bands corresponding to these proteins showing the largest apparent change in expression on treatment with IFNγ were cut and sent for MS analysis. The protein down regulated by IFNγ was identified as AII by MALDI-MS analysis of peptides generated by trypsin degradation of the excised Coomassie stained gel band. The other proteins of 66 and 46 kD were identified as Selectin and IFN induced protein 53 and Selectin, respectively. As

can be observed from Fig.4.7A these three proteins appear to be expressed at the same level on the surface of normal and cancer cells and show a similar pattern of regulation in response to IFNγ stimulation in both cell lines. The expression of Selectin and IFN induced protein 53 is increased by approximately 2 (17 ± 2, increasing to 35 ± 4) and 4 fold (20 ± 1 rising to 61 ± 2) respectively, following stimulation with IFNγ. The significance of this data was confirmed statistically using two-way ANOVA analysis for both selectin and IFN induced protein 53. Cell line was found to be an insignificant factor in the expression of both proteins (P=0.41 and P=0.24 respectively), however IFNγ treatment was shown to have a significant effect on expression (P=<0.0001 in both cases). There was no interaction between cell line and IFNγ treatment in either case (P=0.41 and P=0.54 respectively).

AII expression decreased by 3 fold (32 ± 2 to 9 ± 1.3) on stimulation with IFNγ in both cell lines. This was confirmed by immunoblotting of the three replicate gels with an anti-AII antibody, a representative result and be seen in Fig.4.7B. Statistical analysis of these results using the students t–test indicates that not only is AII expression significantly down regulated by IFNγ, with a P value of 0.001 in 1542 NPX cells and 0.000 in 1542 CP3TX cells, but also that the down regulation of surface expressed AII in response to IFNγ occurs to a greater degree in 1542 CP3TX cells (P=0.001).

**4.3 Discussion**

The aim of this study was to compare the effects of IFNs on protein expression in 1542 NPTX and CP3TX cell lines. The initial section of this research focused on the comparison of proteins involved in the interferon signalling pathway. The rationale for this study was that identification of differing responses to IFNs may lead to a therapeutic use of these cytokines. The expression of various key proteins in IFNγ signalling such as p48, STAT1 and the IFNγR, on exposure to various conditions (IFNγ, α and EGF) was determined. These experiments also allowed examination of the cross talk between the IFN signalling pathway and that of the mitogen EGF. The second section focussed on the analysis of cell surface proteins differentially modulated by IFNγ in 1542 NPTX and CP3TX cells.

4.3.1 IFN signalling

Avidin blotting of lysates from cells exposed to various stimulation conditions revealed several proteins modulated by IFNs. It is possible that these represent cell surface proteins regulated by IFNs, confirming that these cytokines can modulate the cell surface composition of 1542 NPX and CP3TX cells. Additionally these results demonstrate that there are different patterns of protein expression in the 1542 NPX and CP3TX cell lines under different stimulation conditions. Before any protein identifications can be made, however, pre-fractionation using affinity chromatography should be employed to remove contaminating cellular proteins that may confound true cell surface patterns of expression.

The differences in IFN modulated protein expression in 1542 NPTX and CP3TX cells were examined by analysis of the transcription factor p48. This protein was chosen, as it is a part of a complex responsible for the transcription of interferon inducible genes, many of which have been found to be differentially expressed in these cell lines (Nagano et al., 2003). In this study, p48 was found to be over expressed in the cancer cell line. This finding is possibly indicative of the cancer cell being more sensitive to p48-inducing factors such as EGF, which is present in the complete KSFM. This sensitivity could result from over expression of the EGF receptor making the cell more responsive to normal circulating levels of EGF. The increased expression of p48 could be a downstream effect directly or indirectly due to the cancer cell acquiring a more mitogenic phenotype and thus a survival advantage. The induction of p48 expression by EGF and IFNs indicates that signalling from the EGFR may in some part follow a similar pathway to that of IFNs, suggesting the possibility of cross talk between these pathways.

Additional differences in the pattern of expression of p48 were observed between the isogenic pair of cell lines, indicating that there could be an alteration in crosstalk between the IFN and EGF signalling pathways in the cancer cells. In normal cells in the absence of EGF, more p48 is expressed on stimulation with IFNα than with IFNγ, as would be expected if p48 was required for transcription of genes containing ISRE's. The situation

is the converse, however in the 1542 CP3TX cell line, where more p48 is expressed on stimulation with IFNγ. Although the type I and II IFN pathways are similar, sharing a number of key proteins, p48 is not thought to be involved in IFNγ inducible gene transcription. This converse expression of p48 in cancer cells could be advantageous, as it may lead to the avoidance of IFN signalling and the subsequent cell changes leading to heightened immune surveillance. Further studies would be needed however, to confirm this hypothesis. These studies could include quantitative analysis of other signalling factors associated with IFN signal transduction, such as STAT2.

In addition to the above, differences in IFN signalling between 1542 NPTX and CP3TX cells are also seen in the expression of the STAT1 β subunit. Unlike the α subunit which remains at a constant expression level in all conditions in both cell lines, the β subunit appears to be modulated by IFNs α and γ. In the normal cell line the level of expression of the STAT1 β subunit increases by the same amount in response to α or γ alone or in combination. In the cancer cell line this is not the case, as while both cytokines lead to increased expression of STAT1 β, a higher level of induction is seen with IFNγ. The potentiation of p48 and STAT1 β expression patterns in both cell lines by EGF, suggest that there is some cross talk between the mitogenic and IFN signalling pathways. The aberrant expression patterns of IFN signalling molecules observed in the 1542 CP3TX cell line may also be attributable to convergence with the EGF or some other antagonising signalling pathway. Further exploration of the interactions between these pathways is required before their effects on each other can be clearly delineated.

As mentioned previously IFNγ is capable of modulating cell surface proteins, including its own receptor IFNγR. In IFNγ stimulated cells expression of IFNγR has been localised to lipid rafts, along with the signal transducer STAT1 molecules (Bacso et al., 2002, Sehgal et al., 2002). Expression of the IFNγ receptor was examined in the presence and absence of IFNγ in several prostate cell lines. The rationale for this study was that a lack of the upregulation of IFNγR seen in normal cells, could lead to a reduction in responsiveness to IFNγ in the cancer cells and the avoidance of immunosurveillance.

The IFNγR protein appeared to be most strongly in the unstimulated state in the normal cell line Pre 2.8, with 1542 NPTX and CP3TX also showing an apparent high level of expression. The other cancer cell lines, PC3, DU145 and LNCaP expressed IFNγR at a lower level. On stimulation, the 1542 NPTX cell line appeared to show the increased expression of the IFNγR expected of a normal cell line. However, no increase in expression was seen in Pre 2.8 cells. Most of the cancer cell lines appeared to show upregulation of the IFNγR protein, there was however, a degree of variation in this increased expression of IFNγR in the cancer cells. The 1542 CP3TX and PC3 cell lines appear to express the greatest level of IFNγR induction, there then followed DU145 with a lower level of induction by IFNγ, then LNCaP which showed a reduction in IFNγR expression on exposure to IFNγ. This may indicate that a loss of IFNγR regulation by IFNγ represents a step towards a more metastatic phenotype. The reduction in IFNγR expression observed in LNCaP could be advantageous to a tumour cell as not only would it avoid immune detection but also the growth suppressive effects of IFNγ. These observations however, should be taken as no more than inferences for further study, as the lack of specificity of the IFNγR antibody made assessment of the significance of the results impossible. Further replicates of this study are required, including both positive and negative controls to isolate the bands that represent the IFNγR. This could be further verified by protein/peptide competition for binding sites on the protein of interest followed by comparative immunodetection with pure lysate and both negative and positive controls. This protocol could be used for any immunoblot where unexpected multiple bands, suggesting non-specificity, are observed. In addition, immunohistochemical analyses of IFNγR expression in differing grades of prostate cancer clinical samples may provide a more conclusive link between IFNγR expression and malignant progression in prostate cancer.

### 4.3.2 Cell surface proteins

The glycoprotein gp96 was chosen for further analysis to examine the effect of IFNγ on a cell surface protein involved in immune display that is upregulated in 1542 CP3TX cells. Studies of total cell lysate and immunofluorescence in a panel of prostate cell lines in the

presence and absence of IFNγ confirmed that gp96 expression levels were elevated in the cancer cell line. Cell surface analysis of gp96 was performed using the optimised protocol from chapter 3. This experiment resulted in confirmation that gp96 is over expressed in the 1542 CP3TX cell line and that expression can be reduced by exposure to IFNγ. Stimulation with IFNγ resulted in an increase in gp96 expression in 1542 NPTX cells, also confirming previous findings. Further confirmation of this effect resulted from 2DGE where it appeared that the increase in gp96 expression in the normal cells was due in part to an increase in the number of isoforms of the protein, this reaction was not observed in the 1542 CP3TX cells. The appearance of these isoforms may have been due to non-specific staining of other proteins, however this effect was not seen any other applications of the antibody probe.

gp96 is a molecular chaperone responsible for the transport of peptides to the MHC 1 proteins on the cell surface. In addition, gp96 has been reported to be a primitive antigen display molecule in its own right, able to elicit cytotoxic responses (Robert et al., 1999, Manjili et al., 2002). Consequently it is difficult to account for the over expression of this protein in cancer cells, as increased antigen display may be a selective disadvantage. However, studies have discovered that gp96 over expression in cancer cells is linked to inhibition of apoptosis (Chen et al., 2002). It is possible, therefore, that in the cancer cell line there is a trade-off in gp96 expression between the negative effects of antigen display and the advantage conferred by the inhibition of apoptosis. Further evidence for this could be seen in the fact that 1542 NPTX cells produce a number of gp96 isoforms on stimulation with IFNγ, in contrast with the reduction seen in the single gp96 isoform in 1542 CP3TX cells. These results may suggest that in the normal cells multiple isoforms of gp96 are produced to transport a number of different peptides for antigen display on stimulation with IFNγ, possibly to facilitate the immune response. In the cancer cells this does not occur, the response being to down regulate gp96 and thus potentially avoid immune surveillance.

The application of the cell surface analysis protocol developed in chapter three allowed the identification of several proteins displaying apparent differential expression when

stimulated with IFNγ. The two isogenic cell lines had similar protein expression patterns when stimulated with IFNγ, both displaying upregulated levels of interferon-induced protein 53 and selectin and exhibiting a down regulation in AII following 24 hour treatment with IFNγ. The down regulation of cell surface AII in both 1542 NPX and CP3TX cell lines following 24 hour exposure to IFNγ was confirmed by western blotting. The results of this experiment however, revealed a difference in response to IFNγ between the two cell lines. In the 1542 CP3TX cell line a greater degree of AII down regulation in response to IFNγ was observed in comparison to that of normal cell line. This may suggest that, in vivo, IFNγ could cause a greater down regulation of AII and possibly invasive potential in the cancer cells. AII was chosen for further study due to its potential role in cell invasion and therefore its possible therapeutic target value.

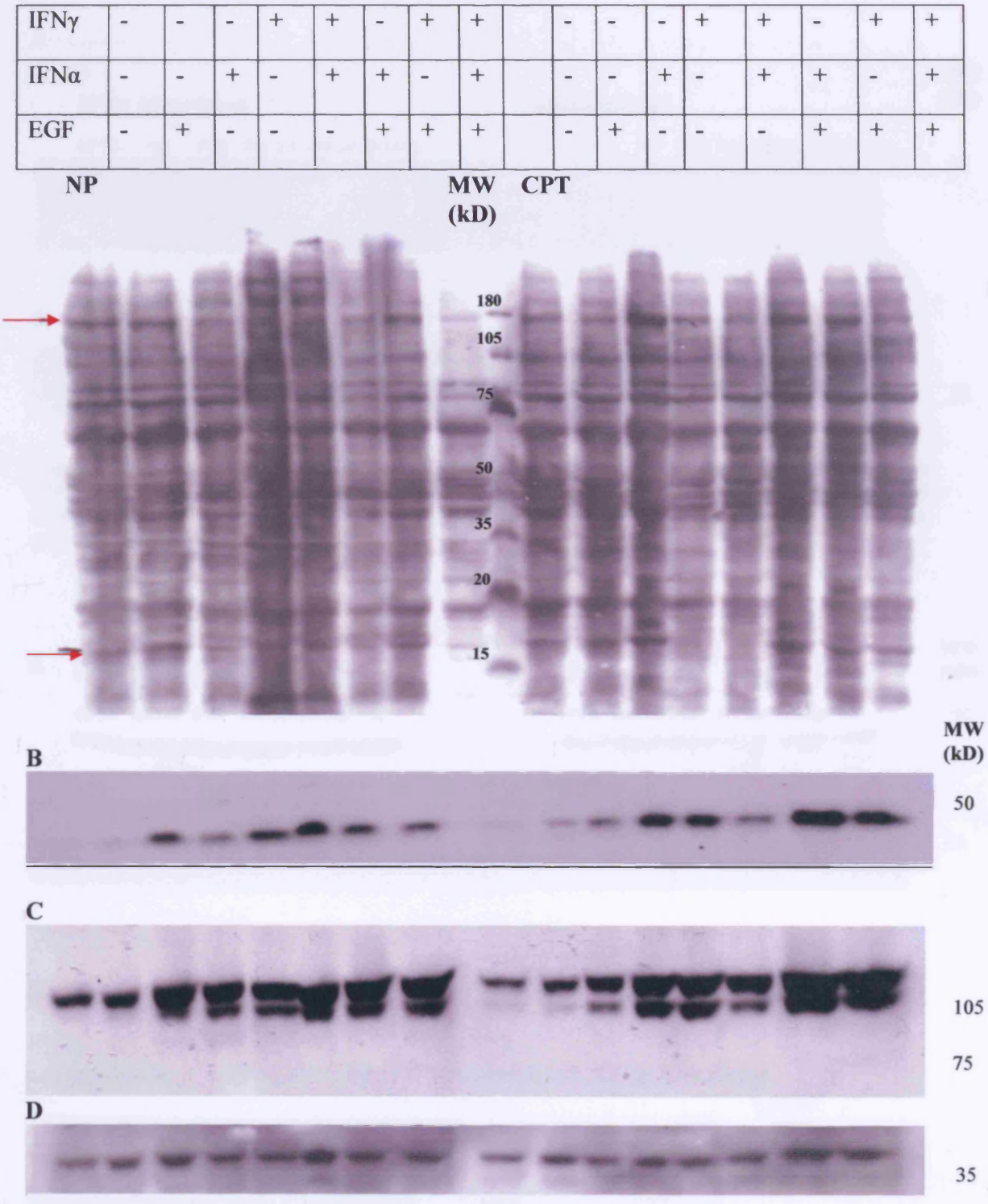



**Figure 4.2.** A comparison of IFN signalling molecules in 1542 NPTX CP3TX cells on stimulation with various factors showing; **A** an avidin blot of total cell lysate following cell surface labelling with LC biotin, **B** the expression of p48, **C** expression pattern of STAT1, **D** loading control (β actin). The table at the top of the figure denotes the position of each of the samples conditions used in this study and relates to all four gel representations.

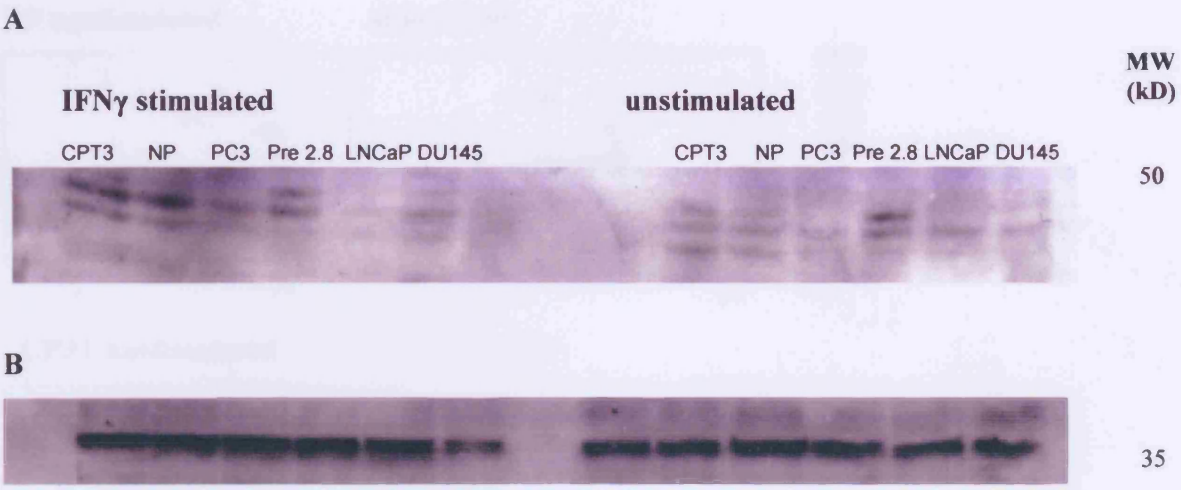
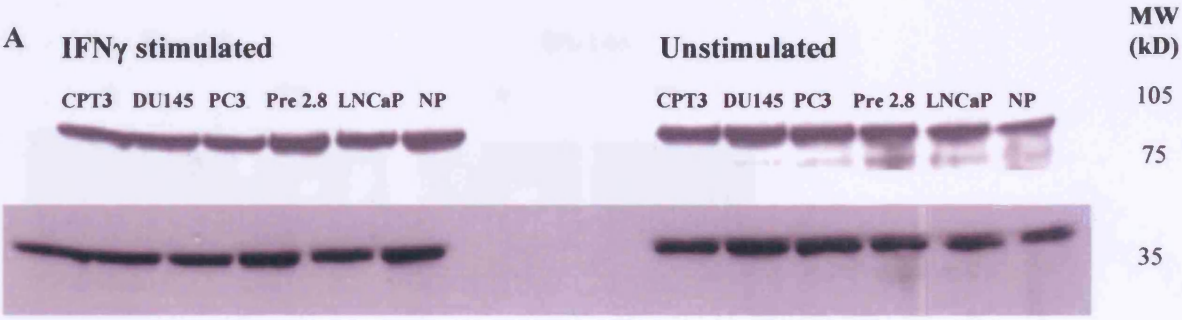



**Figure 4.3 A** IGFR expression in several prostate epithelial cell lines plus or minus 24 hr stimulation with IFNγ, **B** β actin expression as a loading control. IGFR expression is possibly higher in normal cell lines and appears to increase with IFNγ treatment in the majority of cancer cell lines. A decrease in expression of this protein may be observed in LNCaP.



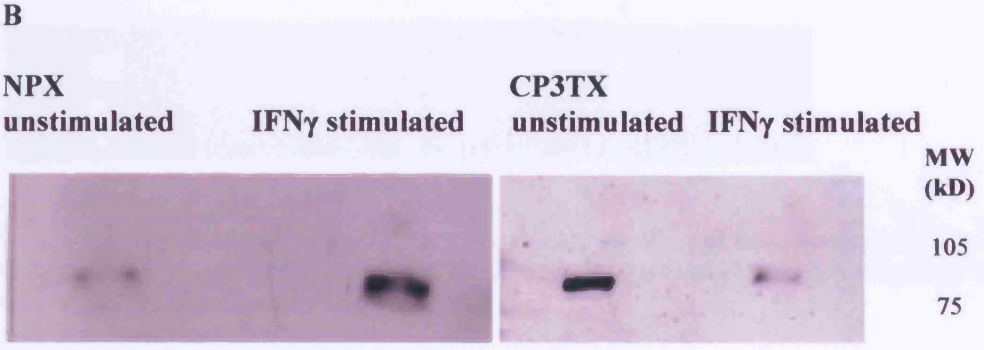




**Figure 4.4** Anti-gp96 immunoblot of **A** panel of prostate cell lines, **B** 1542 NPTX and CP3TX cell surface fraction, plus or minus stimulation with IFNγ.

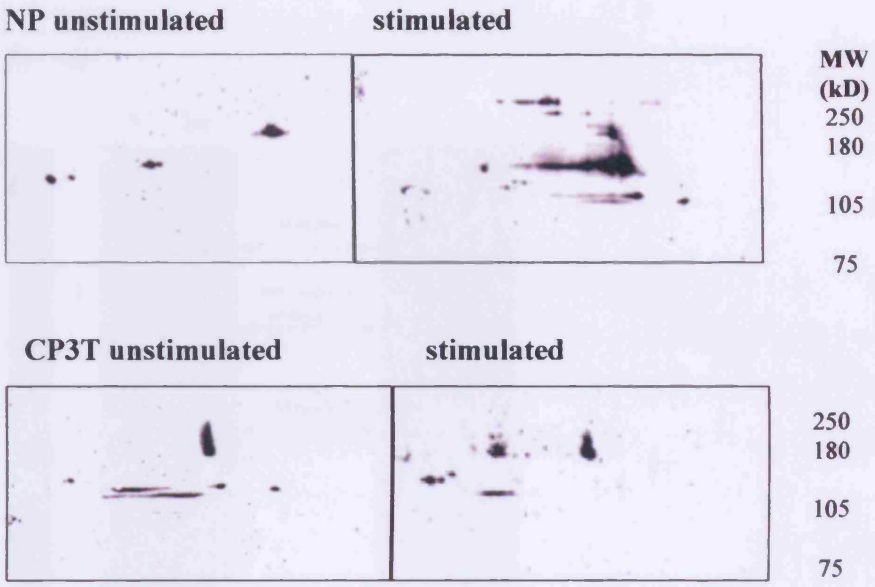



**Figure 4.5.** Anti-gp96 immunoblot of 2D analysis of 1542 NPX and CP3TX cell lines with or without 24 hour stimulation with IFN gamma. On stimulation NP cells appear to produce a multitude of gp96 isoforms leading to a higher expression level, this is not seen in CPT cells.

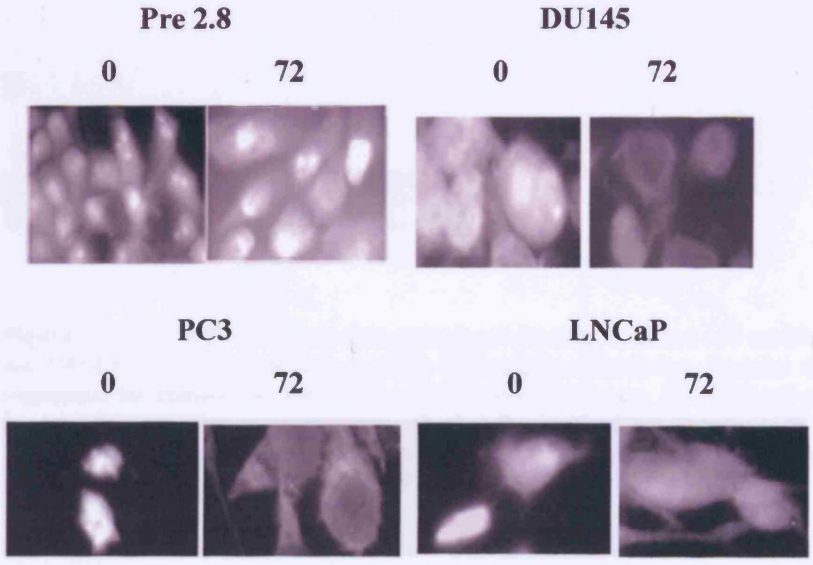



**Figure 4.6** gp96 immunofluorescence in a selection of prostate cell lines in the absence or presence of IFNγ treatment, at a magnification of x60. These results suggest that gp96 is down regulated in cancer and upregulated in normal prostate epithelial cells.

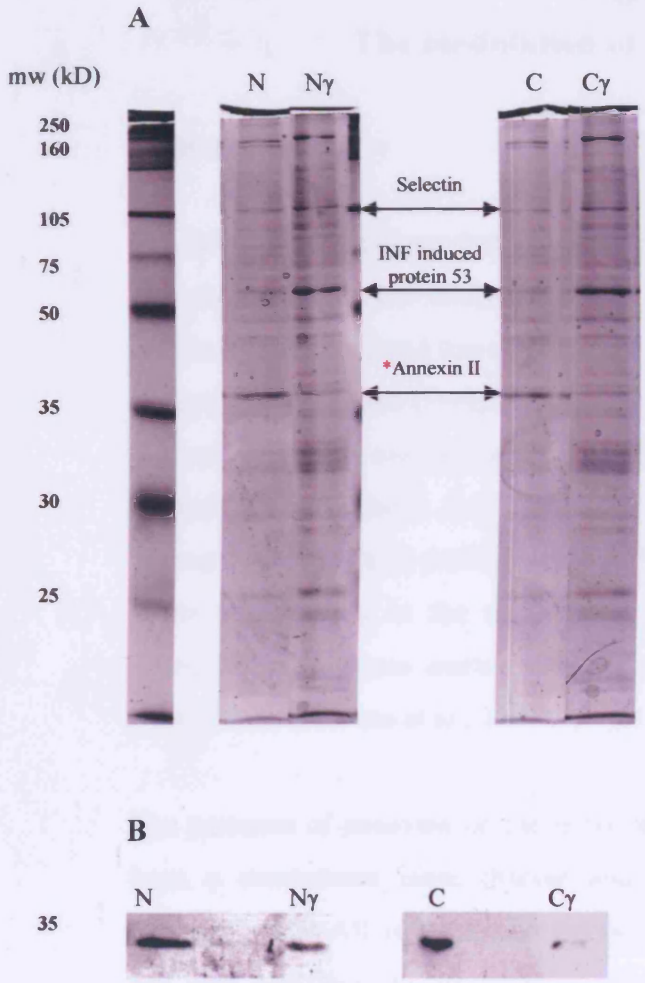



**Figure 4.7. A** SDS PAGE coomassie stained gel showing total and cell surface fractions of 1542 NPX (N) and CP3TX (C) cell lysate plus or minus IFN gamma stimulation. Proteins showing apparent differential expression on stimulation with IFNγ are indicated by arrows. IFN induced protein 53 and Selectin both increase in expression on stimulation, whereas the level of AII decreases. **B** A duplicate gel probed with anti-AII. This replicate experiment confirms AII is down regulated by IFNγ on the cell surface, this effect is most apparent in the 1542 CP3TX cell line.

# Chapter 5
# The modulation of cell surface AII by IFNγ

## 5.1 Introduction

The 36kD calcium dependent phospholipid binding protein AII, identified in chapter 4, was chosen for further study as several studies suggest that this protein has a potential role in cell invasion and therefore may provide a target for the prevention of invasion and subsequent metastasis (Podgorski and Sloane, 2003, Mai et al., 2000b, Diaz et al., 2004). The role of AII in invasion is thought involve its function not only as a co-receptor for several endopeptidases (e.g. plasmin) (Diaz et al., 2004), ECM components and pathogens (Pietropaolo and Compton, 1997, Choi et al., 2000, Mai et al., 2000b), but also in its participation in the remodelling of the basement membrane by activation of extracellular proteases and/or by direct interactions with components of the ECM in tumour cells (Falcone et al., 2001).

The presence of annexins on the outer leaflet of the plasma membrane has historically been a contentious issue (Siever and Erickson, 1997). However, there are many indications that AII is present on the outer leaflet of the plasma membrane lipid bilayer and may contribute to the invasive potential of the cell (Emoto et al., 2001). In the clinical setting, AII over expression on the cell surface has been reported in a number of carcinomas including brain (Nygaard et al., 1998), breast (Schwartz-Albiez et al., 1993), lung (Cole et al., 1992, Brichory et al., 2001b), pancreas (Paciucci et al., 1998) and colon (Emoto et al., 2001). In such cancers, surface AII over expression has been linked to a more aggressive tumour phenotype and concurrent poor prognosis (Mai et al., 2000c). The link between AII expression and an invasive phenotype was recently explored in macrophages where it was found to regulate their invasive and degradative phenotype. In this study the ability of AII to bind plasmin was inhibited by treatment with an antibody to AII and this prevented migration of cells through a simulated ECM (Falcone et al., 2001). Recent studies have provided further evidence for the role of AII in cellular progression to a more invasive phenotype. These studies have focussed on the ability of

AII to bind to proteases such as pro-cathepsin B, tissue plasminogen activator and plasmin (Mai et al., 2000b). Binding to AII activates these protease enzymes, initiating a proteolytic cascade resulting in the degradation of the ECM (Roshy et al., 2003, Podgorski and Sloane, 2003). The literature supports the hypothesis that AII present on the cell surface has a role in movement of the cell through the ECM (Mai et al., 2000b).

This study aims to examine the effect of IFNγ stimulation on cell surface AII expression. The first part of this chapter focuses on the identification and cell surface localisation of AII and the effect of IFNγ stimulation on AII expression. No connection has previously been found between IFNγ stimulation and AII expression. Despite the fact that other cytokines, such as interleukins, have been shown to modulate the expression of AII on the cell surface (Brichory et al., 2001a), the regulation of AII by IFNγ is previously unreported. IL-6 increases the cell surface expression of annexins I and II in lung cancer cells by 3 and 2 fold respectively, although no change was seen in cytosolic expression indicating a cytokine stimulated translocation of AI and II to the cell surface (Brichory et al., 2001a). A similar modulation was seen in leukaemia cells exposed to IL-3 (Matsunaga et al., 2004). The function of AII is explored in the subsequent section, to delineate the effect on invasive capacity of modulation by IFNγ. Invasive capacity was chosen for analysis because of the potential role AII has in cell invasion and an anti-AII antibody was used to inhibit invasive potential. The final part of this chapter concentrates on the mechanism of the IFNγ induced modulation of surface expressed AII, based on recent research into the transport and cell surface expression of annexin I (Chapman et al., 2003). Both annexins I and II are transported to the cell surface by some unknown mechanism, as they are not processed or transported through the trans-golgi network (TGN) (Chapman et al., 2003). Annexin I and II lack a cleavable signal sequence at the amino terminus that is usually required for the externalisation of proteins via the TGN. The transport mechanism of such proteins to the cell surface is yet to be discovered, though there is mounting evidence that proteins lacking this signal sequence are transported to the extra cellular face of the plasma membrane by ABC transporters (Chapman et al., 2003, Kuchler and Thorner, 1992). Annexin I has recently been reported to be transported to the cell surface by the ATP binding cassette transporter ABCA1 (Chapman et al., 2003).

The ABC protein family contains over 100 hundred proteins with varied roles, including the transport of ions (Ardehali et al., 2005), peptides (Koch et al., 2004), cytokines (Marty et al., 2005) and steroid hormones (Qian et al., 2001). They are defined by the possession of two nucleotide binding folds and two transmembrane domains forming channel like structures in the membrane (Kuchler and Thorner, 1992). There has been debate as to whether such transporters can facilitate the movement of large molecular weight proteins to the cell surface, however studies have shown that ABCA1 transports IL-β1 to the intracellular face of the plasma membrane (Fielding et al., 2000).

The most well characterised role of ABCA1 is the transport of lipids and free cholesterol to apo-1, this mechanism is severely impaired by mutation of the ABCA1 gene, as in Tangier disease, leading to a build up of cholesterol in organs including, tonsils, liver and spleen (Lawn et al., 1999). Recent research has shown that the ABCA1 transporter is localised to calveolae or lipid raft structures on the cell membrane and that this position is critical for its role in cholesterol transport (Fielding et al., 2000). In a recent study, immunofluorescence demonstrated that AI co-localises with the ABCA1 transporter in these lipid raft structures (Chapman et al., 2003). It was also discovered that exposure to an inhibitor of ABC transporters, glyburide, negates AI expression on the cell surface. Conversely stimulation with dexamethasone increased the surface expression of AI (Chapman et al., 2003). The possibility that this transporter may also be responsible for the translocation of AII to the cell surface is explored in the last section of this chapter.

## 5.2 Methods and Results

### 5.2.1 AII is down regulated on the cell surface in response to IFNγ

As surface exposed AII appeared to be down regulated by IFNγ on normal and cancer cells (See Figure 4.7), the total level of AII expression in these cells was explored. 1542 NPX and CP3TX cells plus or minus 24 hr stimulation with IFNγ were cultured, using 2.25 x $10^6$ cells for each condition and lysed as per protocol (see section 2.2.4) in 500μl of 1% OGP lysis buffer. 50μg of these complete cell lysates representing 1542 NPX and CP3TX cells plus or minus IFNγ stimulation were resolved on 12.5% gels in triplicate. The gels were then subject to western transfer and immunodetection was performed using

an anti-AII antibody. Expression levels were normalised to β actin and quantitated by densitometry. See Figure 5.2.

Similar levels of AII were found in the normal and cancer prostate cell lines (Figure 5.2) and IFNγ exposure did not change the total cellular level of AII. This was verified by two-way ANOVA statistical analysis where the amount of variation in AII expression was found to be within the limits of random sample fluctuations for both cell line and IFNγ treatment comparisons (P=0.34 in both cases) no significant interaction between these variables was noted. This experiment was repeated using a panel of prostate cell lines including the benign prostate cell line Pre 2.8 and three cell lines PC3, DU145 and LNCaP derived from metastatic prostate cancers. The total level of AII expression in the other normal and cancer prostate cell lines were also unaffected by gamma stimulation (Figure 5.3). This was confirmed by a two-way ANOVA analysis which found that the amount of variation in AII expression on stimulation with IFNγ in these cell lines was within the limits of random sample fluctuations (P=0.66). There was however a significant difference in the mean expression of AII when the data was categorised by cell line in an ANOVA analysis (P=<0.0001). This finding was further analysed using the Tukey test, which performs a pair wise comparison to isolate the source of variation within the factor. The LNCaP cell line was isolated as displaying the most significant difference in mean AII expression when subject to pair-wise comparison with the other prostate cell lines (mean difference of 38.7 at a 95% confidence interval). This was in agreement with the preliminary observation that AII expression was absent in the metastatic prostate cancer cell line LNCaP and in accordance with findings by Chetcuti and colleagues (Chetcuti et al., 2001a) who suggest that the AII gene is frequently hyper-methylated in prostate cancer.

To test this hypothesis a sample of LNCaP lysate from cells that had been exposed to the de-methylating agent 5-aza-deoxycytidine was obtained from Dr. A Birtle at the Prostate Cancer Research Centre, UCL. 30μg of this lysate was run alongside the same quantity of untreated LNCaP lysate in duplicate on a 12.5% gel, transferred to nitrocellulose and subject to immunoblotting with an antibody to AII. The results of this study can be observed in Figure 5.4. In both replicates a faint band corresponding to the 36kD

molecular weight of AII, was present in the de-methylated sample. Due to the limited number of replicates statistical analysis was not performed.

5.2.2 Confirmation of surface localization of AII

The lack of response in total cellular AII expression when exposed to IFNγ indicated that the down regulation of AII expression by IFNγ was cell surface specific. As the surface localization of AII was controversial due to the lack of signal peptide, it was necessary to verify the cell surface localization of AII via additional methods. Initially this was done through immunofluorescence on 1542 NPX cells fixed in 4% paraformaldehyde. 2,000 1542 NPX cells were added to each well of a 24 well plate containing glass cover slips and cultured for 72 hrs. The cells were then fixed and immunofluorescence performed using an anti-AII antibody as per protocol (see section 2.2.15) and visualized using an inverted microscope attached to a cooled CCD camera.

Immunofluorescence staining indicated that AII was present on the plasma membrane of 1542 NPX cells, as a generalised pattern of diffuse staining of the membrane was observed (Figure 5.5). Despite the use of 4% paraformaldehyde and lack of permeabilization, there appeared to be some background staining inside the cells. It was therefore necessary to further confirm that this AII expression was on the cell surface and not on the cytosolic face of the plasma membrane.

Surface AII expression was additionally verified by the release of AII from the plasma membrane by interference with its calcium dependent association with phospholipids. This was performed using two methods, by disruption of the extra cellular calcium level and by the addition a calcium chelator. These methods were performed in triplicate on 2.25 x $10^6$ 1542 NPX cells as per protocol (see section 2.2.8). To analyse the efficacy of these techniques 30μg of the resultant samples were run on 15% gels and subject to western transfer. The membranes were then probed with anti-AII antibody.

The results demonstrate that AII can be eluted from the cell surface with minimal cell disruption by manipulating the extra cellular calcium levels by either of the two methods (Figure 5.6 A, B). The lowest concentration of $CaCl_2$ gave the highest yield of AII; this concentration is lower than that usually found in the medium. The observation that

calcium concentration has a significant effect on the presence of AII in the elution extract was confirmed as significant via a one-way ANOVA analysis (P=<0.0001). To assertain the concentration generating the most significant effect a Dunnett test was performed, which compares the experimental groups to the baseline in this case the 0mM calcium concentration. All concentrations were found to have a significant effect on AII expression with the largest effect seen in the comparison of 0.5mM to the baseline (a difference of 46.3 at a confidence interval, CI, of 95%).

The extra cellular calcium concentration was also lowered by use of the calcium chelator EDTA, the effect of which can be observed in Figure 5.6B. The apparent increase in the presence of AII in the extra cellular media following EDTA treatment was confirmed as significant by a one-way ANOVA analysis (P=<0.0001). To confirm the concentration generating the most significant effect a Dunnett test was performed. The optimal EDTA concentrations for AII yield, those producing the most significant deviation from the baseline, were 0.5 to 1mM with difference values of 49.6 and 48.6 respectively, at the 95% confidence limit. The 0.5mM concentration has been used in other studies to liberate cell surface AII (Wright et al., 1995, Pietropaolo and Compton, 1997) and was therefore the elutant of choice for further experiments. Elution of surface bound AII with EDTA solutions above this concentration appeared to yield a second AII band of a lower molecular weight.

### 5.2.3 Timescale of surface down regulation by IFNγ

Time course experiments were conducted utilizing several techniques to specifically analyze surface bound AII. The first of these techniques was immunoprecipitation with an anti-AII antibody. For this experiment, 1.5 x $10^6$ 1542 CP3TX cells were cultured and stimulated with IFNγ for 0, 1, 2, 6 and 8 hours. These were then labelled with SS biotin, lysed in 1% OGP and a Lowry protein assay performed. 500μg of each of these lysates was pre-cleared as per protocol (see Appendix 8) with 50μl of Protein A:G beads (Amersham). The sample was then incubated with anti-AII antibody for 1 hr at 4°C and immunoprecipitation was then performed as per protocol (see Appendix 8) using 50μl of Protein A:G beads. The samples were then run on a 12.5% gel under non-denaturing conditions and subject to western transfer. The blot was then probed with streptavidin-

HRP and subject to densitometry. As can be observed in Figure 5.7A, this process detected a band corresponding to the correct weight for AII, which was down regulated by IFNγ. The level of cell surface AII expression decreased by approximately 2 fold (43 ± 3 decreasing to 23 ± 1) following 2 hrs and was absent after 8 hrs stimulation with IFNγ. This was verified through statistical analysis using one-way ANOVA analysis that resulted in the identification of a significant difference in mean AII expression with exposure to IFNγ (P=<0.0001). To identify which time point contributed the most significant difference in AII expression a Dunnett test was performed, with the exception of the 1 hour time point, all other data groups displayed a significant difference when compared to the baseline (2vs.0 hrs = -19.3, 6vs.0 hrs = -32.3). The most significant difference was at the 8 hr time point (-44.0 at 95% CI) confirming the earlier observation that this represented the maximal decrease in AII expression.

To analyze the effect of IFNγ on the binding partner of AII, p11, the membranes from the above experiment were stripped and probed with anti-p11 antibody (See Figure 5.7B). The bands were then analysed via densitometry and the data subject to statistical analysis using one-way ANOVA, which indicated that there was a significant difference in p11 expression over time, in cells exposed to IFNγ (P=<0.0001). To identify the length of exposure with most effect on p11 expression a Dunnett test was performed. This indicated that data from all time points with the exception of 1 hour, displayed a significant difference (2vs.0 hrs =-8.0, 6vs.0 hrs =-11.3) to the baseline (0 time point), with the greatest difference seen at 8 hours (-37.0 at 95% CI) where expression of both AII and p11 appears to be lost.

In concurrence with the effect of IFNγ on AII, a band of approximately 65kD was seen to be fractionally down regulated at 2 hrs and lost at 8 hrs stimulation with IFNγ. This band was the wrong molecular weight to be monomeric p11 or the AII/p11 heterotetramer and therefore may represent non-specificity of the antibody. The same antibody was, however, also able to detect a band of the correct monomeric weight for p11 in total lysate samples exposed to denaturing conditions (see Figure 5.7C). A 65kD band was additionally detected in a p11 immunoblot of cell surface proteins liberated by calcium treatment (Figure 5.7D), indicating that this may represent some other form of p11

complex possibly with AII. Unfortunately, the images obtained from these blots had high background interference and consequently the bands were not able to be accurately quantified via densitometry, which made statistical analysis of the results impossible.

The timescale of surface AII regulation by IFNγ was also examined by timecourse studies utilizing the calcium depletion methods for the release of AII. In the first instance 750,000 1542 CP3TX cells were cultured and stimulated with IFNγ for each 0, 1, 2, 4, 6 and 8 hours. The surface AII was then liberated using 0.5mM $CaCl_2$ as per protocol (see section 2.2.8). 30μg of each of the samples was loaded onto a 15% gel, run and transferred as per protocol (see section 2.2.10). The membrane was then probed with anti-AII and anti-plasminogen antibodies, the results were analysed by densitometry a representative image of which can be observed in Figure 5.8A.

A one-way ANOVA analysis was performed on these data to elucidate whether exposure to IFNγ had a significant effect on surface AII expression, this analysis indicated that there was a significant difference in mean AII expression following various lengths of exposure to the cytokine ($P<0.0001$). A further Dunnett test was used to identify those time points at which AII expression deviated most significantly from the baseline. The results confirm that there is a significant reduction in surface AII expression within 1-2 hrs (a difference of -14 at both time points when compared to the base line) and a loss of expression within 4-6 hrs of IFNγ stimulation (with differences of 41 and 44.3 from the base line respectively at 95% confidence limits). Immunoblotting for pro-cathepsin B, an endoproteinase requiring AII to bind to the cell surface, revealed a decrease in the level of surface associated pro-cathepsin B at the same time point as the majority of AII is lost. This was analysed via densitometry and confirmed by statistical analysis using one-way ANOVA followed by a Dunnett test. Thee data disclosed that interferon gamma treatment had a significant effect on the amount of pro-cathepsin B present on the cell surface ($P=<0.0001$) and that all time points displayed a significant difference in expression of the protein when compared to the baseline (-13.3 for all time points at a 95% CI).

To further confirm these results and clearly delineate the timescale of surface AII modulation, a similar study was conducted using EDTA release of this protein. 750,000 1542 CP3TX cells were grown to 70% confluence, stimulated with IFNγ at a concentration of 500U/ml for 0, 20, 40, 60, 120, 240, 360 minutes and the cell surface AII eluted with EDTA as per protocol (see section 2.2.8). 30μg of each sample was then run on a 15% gel, subject to standard electrophoresis and western transfer. This membrane was then probed with anti-AII and anti-plasminogen antibodies. This experiment was repeated in triplicate, a representative result can be seen in Figure 5.8B. These results were quantified by densitometry and analysed using one-way ANOVA, within which the data revealed that there was a significant difference in the mean expression of AII on exposure to IFNγ over a 360 minute timescale ($P=<0.0001$).

These results do not replicate the reduction in AII surface expression after 1 hr seen in the $CaCl_2$ release time course. However, the majority of surface expression is lost at 4 hrs with a complete loss at 6 hours of stimulation with IFNγ. This was confirmed by the Dunnett test which indicated that there was no significant difference in mean AII expression until 240 minutes of interferon exposure had elapsed (a difference of -24.8 from the base line) and that the maximal difference was observed at 360 minutes (a difference of -41.8 at 95% CI from the base line). The apparent retention of AII expression seen in the results of this experiment may represent an increased efficiency of EDTA to elute AII from the surface. Plasminogen immunoblotting however does suggest some change in surface AII within this timescale, whether in expression or conformation, as after 20 minutes stimulation with IFNγ, plasminogen which requires AII to bind to the surface, is lost (Figure 5.8B). These results were analysed by densitometry and confirmed by one-way ANOVA statistical analysis. These data suggest not only a significant effect of interferon exposure on plasminogen present on the surface of cells ($P=<0.0001$) but that also all time points displayed significant mean differences when compared to the baseline, ranging from -6.8 for 20 vs. 0 minutes, to -15.5 for 360 vs. 0 minutes. This indicates that there is a two-stage loss of plasminogen from the cell surface with a portion lost at 20 minutes and the remaining protein lost at 360 minutes corresponding with the total loss of AII from the cell surface at this time point.

To explore the time dependent effect of IFNγ on the cell surface expression of AII in other prostate cell lines, an EDTA time course experiment was conducted using the other isogenic prostate cell lines 1532 NP/1532 CP3TX and 1535 NP/1535 CP3TX. 3 x T-80 cell culture flasks of each cell line each containing 750,000 cells, were grown to 70% confluence and stimulated with IFNγ for 0, 1, 2, 3, 4 and 6 hours. The surface AII was eluted using the EDTA release method, 50μg of sample was loaded onto a 12.5% SDS PAGE, run and then transferred to a nitrocellulose membrane. This membrane was then probed with the anti-AII antibody and the expression of AII analysed via densitometry. This experiment was performed in triplicate, representative results of which can be observed in Figure 5.9.

The results in Figure 5.9 suggest that both isogenic pairs of cell lines express AII on the cell surface. Both 1535 NPX and CPT cells express high levels of AII and appear unaffected by IFNγ treatment. This was confirmed by two-way ANOVA analysis which found no significant difference in AII expression when densitometry data was categorised by cell line ($P=0.7$) or time point ($P=0.51$), with no significant interaction between these factors ($P=0.77$). The other isogenic pair, 1532 NP and CPT however, did show a surface down regulation of AII on stimulation with IFNγ, similar to that observed in 1542 NPX and CP3TX cell lines. The 1532 CP3TX cell line appears to express less AII on its cell surface than the normal cells in the resting state. On stimulation however, 1532 NP and CPT cell lines have the same pattern of expression, although down regulation was more marked in the cancer cell line. This was confirmed by statistical analysis using two-way ANOVA, which indicated that both cell line and exposure to IFNγ factors had a significant effect on AII expression ($P<0.0001$ for both factors), the enhanced down regulation in the cancer cell line was also indicated by the presence of significant interaction between the two factors ($P=0.0037$).

In these cell lines the level of surface AII decreased significantly at 3 hrs, but appeared to become re-expressed at a lower level following 4-6 hrs stimulation with IFNγ. The significance of this pattern of expression was analysed via one-way ANOVA followed by a Dunnett test to isolate the time point most different from the baseline. All time points, with the exception of 1 hour, displayed significant mean AII expression differences, with

the most significant difference at 3 hours (-17 at 3vs. 0 hrs, -14 at 4 and 6vs. 0 hrs). This pattern of expression, seen in all replicates, suggests that in 1532 cells surface AII expression is lost at a point comparable to that seen in the 1542 lines, however, expression is regained by some unknown mechanism that does not occur in the 1542 cell lines.

5.2.4 Immunofluorescence analysis of the modulation of cell surface AII by IFNγ

The relationship between AII, p11 and the cell surface was examined further by immunofluorescence of these proteins in 1542 NPX and CP3TX cells. The two cell lines were seeded into a 24 well plate containing cover slips at a density of 2,000 cells per well and cultured for 48 hrs. IFNγ was then added to half the cells and incubated for 24 hrs. Immunofluorescence was then performed as per the live cell protocol (see section 2.2.15) using AII and p11 antibodies, in addition to phalloidin to stain actin and cholera toxin to stain GM1 gangliosides, which are a present in high abundance in lipid rafts. Following staining the cells were photographed under x40 magnification using a confocal microscope. Representative images can be observed in Figure 5.10.

The immunofluorescence staining indicated that AII was indeed present on the surface of 1542 NPX and CP3TX cells and that stimulation with IFNγ reduced the level of AII on the cell surface. A generalised pattern of AII staining could be observed in both 1542 CP3TX and NPX cells in their unstimulated state. The pattern of staining in these cells, however, differed greatly following 24 hour stimulation with IFNγ. The staining in 1542 NPX cells changed following stimulation from a generalized to focal staining of the membrane, to a pattern localised to areas corresponding to lipid raft structures. These lipid rafts appeared to congregate into large semi-circular structures polarised to one side of the cell. In IFNγ stimulated 1542 CP3TX cells, however, AII staining was vastly reduced and not localised to lipid raft structures. In these cancer cells lipid rafts appear to be more prevalent in the unstimulated state than in the corresponding normal cell line, on stimulation they appear remain at the same level or decrease in size and number.

Cell surface staining for p11 in unstimulated 1542 NPX and CP3TX cells was more discrete than that observed with the AII antibody. Unfortunately, it was impossible to use both antibodies in conjunction as both were derived from mice and of the same isotype. The cellular localisation of p11 in unstimulated cells closely mirrored that of the lipid raft structures in both cell lines. On stimulation, there was an accumulation of p11 in the hyper-enlarged lipid raft structures in the 1542 NPX cells, although the overall intensity of staining appeared to decrease. In stimulated 1542 CP3TX cells p11 staining was vastly reduced, possibly due to a reduction in lipid raft structures. When compared, AII and p11 proteins appear not to co-localise in unstimulated cells from either cell line (compare top images Figure 5.10 and Figure 5.11). However, under stimulation the proteins adopt a more similar pattern of staining (compare bottom images Figure 5.10 and Figure 5.11). This could possibly be due to a loss of monomeric AII on the cell surface, leaving only the heterotetramer containing p11 bound to lipid rafts, when stimulated with IFNγ.

5.2.5 The influence of IFNγ on cell invasive capacity

The functional effect of AII surface down regulation by IFNγ on prostate epithelial cells was explored by means of cell invasion assays. 1.5 x $10^6$ of each NP, 1542 CP3TX and LNCaP cells were cultured to 70% confluence at which point half of the flasks were stimulated with 500U/ml IFNγ for 24 hrs. All cells were then removed from the growing surface and subject to the cell invasion assay protocol included in section 2.2.16. The migrants were then counted, these data pooled from six replicate experiments, tabulated and subject to the student's t-test. A graph representing this data can be observed in Graph 5.1.

These cell invasion studies disclosed a statistically significant (to 95% confidence limits) correlation between IFNγ stimulation and invasive capacity. Both 1542 NPX and CP3TX cells showed a reduction in invasive capacity when IFNγ was present in the media, this reduction was statistically significant in both cell lines ($p=0.0024$ in NPX cells and $P=0.029$ in CP3TX cells). In LNCaP cells, that express no AII, there was reduced invasive capacity and no reduction on stimulation with IFNγ.

The above experiment demonstrated that IFNγ affects the invasive capacity of cells expressing AII. The involvement of AII in retarding cell invasion was confirmed by conducting an Anti-AII invasion assay. This assay followed a similar protocol to that featured above with the exception that only 1542 CP3TX cells were used and that an anti-AII antibody was added (see section 2.2.16). The data resulting from six replicate experiments can be observed in Graph 5.2.

The results of the anti-AII invasion assay indicated that there was a statistically significant reduction ($p=0.021$) in invasive capacity in 1542 CP3TX cells when anti-AII mAb was present in the media. A control assay following the same protocol but using mouse IgG confirmed that this result was not due to a non-specific antibody interaction (Graph 5.3). The functional effect of AII expression on invasive capacity was explored further by re-expressing the protein in the LNCaP cell line. For this experiment, the LNCaP cells were seeded into a 24 well plate at a density of 71,250 cells per well then subject to transformation as per protocol (see section 2.2.17). GFP expression was then analysed and photographed (See Figure 5.12). Following a subsequent round of transformation, cell invasion assays were performed in six replicate experiments as per protocol (see section 2.2.16). The data from this experiment are represented in Graph 5.4. Figure 5.12can be observed in Figure 5.12.

The photograph in Figure 5.12 demonstrates that GFPAII expression in transfected LNCaP cells localises mainly to the perinuclear area. However, at higher magnification in the migrant cell pictured in Figure 5.13, cell borders can be observed which suggests a proportional membrane localisation for the GFPAII. This membrane expression of AII may be responsible for the increased invasive potential of GFPAII transfected LNCaP cells observed in Graph 5.4. This graph demonstrates that there is a significant ($P=0.0019$) improvement in invasive capacity with GFP AII transfection when compared to that of the vector only.

5.2.6 Analysis of AII membrane transport in prostate epithelial cells

The pathway by which AII is transported to the cell surface is unknown; however, recent studies have suggested that AI is transported to the cell surface via ABCA1. To determine whether this transport pathway is also responsible for the externalisation of AII, several studies were performed. Firstly following the protocol of Chapman et al 2003, 1542 CP3TX cells were exposed to glyburide an agent known to inhibit ABC transporters and dexamethasone known to stimulate the externalisation of AI. Six T-80 flasks each containing 750,000 1542 CP3TX cells were cultured to 70% confluence and then treated with or without glyburide, in the presence or absence of dexamethasone. The stimulation of the cells was performed as follows; glyburide was added to the cell media 24 hrs before lysis at a concentration of 100μM, this agent is dissolved in DMSO the concentration of which was kept below 0.01% and a DMSO control included. Dexamethasone was added 3 hours before lysis at a concentration of 0.1μM, this compound is soluble in EtOH, which was kept below 0.1% and an EtOH only control included.

Following culture and stimulation the cells were washed x3 with 1x PBS and 1ml of 0.5mM EDTA was added for 1 minute to liberate the surface bound AII. The cells were then lysed in 1% OGP as protocol (see section 2.2.4). The samples were then vacuum dried where necessary and protein concentrations quantified using the Bradford assay protocol (see Appendix 2). The samples were then loaded at a concentration of 50μg of protein per well onto a 12.5% gel and subject to standard electrophoresis and western transfer. This was repeated in triplicate and the membranes were probed with anti-AII antibody, a representative result of which can be observed in Figure 5.14.

These results were quantified via densitometry and normalised against their respective controls i.e. DMSO or ETOH and analysed statistically using two-way ANOVA analysis. Both factors dexamethasone and glyburide, were found to have a significant effect on intracellular and extra cellular AII expression ($P<0.0001$ in all cases) and there was a significant interaction between the two factors ($P<0.0001$). Glyburide treatment

decreased the proportion of AII on the surface of 1542 CP3TX cells and increased that present in the total cell fraction, in comparison to the DMSO control. Dexamethasone slightly increased the amount of AII on the cell surface but greatly reduced that found in the total cell lysate. When both agents were used together the same AII pattern of expression as with dexamethasone alone was found in both surface fraction and cell lysate.

To observe the timescale of the effect of glyburide on surface AII, a time course using this inhibitor was conducted. 12 T-80 cell culture flasks each containing 750,000 1542 CP3TX cells were cultured to 70% confluence ($1.5 \times 10^6$ cells for each time point) and exposed to 100μM glyburide for 0 (DMSO only), 20, 40, 60, 120 and 240 mins. The cells were washed three times in 1x PBS then 500μl of 0.5mM EDTA was used to liberate surface AII from both flasks. The sample was then vacuum dried and the cells lysed in 1% OGP. The EDTA and OGP samples were loaded onto a 12.5% gel at concentrations of 50μg and 30μg per well respectively and subject to western transfer as per protocol. AII expression was quantitated by immunodetection and densitometry. This experiment was conducted in triplicate and a representative result can be observed in Figure 5.15A. Statistical analysis of these results, using one-way ANOVA, indicated that the reduction in AII expression on the cell surface showed a significant correlation with time of exposure to glyburide ($P<0.0001$). The greatest loss of expression was observed at the 120-minute time point and expression appeared to then remain at a low level for the duration of the time course (see 5.15A). This was further confirmed by the Dunnett test which indicated that the only significant difference in surface AII expression was seen at time points 120 and 240 (a difference of -19.4 in both cases). A one-way ANOVA analysis also confirmed that glyburide had a significant effect on AII expression within the cell ($P<0.0001$). AII expression in the total lysate increased slightly at 40 mins, with total AII expression remaining at this level for the remainder of the time course. This was also indicated by the Dunnett test which disclosed significant difference in mean AII expression from the 40 minute time period onwards (average difference of 8.2 at all time points except 20 mins). The intra and extra cellular expression of AII, measured by

densitometry of immunodetected bands, appears to follow an inverse trend on exposure to glyburide as can be observed in Graph 5.5.

To confirm that glyburide treatment does negate AII surface expression 1.5 x $10^6$ 1542 CP3TX cells were cultured plus or minus 100μM glyburide for 24 hours, then washed and the surface AII EDTA liberated. These samples were run at a concentration of 50μg per well on a 12.5% gel. This was repeated in triplicate, a representative result can be seen in Figure 5.15B. In this figure, it can be observed that the expression of surface AII is lost following 24 hr exposure to 100μM glyburide (Figure 5.15B). These data were quantified and subject to statistical analysis via the students t-test which resulted in a significant difference (P=0.0005) in AII expression between those cells exposed to glyburide and those exposed to the control DMSO.

In a previous experiment, it was noted that dexamethasone could increase the level of AII expressed on the cell surface, to observe whether this effect could negate the suppressant effect of IFNγ, the following experiment was conducted. 4 x T–80 cell culture flasks each containing 750,000 cells were cultured to 70% confluence and stimulated with or without 0.1μM dexamethasone, plus or minus IFNγ for three hours. The cells were then washed and the surface AII liberated by EDTA release, following which the cells were then lysed in 1% OGP. 20μg of each sample was run on a 10% gel and subject to western transfer. This was repeated in triplicate a representative result of which can be observed in Figure 5.16.

These results were quantified and subject to two-way ANOVA statistical analysis. The results of this confirm that three hour exposure to IFNγ decreases surface AII significantly (P<0.0001) and that dexamethasone significantly increases the level of this protein on the cell surface (P=0.002). When both agents are applied together however the effect appears similar to that of IFNγ alone, however ANOVA analysis shows there is significant interaction between the two factors (P<0.0001).

To analyse whether the glyburide and IFNγ dependent decrease in AII expression was due to a down regulation in ABCA1 expression, the level of ABCA1 on exposure to these agents was analysed in a timecourse experiment. 750,000 1542 CP3TX cells were prepared and stimulated with IFNγ for 0, 1, 2, 4, 6 hours. Two separate flasks each containing 750,000 cells were prepared for the glyburide exposure of 0 and 24 hours. The cells were washed three times, lysed in 500µl 2DLB and 40µg of each loaded onto a 10% gel, run and subject to western transfer as per protocol (see section 2.2.10). The membranes were then probed with anti-ABCA1 antibody. This experiment was repeated in duplicate, an image of which can be seen in Figure 5.17. These results show that there is no change in ABCA1 expression either on stimulation with IFNγ or inhibition with glyburide.

The activity of the ABCA1 transporter is known to be affected by phosphorylation of threonine residues in a PEST sequence, see Figure 5.18 (Wang et al., 2003). Phosphorylation of this site is thought to lead to targeted degradation by calpains. The hypothesis that phosphorylation could be a mechanism for down regulation of the activity of ABCA1, subsequently lowering the level of AII, was tested via the following experiments. Firstly, isoelectric focussing (IEF) analysis was performed on 40µg of 1542 CP3TX cell lysates from the above experiment, using Ampholine PAGplate gels pH 5.5-8.5 (Amersham). The samples were loaded onto two sample application strips placed on the negative edge of the gel and run as per manufacturer's instructions (see Appendix 9). The gels were then transferred and probed with anti-ABCA1 antibody. Unfortunately, the IEF separation time was not sufficient in this first experiment to resolve the protein sufficiently; however, the poorly resolved band did appear to migrate towards the negative terminal between 2 to 4 hrs, which could be suggestive of phosphorylation. A second replicate of the experiment, which was subject to IEF as listed in the 2DGE protocol, was sufficient to resolve bands. These results indicated that following 1 hr of stimulation with IFNγ the band recognised by the ABCA1 antibody, shifts slightly towards the negative electrode and several additional bands appear. These additional bands are lost between 4-6hrs and may correspond to degradation products produced by

labelled IFNγ timecourse of 1542 CP3TX cells conducted by Dr S Naaby-Hansen (see Figure 5.19). Densitometry of these results revealed that ABCA1 expression decreased to 70% following 1 hour and was further reduced to below 50% after 4 hour stimulation with IFNγ in unlabelled cells and that this reduction was accompanied by an accumulation of lower molecular weight forms which may represent cleavage products. The results of the radio labelling experiment indicated that ABCA1 and the lower molecular weight forms became increasingly phosphorylated following 4 hrs of exposure to IFNγ (see Fig.5.19B).

To delineate the involvement of calpain II in the modulation of ABCA1 activity and subsequent down regulation of AII, calpain II inhibition studies were performed. 18 x T-80 flasks of 1542 CP3TX cells each containing 750,000 cells were cultured to 80% confluence, at which point 12 flasks were treated with 30μM calpain II inhibitor (Calbiochem) dissolved in DMSO, for two hours. The final concentration of DMSO was kept to 0.001% in the media. Following this, the untreated and calpain II inhibitor treated cells were stimulated for 0, 1, 2, 4, 6 hours with 100U/ml IFNγ. The cells were then washed three times in PBS, the surface AII was eluted with 0.5mM EDTA and 50μg of this sample was run on a 12.5% gel and transferred to a nitrocellulose membrane. The membrane was probed with anti-AII. This was replicated in triplicate and analysed via densitometry, a representative result can be seen in Figure 5.20.

IFNγ stimulation of cells without calpain II inhibition leads to a down regulation of surface AII at 4 hrs. However, when cells were pre-treated with calpain II inhibitor the level of AII surface expression remains the same even after 4 hour stimulation with IFNγ. This can be observed in Graph 5.6. These results were analysed statistically by two-way ANOVA which indicated a significant difference in AII expression with temporal exposure to IFNγ and to the calpain inhibitor ($P<0.0001$ in both cases) and there was a significant interaction between the factors ($P<0.0001$). This suggests that there may be some calpain dependent regulation of surface expressed AII.

The calpain II inhibitor used in this study specifically acts on calpain I and II. The expression of calpain II was examined in the following study to observe any changes in expression pattern on stimulation with IFNγ. The membranes created in the IFNγ stimulated time course study of AII expression in 1532/35 NP and CPT cell lines were stripped and re-probed with an anti-calpain II antibody. The results of this can be observed in Figure 5.21. The results of this study suggest that at least two forms of calpain are present in NP cells of both 1532 and 1535 lines. The corresponding cancer cell lines however, appear not to express both of these molecular weight forms. In 1532 CPT cells the higher molecular weight form is expressed, whereas in 1535 the lower molecular weight form appears to predominate (Figure 5.21). In all cell lines calpain II expression appears to be induced following 1 hour of stimulation with IFNγ. In the cancer cell lines, this expression shows an apparent decrease at 4 hours and is regained at 6 hours. Due to the presence of different calpain isoforms and high background, possibly due to poor specificity of the antibody, statistical analysis of these data was not possible.

## 5.3 Discussion

This study aimed to examine the effect of IFNγ on the cell surface expression pattern of one protein, AII. The aim was addressed by focussing on three areas of study, firstly the modulation of AII on the cell surface by IFNγ, secondly the functional effect of this modulation and finally the transport of AII to the cell surface.

### 5.3.1 The surface specific modulation of AII by IFNγ

Once the down regulation of AII had been confirmed by immunoblotting, subsequent studies sought to examine whether this effect on AII expression was observed on a total cellular expression level. No change was seen in the total level of AII expression following 24 hr stimulation with IFNγ, in any of the cell lines tested. These results suggest that AII down regulation is surface specific involving either the regulation of the translocation of AII to or its release from the plasma membrane. This was examined further in the third section on AII transport.

In the panel of prostate cell lines tested for AII expression, LNCaP was the only cell line lacking expression of the protein. This had been found previously, in a study that suggested AII was ubiquitously lost in PCa (Chetcuti et al., 2001a). The results of the present study however suggest the converse, that AII is expressed in the majority of prostate cell lines. The reason for the lack of AII expression in LNCaP has been postulated as being due to the methylation of the gene promoter. In this study, LNCaP lysate from cells that had been exposed to a de-methylating agent appeared to express a low level of a protein corresponding to the correct weight for AII. Due to the nature of this result in both replicate experiments however, it is difficult to draw conclusions from this study. Although promoter methylation and subsequent silencing of the AII gene in LNCaP is a parsimonious possibility, it is equally possible that the methylation status of the promoter is negligible and that the expression of the protein is due to a distal DNA demethylation leading to the reactivation of a protein upstream in the regulation pathway of AII. This mechanism of gene activation was observed in a recent study of the cell cycle regulator protein p21WAF (Schmelz et al., 2005). To further investigate this possibility it would be necessary to characterize the other genes reactivated by 5-aza-deoxycytidine treatment and analyze their interaction with the AII gene, transcript or protein. There are alternative hypotheses for the expression of AII following 5-aza-cytidine treatment that require further exploration. 5-aza-deoxycytidine is a potent inhibitor of DNA Methyltransferase 3a and as such treatment with this drug does not have a specific effect on the AII gene, it has been noted to have wide range demethylating and gene activating capabilities (Schneider-Stock et al., 2005). These include the activation of genes involved in cell cycle arrest (Schmelz et al., 2005, Lavelle et al., 2003), DNA repair (Arnold et al., 2003, Kim et al., 2002), apoptosis (Pulukuri and Rao, 2005) and transport (Worm et al., 2001). The activation of genes coding for transport proteins may particularly influence expression of AII, as this protein is thought to be transported to the surface via a yet unknown protein chaperone. The most likely protein chaperone candidate is ABCA1, a protein which is part of a multi-drug resistant family, the expression of which is known to be affected by de-methylation with 5-aza-deoxycytidine (Worm et al., 2001). The effect of this drug on the transport of AII could be further explored by the transfection of fluorescent AII mutant constructs, with

deletions in the protein binding domain (commonly the amino terminus), the location and expression of which could be followed post treatment with 5-aza-deoxycytidine. In conjunction, the expression of proteins likely to be involved in AII transport, such as ABCA1, could be examined via immunoblotting pre and post treatment with 5-aza-deoxycytidine.

Due to the debate surrounding the surface localisation of AII, it was necessary to confirm the cell surface expression of this protein. This was achieved by immunofluorescence and through the liberation of AII by altering the level of extra cellular calcium. The initial immunofluorescence studies confirmed a plasma membrane localisation of AII, there was however, background staining within the cells. This is not unexpected as the majority of AII is expressed within the cell commonly localised to the cytoplasm and cytosolic face of the PM. Further evidence was required to support the surface expression of AII and this was provided by AII release through the modulation of the level of extra cellular calcium. Decreasing the level of calcium either by reducing the level of calcium in the extra cellular milieu or by the addition of the calcium chelator EDTA led to the release of AII, proving that this protein was indeed present on the cell surface and bound in a calcium dependent fashion. EDTA extraction proved to be the more efficient method for providing a high yield of AII. The concentration of EDTA most efficient at AII release was 0.5-1mM. To ensure that the AII released by either of these methods was solely from the cell surface and not due to protein leaking from the cell the nitrocellulose membranes could have been probed for an abundant intracellular protein such as β-actin.

Treatment with EDTA at higher concentrations led to the release of an additional protein of approximately 34kD also recognised by the AII antibody. The ability of this isoform to be liberated by higher EDTA concentrations, suggests that it is less sensitive to extra cellular calcium depletion. This protein may represent an isoform of AII modified by cleavage, a phenomenon which has been reported previously (Babiychuk et al., 2002). In a recent study AII was found to be subject to cleavage at the amino terminus yielding a 34kD band, most likely to be facilitated by cathepsin, calpain or another metalloprotease (Babiychuk et al., 2002). The amino terminus of AII is thought to be necessary for both

heterotetramer formation and interaction with cholesterol in the plasma membrane (Gerke and Moss, 1997). It is possible therefore, that cleavage of AII in this region yields an isoform, which cannot form the standard heterotetramer, but is still able to bind phospholipids in perhaps a monomeric confirmation. This cleavage would give two species of AII on the cell surface, a full-length heterotetrameric form capable of binding cholesterol and phospholipids in a calcium dependent fashion and a truncated monomeric form capable of only binding phospholipids. In a previous study these different species of AII were found to occupy different membrane locations, the full length form present in lipid rafts, the truncated form in non-raft associated regions (Babiychuk et al., 2002). This result may suggest that AII is present in a heterotetrameric form bound to cholesterol in the lipid rafts and a monomeric form more generally bound to phospholipids. The localisation of the AII species on the cell surface is explored further in immunofluorescence studies, discussed later in this chapter.

Once the cell surface localisation of AII had been confirmed, further studies sought to characterise the action of IFNγ on surface AII expression in 1542 NPX and CP3TX cells. Initially this was achieved through immunoprecipitation using the AII antibody, which revealed that surface AII is down regulated following 2 and lost after 6 hours stimulation with IFNγ. Immunoblotting of the same membranes with an anti-p11 antibody indicated that the expression of this binding partner of AII followed the same pattern of down regulation by IFNγ as AII. This result was inconclusive however as the band detected with the p11 antibody was of the incorrect molecular weight for monomeric p11. This band can also be detected in eluate from calcium-dependent extractions of AII, when probed with the p11 antibody. This may be the result of a non-specific interaction of the antibody with some other protein, or a p11 complex resolved in the non-denaturing conditions. This complex may involve AII as the two co-immunoprecipitate, however this band cannot correspond to the full heterotetramer, as the molecular weight is incorrect for this complex. Further research would require identification of the constituents of this band via mass spectroscopy.

The elution of AII by manipulating external calcium levels was also used to confirm the timescale of IFNγ action. The studies using the $CaCl_2$ method of extraction, demonstrated that surface AII expression was down regulated between 1-2 hours and lost between 4-6 hours. This confirmed the timescale of regulation seen in the previous immunoprecipitation experiment. The membranes for this experiment were also probed with pro-cathepsin B, a protease that requires surface expressed AII as a binding site for its activation. Immunoblotting with this antibody resulted in the detection of a band corresponding to the correct weight for pro-cathepsin B that followed the same pattern of down regulation and loss as AII. The timecourse study employing EDTA release of AII showed no reduction in surface expression at the earlier time point, but replicated the loss of expression at 4-6 hours. The explanation for this differing result may be that EDTA is more efficient at eluting surface bound AII and therefore masks a partial reduction in AII after 1-2 hrs. There must be, however some change in the expression or conformation of AII in the first hour as when the membranes were probed for plasminogen, expression of this AII binding protein is lost after a 20 minute stimulation with IFNγ.

In summary these studies suggest that AII is down regulated on the surface of 1542 CP3TX cells following 2 hours and lost after 6 hours stimulation with IFNγ. The cell surface down regulation of AII by this cytokine is therefore, extremely fast and likely to be the result of post-translational modification of AII or of proteins in its transport pathway. The concurrent loss of plasminogen and pro-cathepsin B would be expected of a protein using surface AII as a binding site, as this protein would lose its association with the membrane following the loss of AII. The loss of AII as a platform for interaction/activation of proteases and ECM components may explain some of the functional effects on cell invasive capacity seen on stimulation with IFNγ, these effects are discussed later in this study.

The down regulation of surface AII by IFNγ was explored in two other pairs of isogenic cell lines to examine whether this response was a common feature amongst prostate cell lines. The 1535 NP and CPT cell lines appeared to express higher levels of surface AII than the 1542 NPX and CP3TX cells and this expression was unaffected by IFNγ. The

1532 NP and CPT cell lines, however, did appear to show a reduction in expression of AII at a time point comparable to that of the 1542 cell lines, with the exception that 1532 cells regain expression of this protein. This response is possibly due to the activation of a redundant pathway, that the 1542 cells are unable to activate. A similar pattern of reactivation was also observed in recent studies of the highly invasive head and neck squamous cell carcinoma cell line HN5. This research, conducted at the proteomics lab at the Ludwig Institute, focussed on the cell surface expression of AII following exposure to IFNγ. The results of this study indicated that surface AII was lost at 1 hr and regained at 2 hrs following treatment with this cytokine. It appears from these results that IFNγ has a rapid reductive effect on surface AII in a number of cell lines including those known to be from highly invasive cancers. This may be therapeutically advantageous, given the potential role of AII in cell invasion, in limiting the invasive and metastatic potential of such cancers. However, as several of the cell lines show a re-expression of AII further research is required to delineate the response of other cancer cell lines to IFNγ and the signalling pathways involved.

The final part of the study into cell surface AII modulation by IFNγ sought to examine the spatial relationship between AII, p11, lipid rafts and the cell surface, using immunofluorescence. The results of this study suggest that the surface specific down regulation of AII by IFNγ appears also to involve a change in localisation of both proteins. In unstimulated cells from both 1542 NPX and CP3TX cell lines, generalised AII staining can be observed on the surface of the cells, which is reduced when both cell lines are exposed to IFNγ for 24 hrs, confirming previous immunoblotting results. A similar response, in terms of surface AII expression, of these isogenic cell lines to IFNγ was noted in earlier studies. However, differences in the pattern of AII expression were observed when the localisation of AII and lipid raft structures was examined. In the normal cell line, on stimulation with IFNγ, large semicircular structures that co-stain with cholera toxin, actin and anti-AII appear polarised towards the edge of the cell. This enlargement and fusion of lipid raft structures in normal cells following exposure to IFNγ has been previously reported and denoted a "signalosome" containing many IFNγ signalling factors such as IFNγR, STAT's and JAK proteins (Sehgal et al., 2002).

Formation of such a signalling platform could enhance the normal cell's sensitivity to cytokines and therefore heighten immunosurveillance. In 1542 CP3TX, no enlargement or fusion of lipid raft structures is observed. In fact, the cancer cells appear to have a higher number of lipid rafts in the unstimulated state this however, does not increase on stimulation with IFNγ. As lipid rafts are reported to contain a number of signalling factors including EGFR, an increased number of lipid rafts in the unstimulated state could be a selective advantage in the cancer cell, as it may increase the cell's sensitivity to ambient levels of growth factors, increasing the mitogenic potential. However, such structures would become disadvantageous when the cell was exposed to cytokines as they contain all the molecules required for transduction of the growth inhibitory and motility reducing effects of IFNγ. Therefore, suppression of the formation of such signalosomes on induction by IFNγ would be a selective advantage for the cancer cell in avoiding immune surveillance.

In comparison to AII, the staining of its heterotetrameric partner p11 in both 1542 NPX and CP3TX unstimulated cells was more localised, showing discrete co-localisation with lipid raft structures. On stimulation the level of expression of p11 is reduced in both cell lines, more markedly in 1542 CP3TX cells. In 1542 NPX cells p11 appears to accumulate in the enlarged lipid raft structures in a similar pattern to AII, as 1542 CP3TX cells do not possess this structure p11 may be present at low levels in the cytoplasm. The difference in staining between the AII and p11 proteins in unstimulated cells supports the theory that several AII conformations are present on the surface of these prostate epithelial cells. As the only known function of p11 is to bind AII in a heterotetrameric formation and in the above study p11 is only found associated with lipid rafts, it follows that the heterotetrameric form of AII is found in the lipid raft structures. The generalised AII staining seen in unstimulated cells may correspond to monomeric binding of the truncated AII protein to the cell surface. On stimulation with IFNγ this generalised membrane staining of AII disappears, remaining only in the enlarged lipid raft structures in 1542 NPX cells. This could correspond to a loss of the monomeric form from the cell surface either by regulation of the release or translocation of AII to the cell surface. Retention of the AII tetramer on the cell surface may be due to its association with the

lipid raft structures, or its heterotetrameric conformation. 1542 CP3TX cells do not appear to induce the formation of enlarged lipid raft structures on stimulation with IFNγ and therefore may not retain the AII heterotetramer. This may be the cause of the greater reduction in surface AII seen in the earlier AII immunoblotting of cell surface fractions from 1542 CP3TX cells in the presence of IFNγ stimulation, in comparison to their normal counterpart.

### 5.3.2 The functional effect of surface AII down regulation by IFNγ

As AII has been widely reported to play a role in cell invasion, the functional effect of cell surface specific down regulation of AII by IFNγ was examined using cell invasion assays. The results of these studies disclosed a correlation between IFNγ stimulation and invasive capacity. In all cell lines tested that express AII, a reduced invasive capacity was observed following 24 hour treatment with IFNγ. This reduction was statistically significant in both 1542 NPX and CP3TX cells. The finding that the greatest reduction in invasive capacity is seen in normal cells appears anomalous, however normal cells can be extremely invasive (Mareel and Leroy, 2003). The only cell line not to show a decrease in invasive capacity when stimulated with IFNγ was LNCaP, a cell line that does not express AII. This may suggest that AII is responsible for the effect IFNγ has on invasive capacity. To confirm this theory invasion assays in the presence of an anti-AII antibody were performed. The results of this study indicated that AII has a role in the invasive capacity of the cell, as there was a significant decrease in the invasive capacity of 1542 CP3TX cells when the anti-AII antibody was present. No change in invasive capacity was observed when an alternative mouse IgG was used. This effect has been observed previously in macrophages (Falcone et al., 2001) and suggests that AII does play a key role in cell invasion and that invasive capacity can be reduced by treatment with the cytokine IFNγ.

The ability of surface expressed AII to influence the cell's invasive capacity was examined further by transfecting this protein into LNCaP cells, which express no endogenous AII. AII was transfected into LNCaP via a GFP expression vector so that expression could be easily observed. The majority of GFPAII expression in LNCaP was

located in the nuclear and cytoplasmic regions of the cell. However, GFPAII expression was observed on the surface of LNCaP cells. This concurs in part with the findings of a study using the same AII construct in rat basophilic leukaemia (RBL) cells, which found that GFPAII was located in the cell cytoplasm, membranes, but excluded from the nucleus (Hayes et al., 2004). This study also found that AII played a significant role vesicle transport especially macropinosome rocketing in response to stress, where it was found to mediate the interaction between the macropinosome and F-actin (Hayes et al., 2004). In another study using the same AII construct GFPAII was found to localise within the cytoplasm, nucleus and plasma membrane of PC12 cells. AII was found to be enriched at the plasma membrane in a calcium dependant manner, as expression of a mutant construct lacking the type II $Ca^{2+}$ binding sites led to exclusively cytosolic expression of AII (Merrifield et al., 2001).

Although the use of a GFP construct to simulate the endogenous expression of AII has provided interesting data with regard to the possible location and function of the protein there are various problems with this model that must be considered. Firstly fusion of the GFP to the AII gene may interfere with transcription or translation of the nucleic acid decreasing expression. Secondly the GFP tag considerably increases the size of the resulting protein this often leads to misfolding, which may have subsequent effects on not only the function of AII but also its interaction with other proteins and its transport within the cell. The GFP tag could therefore affect both the localisation and function of the AII protein. In addition the GFPAII was expressed in LNCaP cells, which normally do not express the protein and therefore may not possess the transport pathway and/or interacting proteins necessary for AII to be localised and function as it would in its normal state. However, the studies considered above have localised both the GFPAII (Hayes et al., 2004, Merrifield et al., 2001) and endogenous AII (Zobiack et al., 2002, Deora et al., 2004) to both the intracellular and membrane localities suggested in this study. In addition the immunofluorescence analysis of AII in 1542 NPTX and CP3TX cells also suggested that the protein was localised to the cytoplasm and plasma membrane of the cells. In light of this, the results of GFPAII expression in LNCaP cells appear to be an accurate reflection of localisation of AII within prostate cells.

The localisation of the GFPAII construct in LNCaP cells is unsurprising as in cells with endogenous AII expression, the majority of AII protein is observed within the cell. The observation of AII on the surface of LNCaP cells, however, suggests that the transport systems and binding partners required for surface AII expression are present despite the lack of endogenous expression. Once expression of the AII construct had been confirmed, the transfected LNCaP cells were subject to an invasion assay to examine the effect of re-introducing AII on invasive capacity. The results of this assay further verify that AII has a role in invasive capacity, as there was a significant increase in invasive capacity in AII transfected cells when compared to those containing the GFP vector only.

Further studies may require the use of an AII construct with a 'super folder' GFP tag which has been shown to be less prone to misfolding in vitro (Pedelacq et al., 2005). This could be used to further investigate the invasive capacity of AII transfected cells under IFNγ stimulation, or in the presence of anti-AII antibody. Such studies would be required to ascertain whether IFNγ could modulate the level of surface AII and therefore invasive capacity, in LNCaP cells. Other cell lines could also be used to negate any possible problems caused by lack of endogenous AII expression in LNCaP cells, however this would lead to potential problems with AII over expression masking the true function of this protein.

### 5.3.3 IFNγ regulation of AII transport to the cell surface

The transport pathway responsible for the surface expression of AII is unknown, due to the lack of signal peptide it is unlikely that AII is transported via the trans golgi network (Chapman et al., 2003). As recent research has indicated that AI, a protein closely related to AII in structure and function, is transported to the cell surface via the ABCA1 transporter, the possibility that this transporter could also be responsible for the externalisation of AII was explored (Chapman et al., 2003). Treatment with glyburide, an inhibitor of ABCA transporters, was seen to decrease the amount of AII on the surface of 1542 CP3TX cells, whilst increasing the amount of this protein present in the cell extract. The opposite effect was observed when cells were treated with dexamethasone, a protein known to increase cell surface expression of AI, possibly by stimulating the ABCA1

transporter. These results may indicate that glyburide inhibits the ABCA1 transporter, leading to an accumulation of AII within the cell and a depletion of AII present on the outer leaflet of the PM. This effect can be reversed by dexamethasone treatment, which may stimulate the transporter, increasing surface AII levels. Glyburide treatment of cells lead to loss of surface AII following 24 hour exposure, a timecourse of AII expression revealed that glyburide starts to decrease surface levels of AII after 120 minutes and the AII within the cell begins to accumulate at 40 minutes. This could provide further evidence that ABCA transporter activity is required for localisation of AII to the cell surface. A similar time course study was conducted to elucidate whether stimulation of the ABCA transporter with dexamethasone could negate the suppressive effects of IFNγ on AII cell surface expression. The results of this study confirmed that dexamethasone increases the level of surface AII and that treatment with IFNγ alone led to a decrease in surface expressed AII at around 3-4 hours. However, when dexamethasone and IFNγ were applied together there was no prevention of the suppressive effect of IFNγ. This suggests that the mechanism of action of IFNγ on surface AII expression via the ABCA transporter, is different to the action of glyburide or dexamethasone, as the down regulatory effect of glyburide can be negated by dexamethasone whereas that affected by IFNγ cannot.

IFNγ has been reported to regulate ABCA1 expression by transcriptional inactivation (Panousis and Zuckerman, 2000). The possibility that this may be the method of regulation of ABCA1 and subsequently AII expression in 1542 CP3TX cells was examined by analysing the total cellular expression of ABCA1 throughout an IFNγ timecourse. The results of this study indicated that there was no change in ABCA1 expression on stimulation with IFNγ or on exposure to glyburide. This suggests that any regulatory effect IFNγ may have on ABCA1 must be posttranslational. Posttranslational modification of ABCA1 on stimulation by IFNγ was examined by isoelectric focussing; this technique separates proteins according to their isoelectric point and therefore is able to resolve proteins differing in charge due to post translation modifications. The results of this IEF analysis were inconclusive, due to problems with reproducibly transferring proteins from the IEF gel to the nitrocellulose membrane. 2DGE proved ineffective also

as ABCA1 could not be resolved in the second dimension due to its size and hydrophobicity. Though inconclusive, the IEF results did appear to exhibit a pattern suggestive of phosphorylation following 1 hour of stimulation. In addition to this, novel bands appear at this time point and are lost at 6 hours. ABCA1 is known to be phosphorylated on threonine residues within a PEST sequence; this post translational modification leads to targeted inactivation of ABCA1 by calpain cleavage (Wang et al., 2003). The pattern of IEF bands mentioned above may reflect the initial phosphorylation of ABCA1, followed by targeted degradation causing the detection of further bands corresponding to cleavage products. This timescale of phosphorylation by IFNγ was also found in radio-labelled samples subject to immunoprecipitation with the anti-ABCA1 antibody. This study also detected a decrease in ABCA1 expression and a gradual increase of low molecular weight proteins recognised by the ABCA1 antibody. Although this is contradictory to the previous finding that ABCA1 levels are unaltered by IFNγ treated, the finding that both the 220kD and low molecular weight forms of ABCA1 become increasingly phosphorylated following exposure to IFNγ suggests that the decrease in ABCA1 expression is due to degradation and not to transcriptional regulation. The phosphorylation and potential degradation of ABCA1 on exposure to IFNγ exhibits the same timescale of effect as loss of AII cell surface expression. It is possible that down regulation of the ABCA1 transporter via phosphorylation and subsequent calpain degradation following IFNγ stimulation is responsible for the reduction and eventual loss of AII from the cell surface. Further studies of the posttranslational modification of ABCA1 by IFNγ and the subsequent effect on AII expression are required before this mode of AII transport to the cell surface can be confirmed. Analysis of the expression of ABCA1 in response to IFNγ at the mRNA level is required to confirm that degradation is the cause of the reduction in expression seen in Dr. Naaby-Hansen's experiments.

The effect of calpain activity on cell surface AII expression was observed by blocking the action of calpain II in 1542 CP3TX cells. Inhibiting the action of calpain II by pre-treatment with IFNγ prevented the down regulation of surface AII seen at 4 hours with IFNγ treatment alone. These results indicate that regulation of cell surface AII by IFNγ is in some way calpain II dependent. The retention of AII on the cell surface in the presence

of IFNγ may be due to a lack of calpain II cleavage of the ABCA1 transporter, allowing the transport of AII to continue despite post-translational modification by IFNγ. An alternative hypothesis could be that IFNγ is directly regulating calpain II. This was explored by observing the expression of calpain II in the other isogenic cell lines 1535 NP/CPT and 1532 NP/CPT over a timecourse of stimulation by IFNγ. The normal cell lines appear to express two forms of calpain II, whereas the cancer cell lines appear to express a majority of one form or the other. This may indicate yet another IFNγ signalling pathway that is deregulated in the cancer cell lines in comparison their normal counterparts. In all cell lines calpain II expression appears to be induced by 1 hour exposure to IFNγ. In cancer cell lines however a reduction in calpain II expression is apparant at 4 hours. In 1532 CPT cells the cell surface expression of AII is reduced at 4 hours but regained at 6 hours, indicating that a fall in calpain II activity at the former time point may lead to a rise in cell surface AII levels at the latter.

The results discussed above, suggest that both ABCA1 and calpain II have a role in the regulation of cell surface AII expression by IFNγ either directly or indirectly. Figure 5.1 featured below, outlines the possible signalling pathways involved in the cause and effect relationship between IFNγ and cell surface AII expression. The model proposed is that activation of the IFNγR leads to the activation of an unknown kinase, which phosphorylates the threonine residues within the PEST sequence of ABCA1. This posttranslational modification leads to the inactivation of ABCA1 and the cessation of AII transport to the cell surface. It is likely that AII would be transported by ABCA1 in association with cholesterol containing endosomes, which then fuse into the outer leaflet of the plasma membrane.

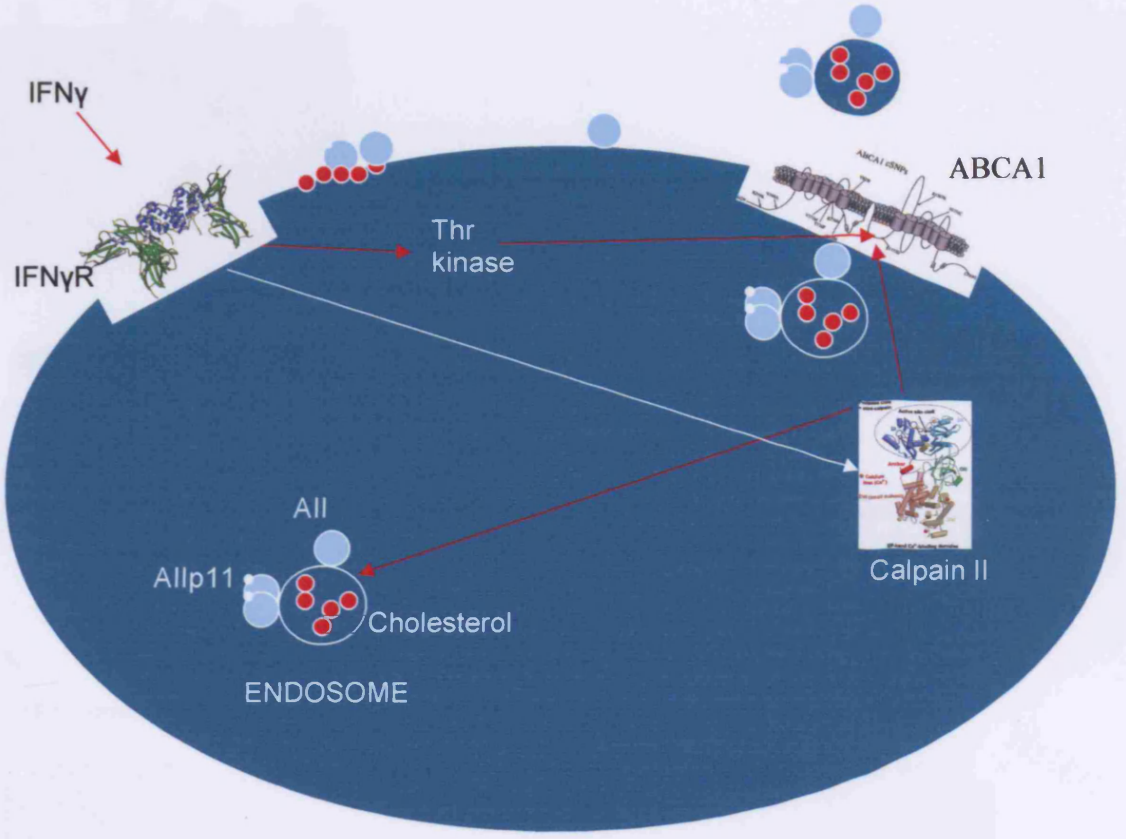



**Figure 5.1**. A model of cell surface AII transport and regulation by IFNγ.

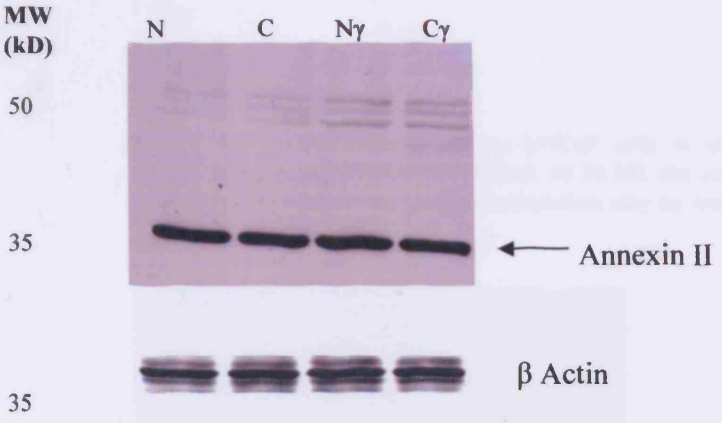



**Figure 5.2.** Total cellular expression of AII in 1542 NPX and CP3TX cells with or without 24 hour stimulation with IFNγ. β Actin expression is included as a loading control. It appears that total cellular expression of AII is unaffected by IFNγ, suggesting that the reduction is solely cell surface specific.

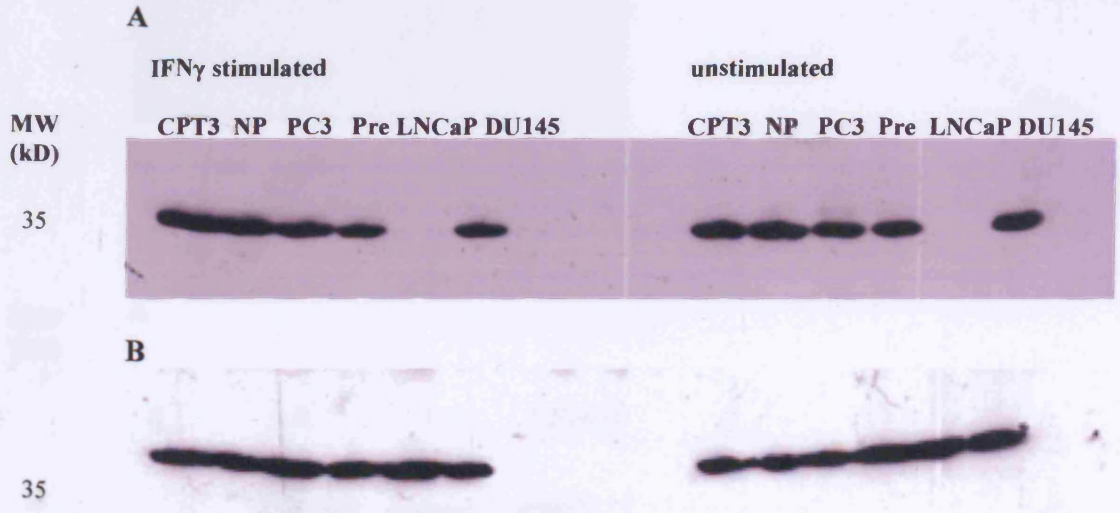



**Figure 5.3**. Total cellular expression of AII in a panel of prostate cell lines with or without IFNγ stimulation. **A** Total cellular expression of all cell lines expressing AII is unaffected by IFNγ stimulation. **B** Expression of the loading control β-actin. LNCaP appears not to express the AII protein.


**Figure 5.4** An AII immunoblot on LNCaP cells in normal state (N) and after treatment with the de-methylating agent 5-aza-deoxycytidine. At 36 kD, the correct weight for AII, a faint band is present in the de-methylated sample suggesting methylation may be responsible for the lack of AII in LNCaP cells.

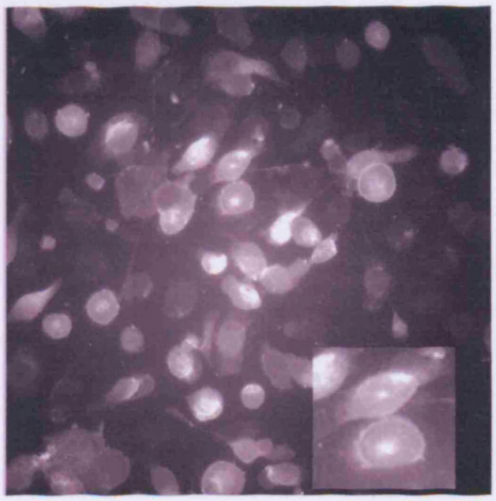


**Figure 5.5.** Annexin II staining in unstimulated 1542 NPX cells. Inlay shows enhanced image of characteristic staining. This image demonstrates that AII is present on the plasma membrane, although some background staining is also seen in the cell cytoplasm.

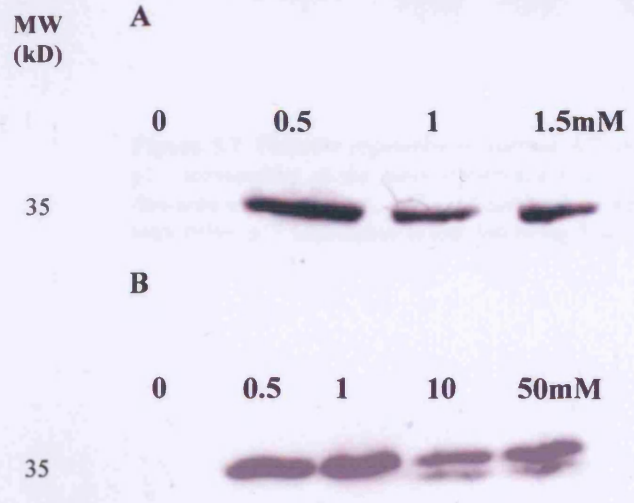



**Figure 5.6.** Anti-AII immunoblot of protein samples obtained by washing with different concentrations of **A** $CaCl_2$, **B** EDTA. A manipulation of external $CaCl_2$ levels either by addition of calcium or by chelation with EDTA, leads to the release of surface bound AII. High concentrations, above 10mM, of EDTA lead to the release of two AII isoforms.

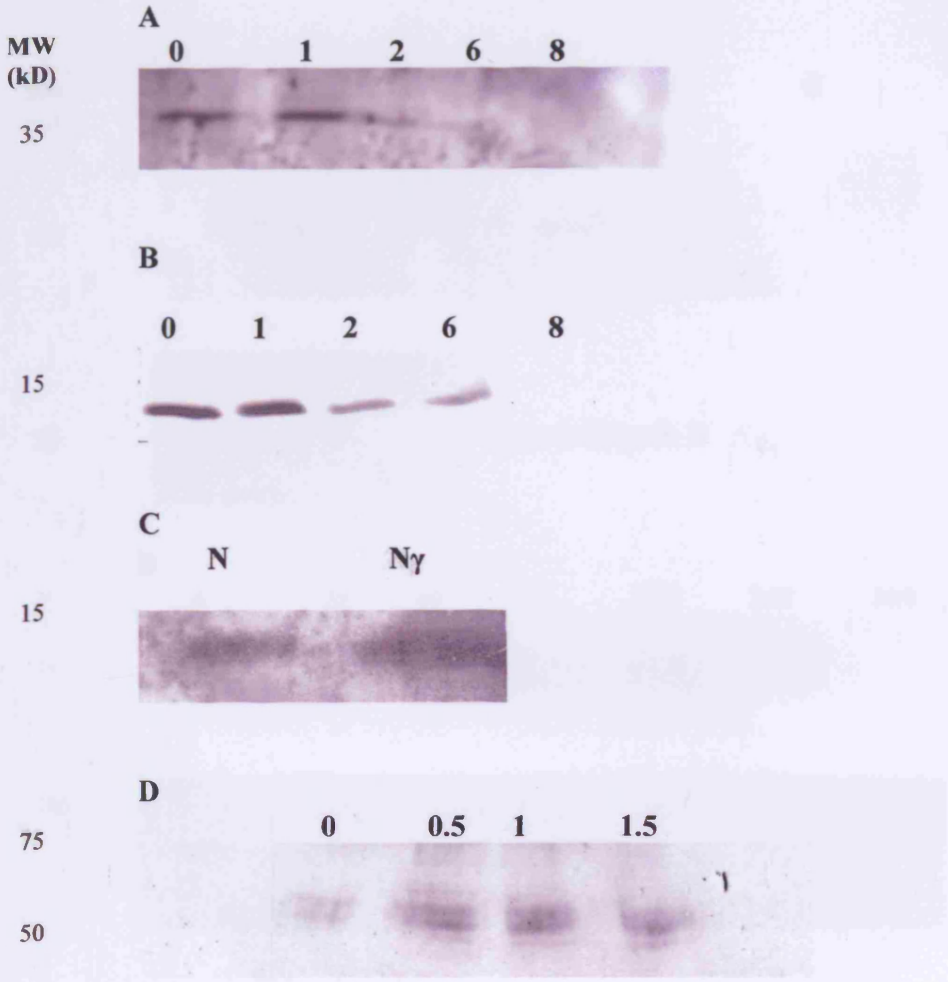



**Figure 5.7**. Periodic regulation of surface AII and p11 by IFNγ **A** Avidin blot of anti-AII IP timecourse **B** p11 immunoblot of the same membrane **C** p11 expression in total cell lysate **D** p11 expression in $CaCl_2$ liberated protein extract. A loss of cell surface AII expression occurs between two and six hours stimulation with IFNγ. p11 expression is lost following 8 hours stimulation with the same cytokine.

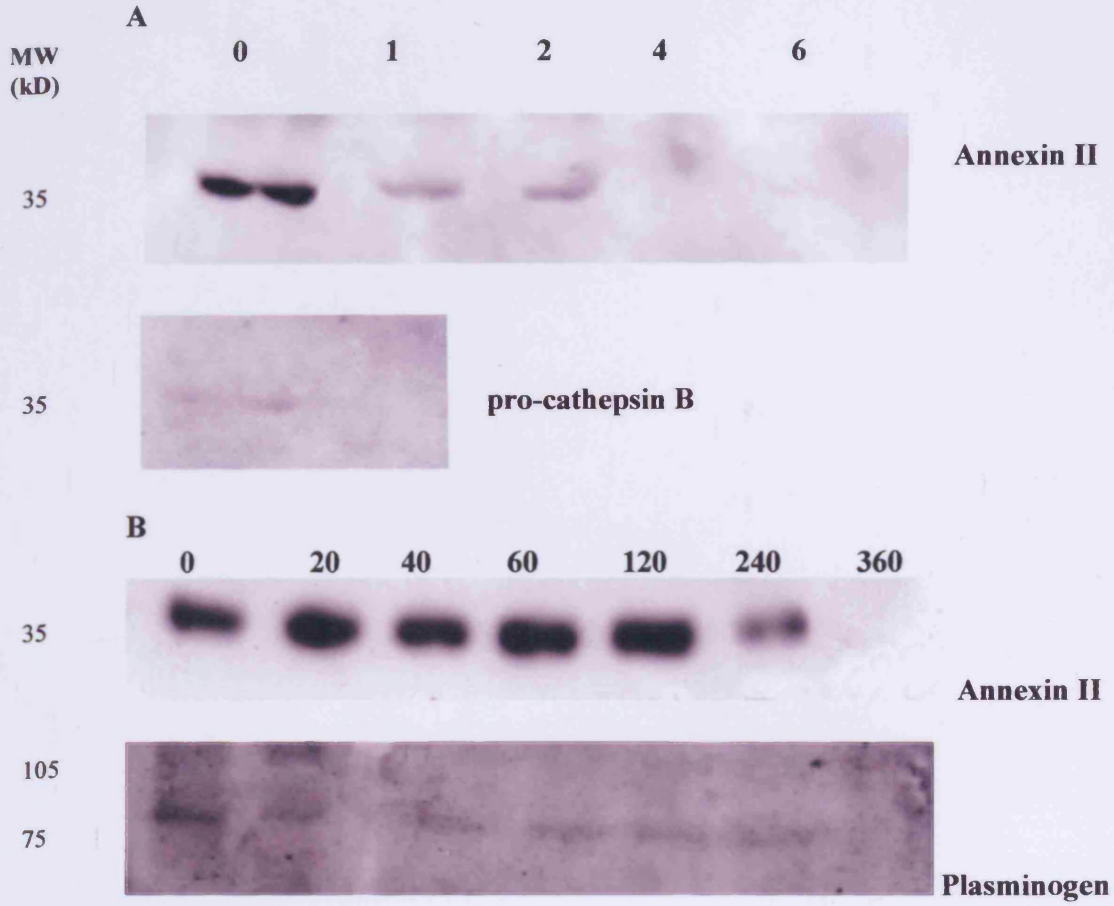



**Figure 5.8.** Verification of cell surface AII modulation by IFNγ by time courses using **A** $CaCl_2$ release **B** EDTA release. AII cell surface expression is lost between 4-6 hours stimulation with IFNγ; AII binding proteins are lost from the cell surface following 1 hour exposure to the cytokine.

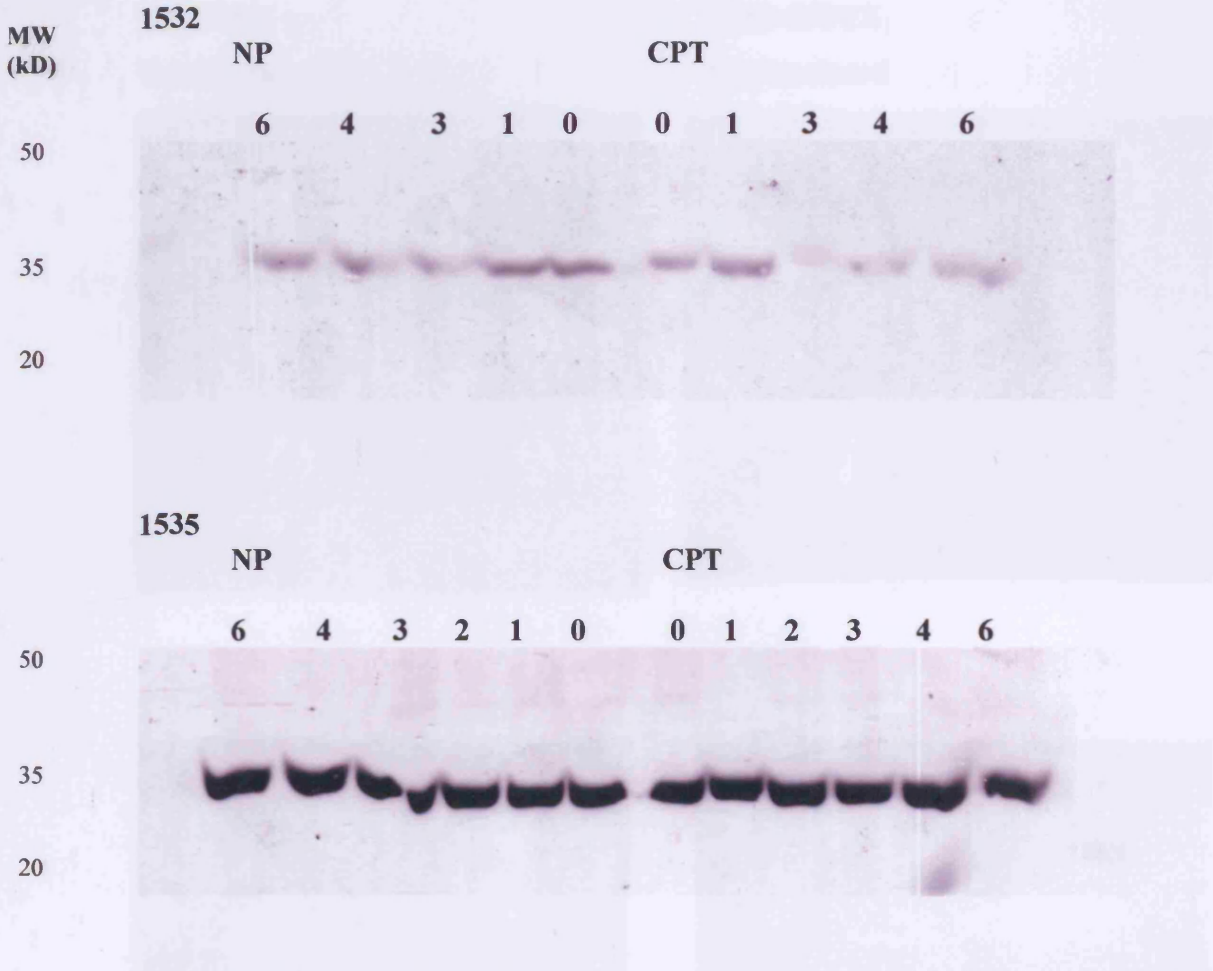



**Figure 5.9**, Time dependent modulation of surface AII by IFNγ in two pairs of isogenic cell lines. The 1535 cell lines appear to express more surface AII than 1542 cells and expression is unaffected by IFNγ. In the 1532 cell lines, cell surface expression is reduced following 3 hour stimulation with IFNγ, comparable to the timescale of down regulation seen in 1542 cell lines.

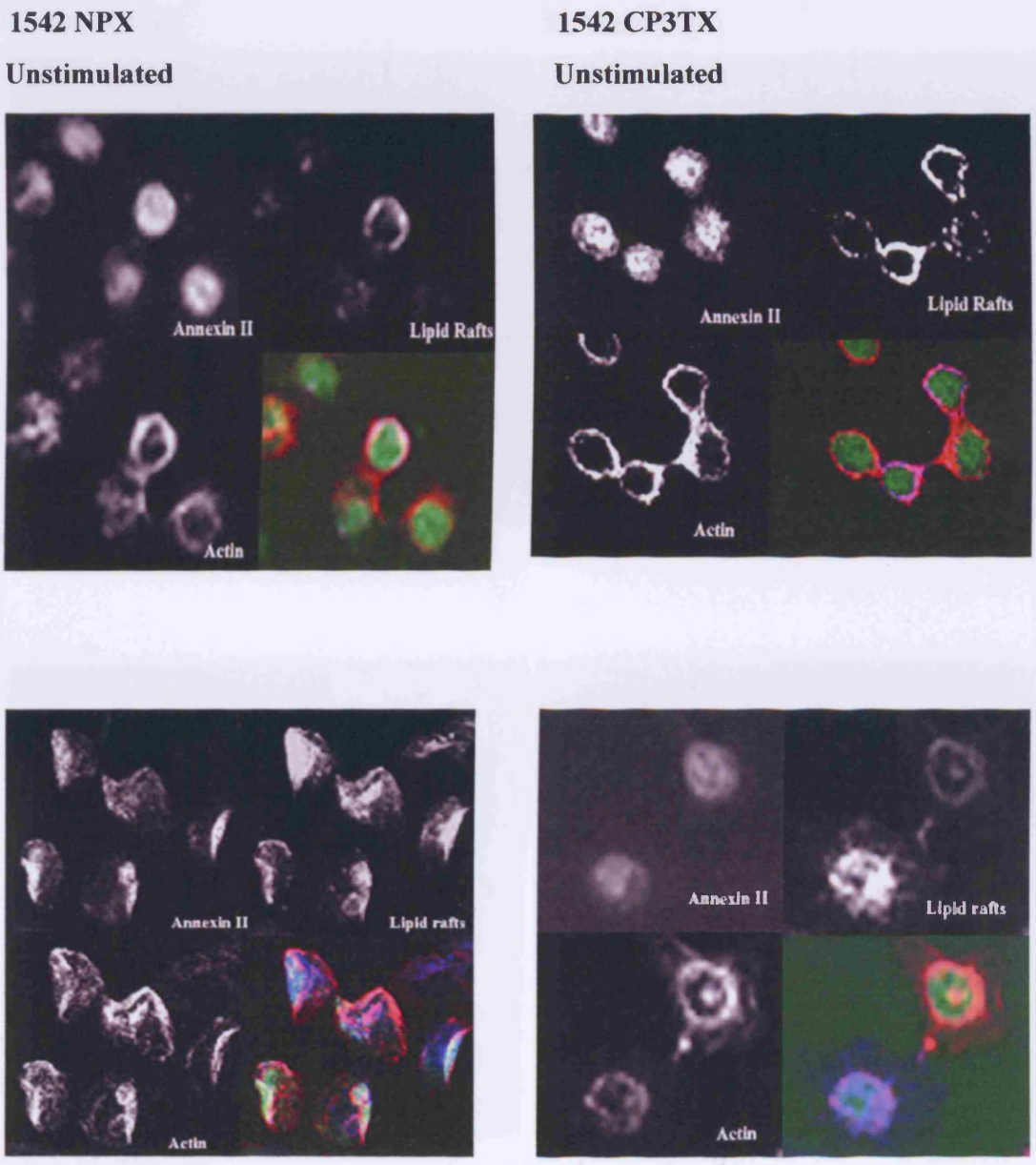



**Figure 5.10**. Anti-AII, cholera toxin and actin staining in live 1542 NPX and CP3TX cells plus or minus 24 hr stimulation with IFNγ. The separate colour channels representing each protein are shown in black and white for increased resolution, the colour picture is a merged image of all three fluorophores. In the colour image AII expression is shown in green, cholera toxin corresponding to lipid raft regions in blue and actin in red. It can be observed that in unstimulated cells from both cell lines AII staining is generalised on the cell surface. On stimulation, however, AII expression is reduced and localised to enlarged lipid raft structure in the normal cells, which are not observed in the CPT cells.

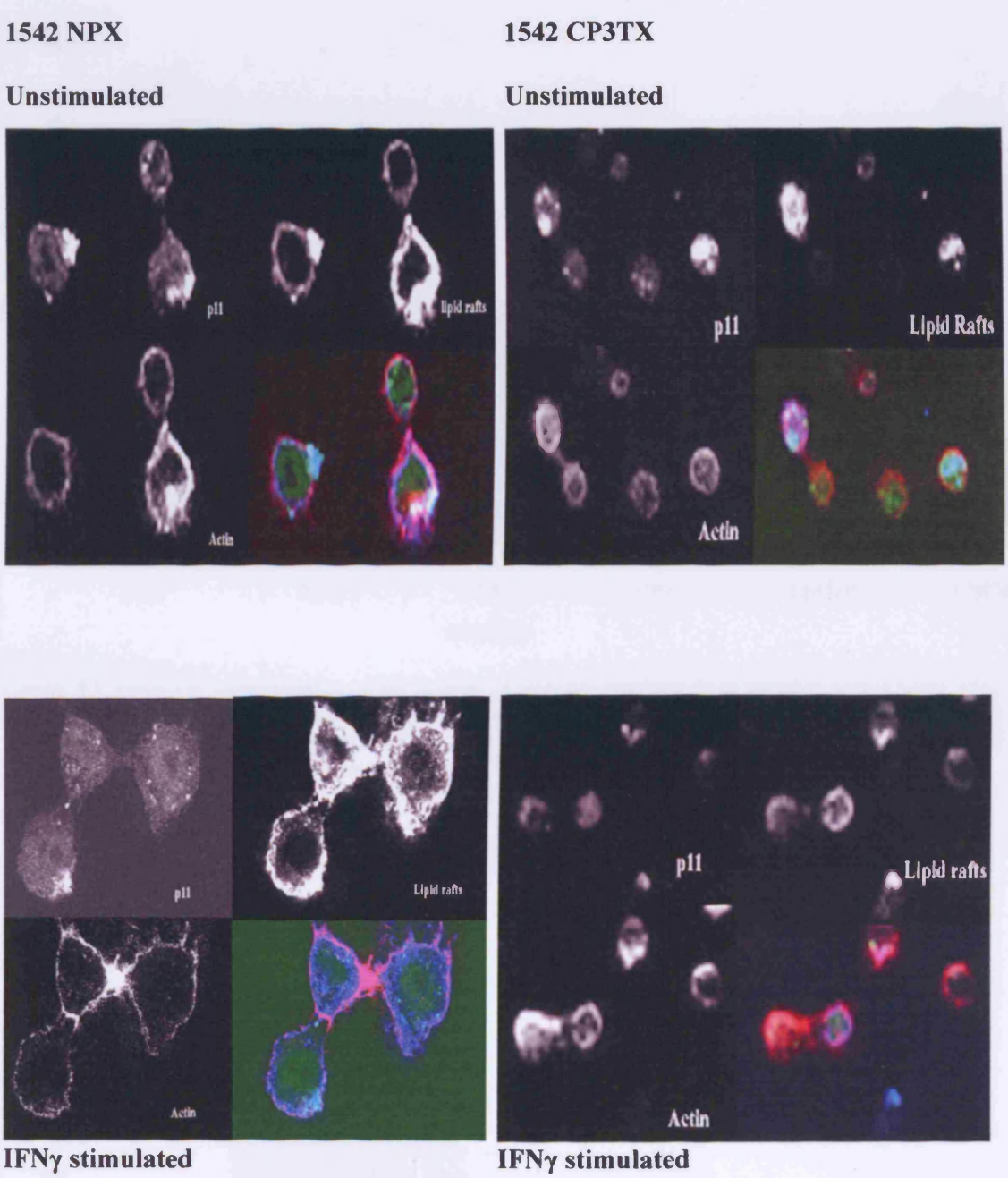
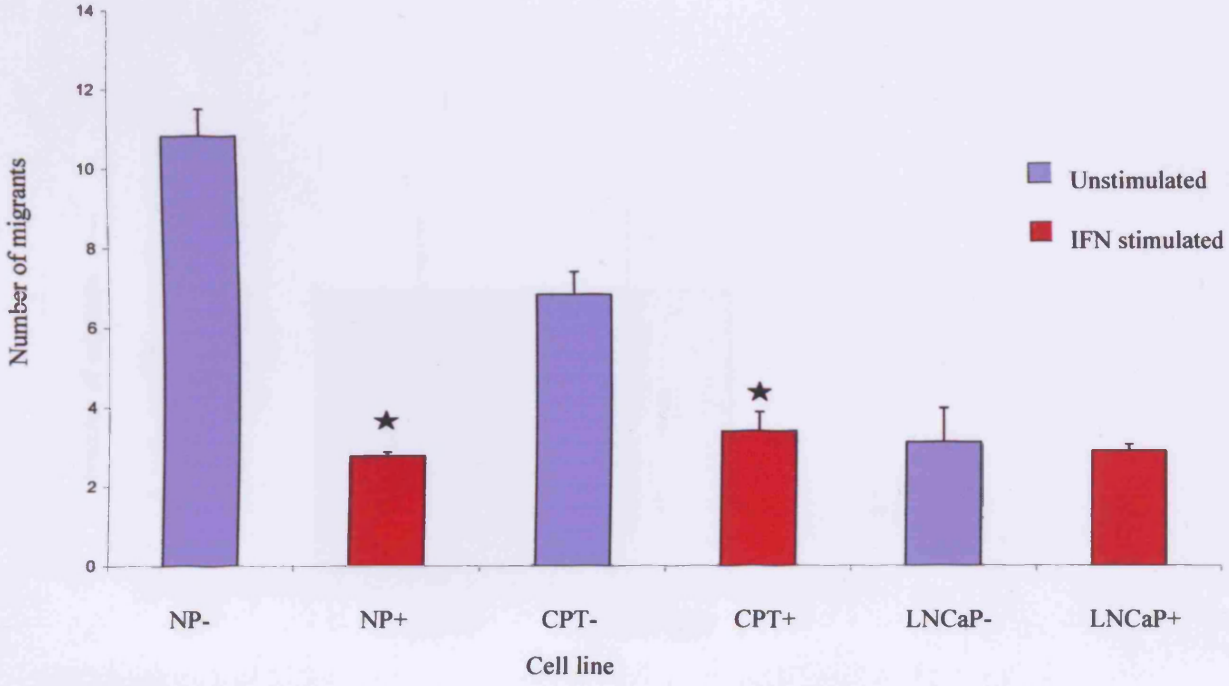
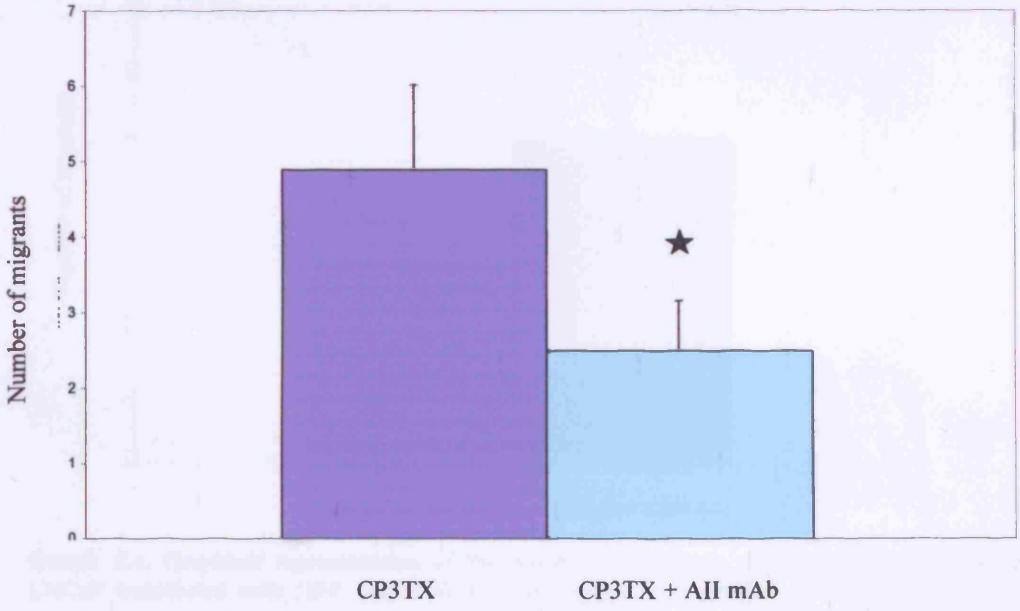


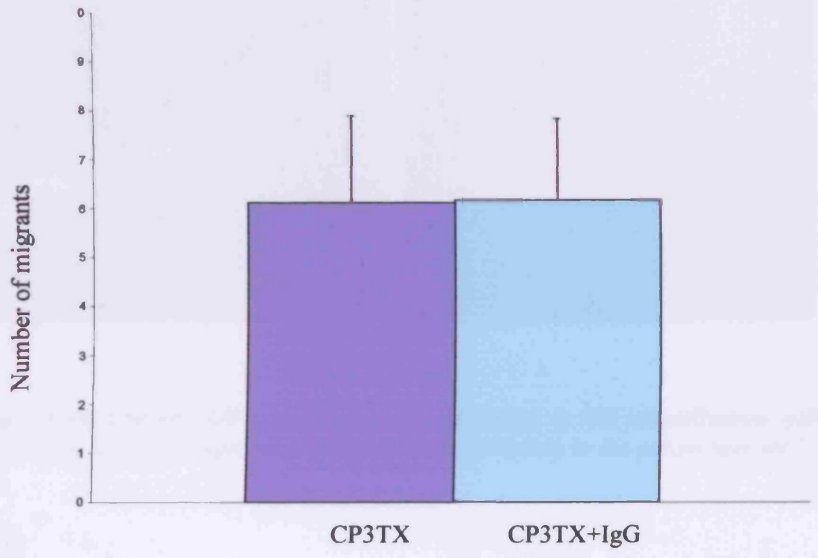

**Figure 5.11.** Anti-p11, cholera toxin and actin staining in live 1542 NPX and CP3TX cells plus or minus 24 hr stimulation with IFNγ. The separate colour channels representing each protein are shown in black and white for increased resolution, the colour picture is a merged image of all three fluorophores. In the colour image p11 expression is shown in green, cholera toxin corresponding to lipid raft regions in blue and actin in red. It can be observed that in unstimulated cells from both cell lines p11 staining co-localises with that of the cholera toxin indicating p11 is present solely in lipid rafts. On stimulation p11 expression is reduced and localised to the enlarged lipid raft structures in the normal cells and almost completely absent in the CPT cells.



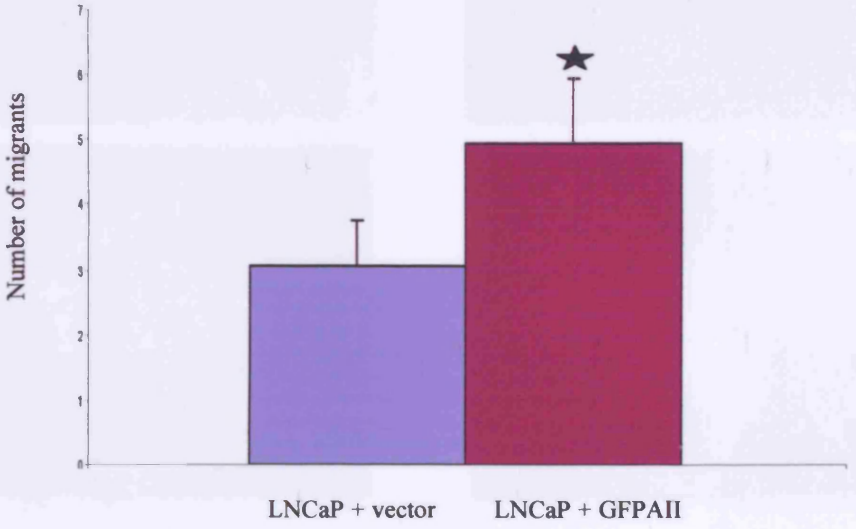

**Graph 5.1**. Graphical representation of the number of migrants resulting from invasion assays using 1542 NPX, CP3TX and LNCaP cells plus or minus stimulation with IFNγ. Asterisk indicates a result significant to P=0.05 confidence limits. It can be observed from this graph that IFNγ reduces the invasive potential of normal and cancerous prostate epithelial cells.




**Graph 5.2**. Graphical representation of the number of migrants resulting from invasion assays using CPT cells plus or minus anti-AII antibody. Asterisk indicates a result significant to P=0.05 confidence limits.




**Graph 5.3.** Graphical representation of the number of migrants resulting from invasion assays using 1542 CP3TX cells plus or minus anti-mouse IgG. The invasive capacity of 1542 CP3TX cells is not affected by treatment with a non-specific antibody of the same isotype as anti-AII.




**Graph 5.4.** Graphical representation of the number of migrants resulting from invasion assays using LNCaP transfected with GFP AII or GFP vector alone. Transfection of AII into LNCaP significantly increases the invasive potential of these cells. Asterisk indicates a result significant to P=0.05 confidence limits.

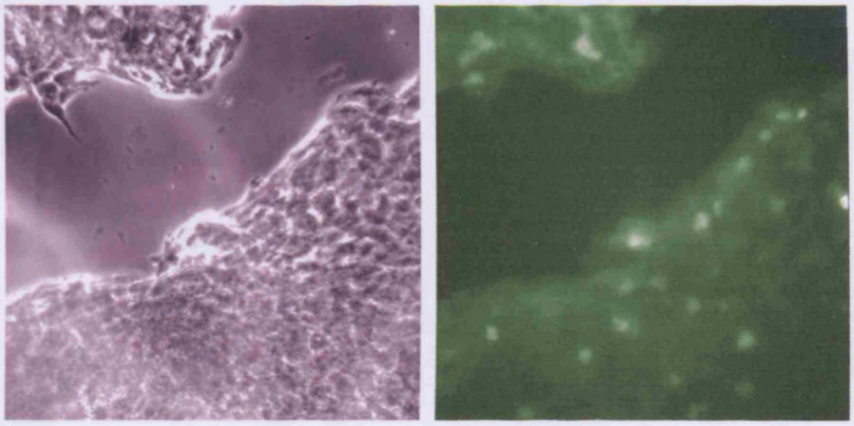


**Figure 5.12.** LNCaP GFP expression, cells are shown at x40 magnification under phase and fluorescent microscopy. GFP AII expression appears localised mainly to the perinuclear and cytoplasmic regions of the cell.

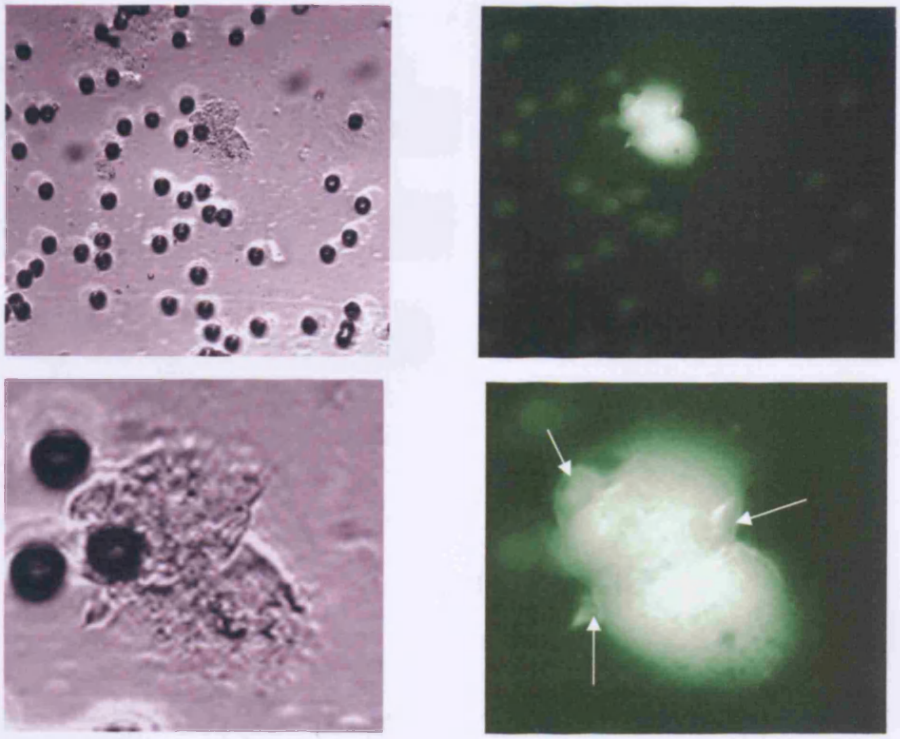
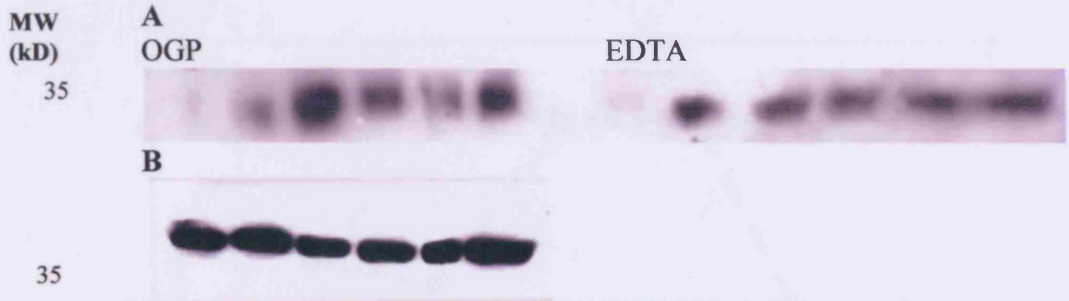


**Figure 5.13.** A migrant GFP AII expressing LNCaP cell shown at x40 and x60 magnification in phase and fluorescent microscopy The majority of GFP AII expression is localized in the cytoplasm, however, cell surface expression can be observed at x60 magnification (see white arrows).




| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dexamethasone | + | + | - | - | - | - | | - | + | + | - | - | - |
| Glyburide | + | - | + | - | - | - | | + | - | + | - | - | - |
| EtOH | + | + | - | - | + | - | | - | + | + | - | + | - |
| DMSO | + | - | + | - | - | + | | + | - | + | - | - | + |

**Figure 5.14**. AII expression on the cell surface and in 1542 CP3TX total lysate following exposure to glyburide and dexamethasone. **A** AII expression analysis shows treatment with glyburide decreases the cell surface level of AII, whilst increasing the AII level inside the cell. Dexamethasone has the opposite effect. Treatment with both agents results in the same pattern of expression as with dexamethasone alone. **B** represents a β actin loading control for the total lysate samples.

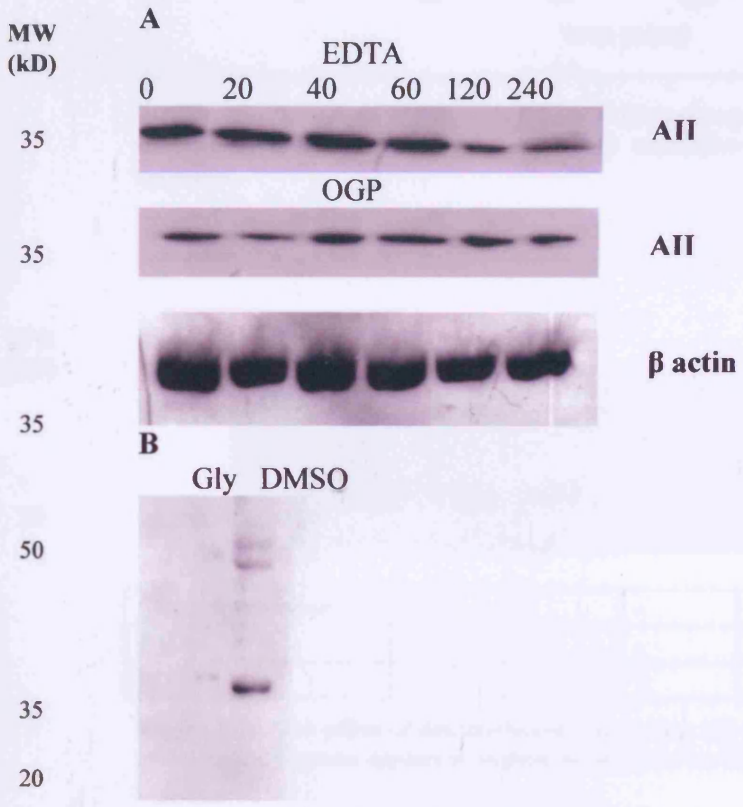
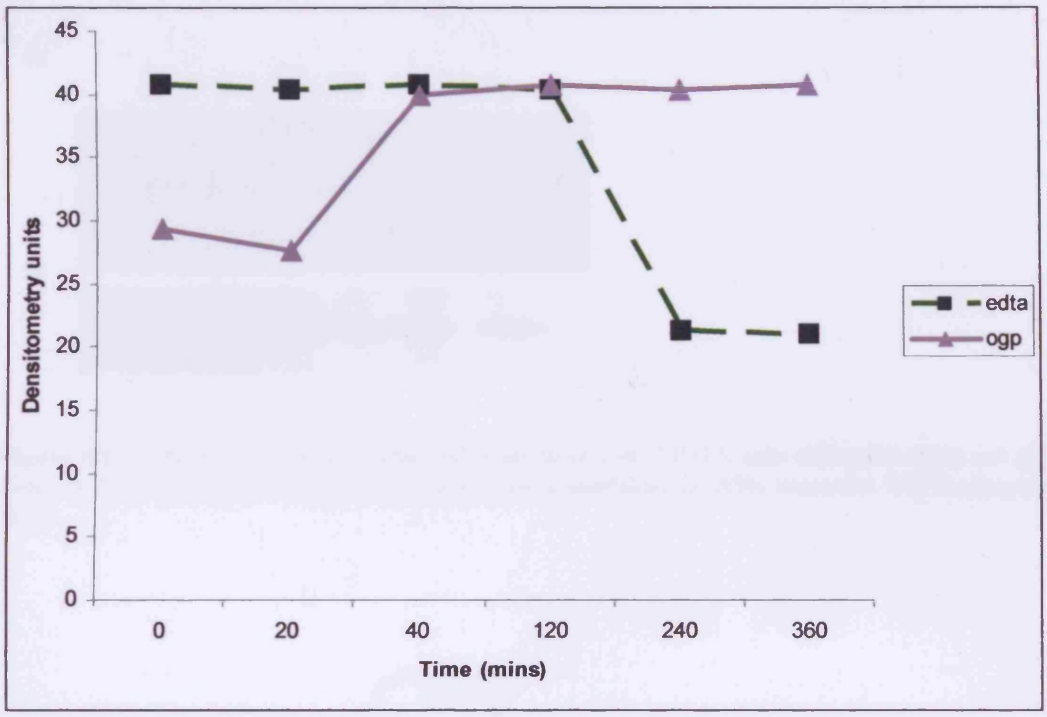



**Figure 5.15**. The effect of glyburide on surface AII expression. **A** Time course AII cell surface and total cell lysate expression in 1542 CP3TX cells exposed to glyburide. A loading control is shown for the total lysate samples. **B** Cell surface expression of AII following 24 hour exposure. Glyburide treatment decreases the cell surface level of AII after 120 minutes of treatment; expression of the protein is lost after 24 hours. The level of AII inside the cell increases after 40 minutes of glyburide treatment.




**Graph 5.5.** The temporal effect of glyburide treatment on the extra and intracellular expression of AII. As duration of drug exposure increases surface expression of AII decreases and intracellular expression increases.

MW (kD)

35

| | | | | | |
|---|---|---|---|---|---|
| Dexamethasone | - | - | - | + | + |
| EtOH | - | + | - | + | - |
| IFNγ | - | - | + | + | - |

**Figure 5.16.** The effect of dexamethasone on surface AII plus or minus IFNγ stimulation in 1542 CP3TX cells. Dexamethasone appears to slightly increase the surface expression of AII.

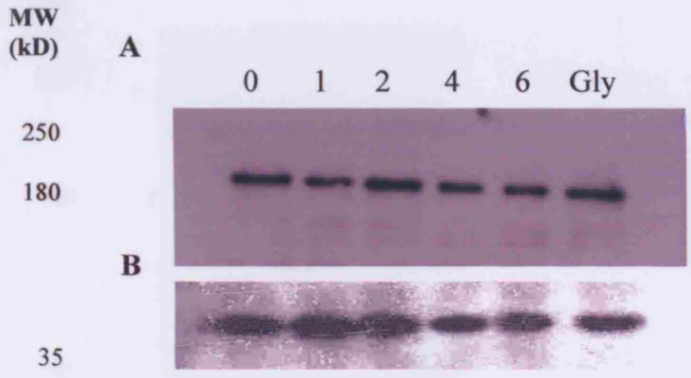



**Figure 5.17**. ABCA1 expression in total cell lysate from 1542 CP3TX cells exposed to IFNγ and glyburide (Gly). **A** The expression of the ABCA1 transporter is unaffected by IFNγ treatment. **B** A loading control β actin.

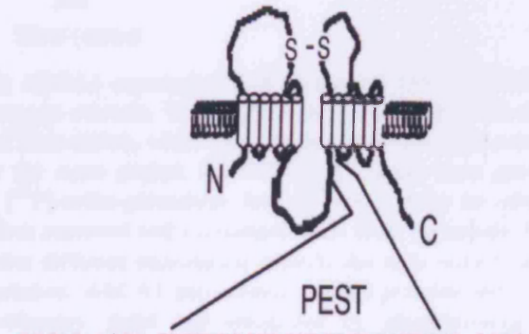



**Figure 5.18.** The PEST sequence of ABCA1. (Wang et al., 2003)

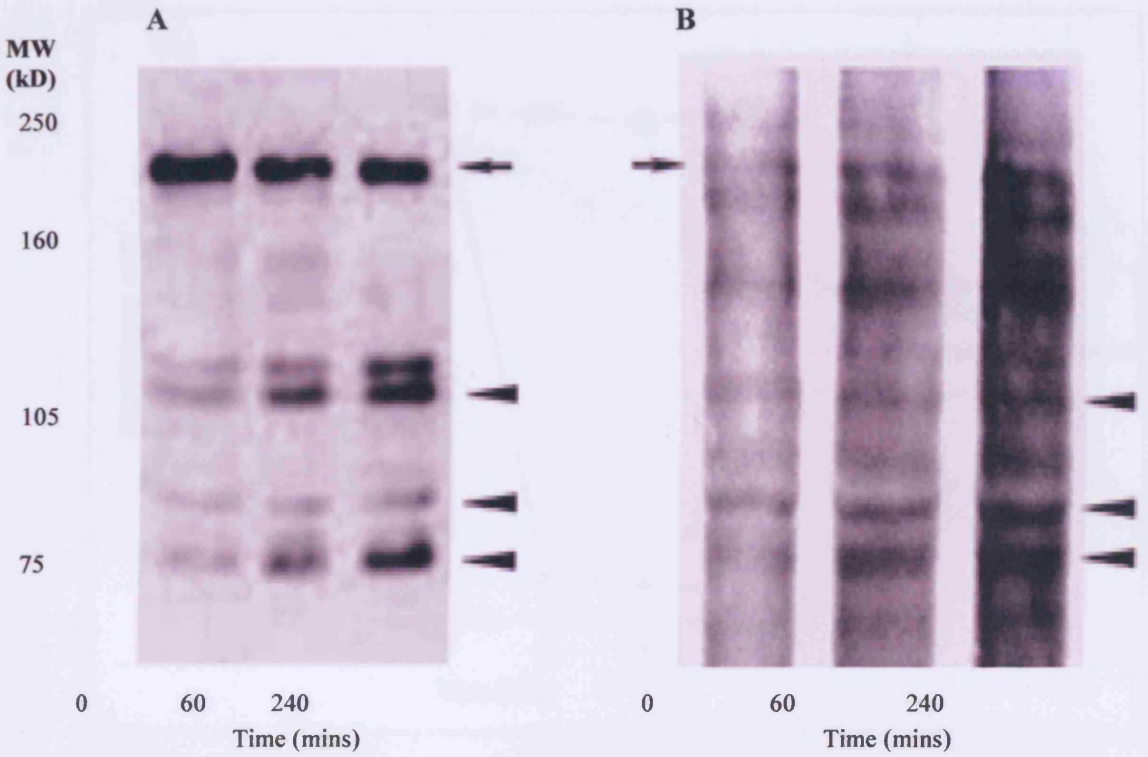



**Figure 5.19. A** ABCA1 expression in IFNγ treated 1542-CP3TX cells demonstrated by immunostaining of whole cell detergent extracts. The expression of the 220kD full-size ABC-transporter A1 is down-regulated over 4 hours of stimulation, while the abundance of low molecular weight (LMW) ABCA1 forms gradually increases over the same period. **B** 1542-CP3TX cells were grown to 70% confluence and metabolically labelled with [$^{33}$P]-ortho-phosphate for six hours prior to stimulation. The isotope containing culture medium was then removed and exchanged with cold, phosphate-free medium and the cells treated with 500 U/ml IFNγ. After different stimulation periods the cells were lysed in RIPA buffer and ABCA1 isolated by immunoprecipitation. ABCA1 and co-precipitated proteins were then separated by SDS-PAGE, transferred to PVDF membranes, dried and visualized by phosphoimaging on a Molecular Imager FX scanner (Biorad). The two LMW phospho-proteins indicated by arrowheads are of similar size as two of the LMW ABCA1 antigens detected by immunoblotting of whole cell extracts.

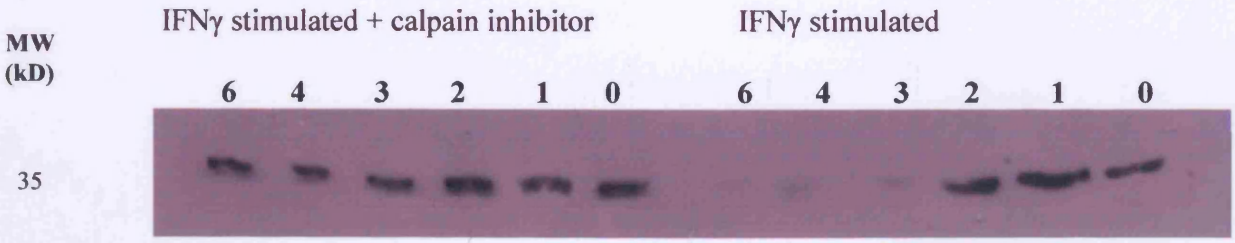
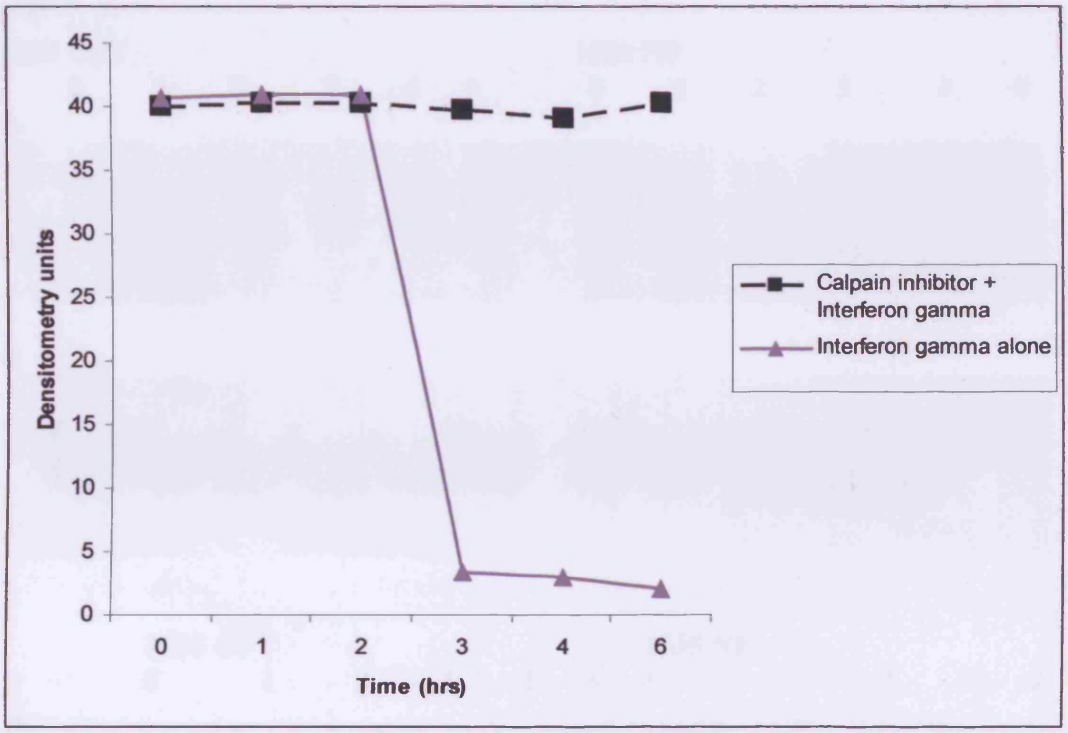



**Figure 5.20**. Time course of cell surface AII expression in IFNγ stimulated 1542 CP3TX cells plus or minus Calpain inhibitor. Calpain inhibition prevents the down regulatory effect of IFNγ stimulation surface AII expression in these cells.




**Graph 5.6**. The temporal effect of calpain inhibitor on AII expression in response to IFNγ. Treatment with calpain inhibitor appears to prevent loss of surface expression of AII normally seen following 3-4 hours treatment with IFNγ.

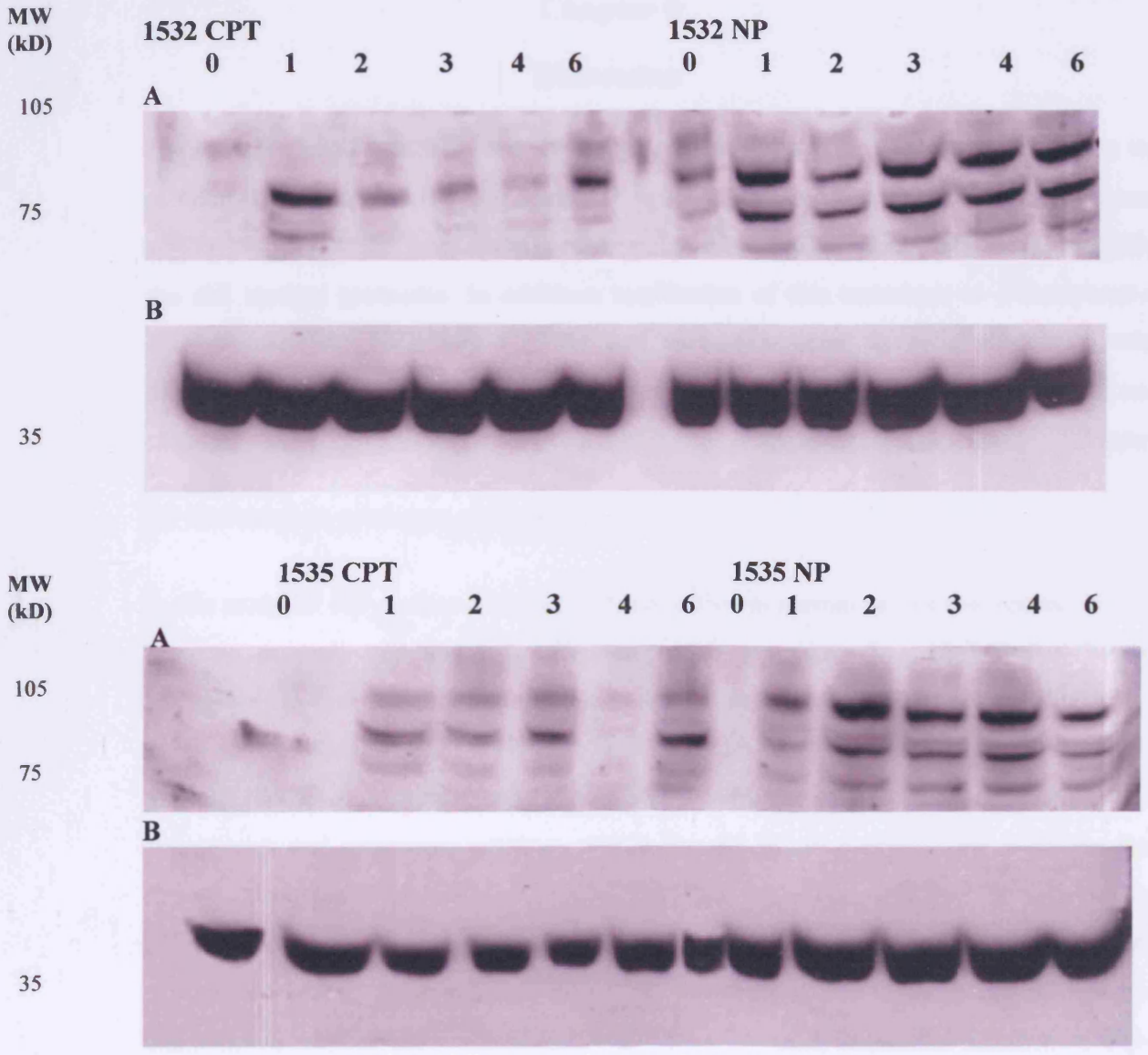



**Figure 5.21**. Time course analysis of calpain I expression on stimulation with, IFNγ in 1532 and 1535 NP and CPT cell lines. In both cases **A** represents immunodetection of calpain I and **B** the loading control β actin. Both normal cell lines appear to express two isoforms of calpain II, whereas the cancer lines express predominantly one form or the other calpain II expression is induced in all cell lines following 1 hour stimulation with IFNγ.

# Chapter 6

# Discussion

The central aim of this study was to analyze the proteins differentially expressed on the surface of normal and cancer epithelial cells using a proteomic approach. This was addressed through the development of a reproducible and efficient method for analysing the cell surface proteome. In addition, application of this technique to a comparative analysis of 1542 NPX and CP3TX cell surface proteins in cells stimulated with interferons led to a comparative analysis of cytokine signalling in these cell lines and characterisation of several proteins of potential use in the management of prostate cancer.

## 6.1 Cell surface proteome analysis

In this study 39 cell surface proteins were identified in normal and cancer epithelial cells from an isogenic cell line pair. This represents a greater than 10-fold enrichment, comparable to that achieved in other studies using gel separation (Zhao et al., 2004) and liquid based chromatography (HPLC) methods (Nielsen et al., 2005). However, despite this apparent level of enrichment there remained evidence of sample contamination from endogenous and exogenous proteins within the gel bands excised. This indicates that further optimisation of the cell surface protein separation protocol is required to efficiently analyse the cell surface proteome. Measures to reduce contamination could include conducting the experiment in a clean room environment, including further pre-fractionation steps to deplete non-membrane proteins using sequential addition of high-salt, carbonate and urea washes similar to those employed by (Nielsen et al., 2005) and separation using techniques offering better protein resolution such as HPLC and 2DGE.

The expression of calnexin and gp96, identified in the cell surface proteome analysis of the 1542 NPX and CPTX cell lines, was analysed through immunoblotting. Due to the lack of antibody availability and the degree of protein complexity in bands excised from 1D SDS PAGE gels, the relative expression of the other 37 proteins could not be analysed. The remaining identified cell surface proteins therefore, require further quantitative analysis to examine their expression in these cell lines. Further analysis

could involve 2DGE, to reduce band complexity and identify any post translational differences in the proteins expressed, immunoblotting with specific monoclonal antibodies and transcript expression analysis via quantitative polymerase chain reaction (QPCR).

Increases in expression of calnexin and gp96 in the cancer cell line were found to be statistically significant. These proteins are molecular chaperones involved in the transport of peptides to MHC I molecules, most often localised to the endoplasmic reticulum. However, it appears that in the 1542 CP3TX cell line, gp96 and calnexin are found expressed at high levels on the cell surface. Calnexin has been found to be over expressed in breast cancer (Li et al., 2001, Yeates and Powis, 1997), multiple myeloma (Munshi et al., 2004), melanoma (Nielsen et al., 2005) and is known to associate with CAM's such as integrin β1 (Yeates and Powis, 1997, Ruoslahti and Reed, 1994). Over expression of calnexin in 1542 CP3TX cell line and its interaction with CAM's suggests that this protein may be involved in cell invasion. A correlation with calnexin over expression and increasing breast cancer grade has been noted previously (Li et al., 2001). In a recent cDNA micro array analysis of prostate epithelial cells, androgens and growth factors were found to significantly up regulate calnexin expression. This may indicate that calnexin has a role in androgen induced proliferation in early tumourigenesis and also in the acquisition of androgen insensitivity through enhanced expression by other mitogenic factors (York et al., 2005). The possible role of this protein in early tumourigenesis and the correlation of expression with disease grade may suggest its utility as a potential diagnostic and/or prognostic marker. Further analysis of calnexin expression, via western blotting and immunohistochemical studies in other cell lines and prostate tissue, will be required before the diagnostic/prognostic potential of this protein in prostate cancer can be assessed.

Over expression of MHC1 peptide chaperones, may appear disadvantageous to the cancer cell as it has been noted in several studies that surface expression of gp96 can elicit anti-tumour immunity (Dai et al., 2003, Robert et al., 2003). However, gp96 over expression has also been associated with protection from cellular stressors such as chemotherapy

(Santin et al., 1998b), irradiation (Santin et al., 1998a), hypoxia, acidosis, nutrient depletion and the inflammatory response (Lee, 1992, Heike et al., 2000, Haverty et al., 1997b). The main protective effect of gp96 is thought to be in the inhibition of apoptosis (Chen et al., 2002, Liu et al., 1998). Protection from such stressors via up regulation of gp96 may provide the cancer cell with a selective growth advantage. As such gp96 may be involved in the early stages of tumourigenesis and therefore of valuable diagnostic marker potential. To examine the broader clinical application of this protein, for example as a diagnostic marker, analysis of its expression in other cell line models and in prostate tissue is required. It would also be of interest to examine the response of prostate cells over expressing gp96 to stressors such as cytotoxic drugs to analyse the suggested protective effect.

### 6.2 Interferon signalling

This study also resulted in the identification of key constituents of the IFNγ signalling pathway that appear to exhibit differential expression patterns between normal and neoplastic cells when stimulated with IFNγ. In accordance with the published literature, these results suggest that 1542 CP3TX cells exhibit differential expression of certain molecules in the IFN signalling pathways in comparison to their normal counterpart, specifically the transcription factor p48 (Nagano et al., 2003). The pattern of induction of p48 expression following interferon stimulation observed in this study is in agreement with that seen in a recent study of transitional cell carcinoma of the bladder where interferon alpha treatment lead to a low level of p48 expression which could be restored by pre-treatment with IFNγ (Matin et al., 2001). Combinatorial use of IFNγ and alpha has also proven effective in increasing p48 and causing subsequent growth inhibition in renal cell carcinoma (Nanus et al., 2000). Defects in the activation of p48 have been noted in hepatocelluar carcinoma cell line MHCC97, where mutations possibly affecting the stability of the protein were shown to negate the anti-proliferative effect of interferon alpha (Wu et al., 2004b). This may suggest that cancer cells gain a selective advantage by suppression of the expression or activity of p48 and thereby decreasing sensitivity to the immunomodulatory effects of IFNs, which include growth inhibition, increased adhesion and heightened immunosurveillance. In clinical trials single cytokine therapy has been

used for advanced prostate cancer, but its efficacy is limited and the toxic effects often severe (Kuratsukuri et al., 2000, van Haelst-Pisani et al., 1992). In general it has been noted that IFNγ is capable of elevating p48 levels in cancer cells unresponsive to other cytokines and as a subsequent effect cancer cells become sensitive to the growth inhibitory effects of interferon alpha (Wong et al., 1998, Matin et al., 2001). In this capacity, the results of this study may suggest that IFNγ could be used as an adjuvant to enhance the effects of IFNα in growth retardation of prostate tumours. Proof of this principle would require trial of the anti-proliferative effect of combinatorial therapy with these cytokines in vitro through MTT assays and in vivo using the TRAMP mouse model. In addition, it would be of interest to verify the results of this study in other prostate cell lines and determine the activity and phosphorylation status of key proteins such as p48 and STATs in normal and cancer prostate cell lines under stimulation with interferons.

### 6.3 Characterisation of gp96

The alteration in interferon signalling in cancer cell lines was found to include the modulation of proteins on the cell surface, of these gp96 and AII were chosen for further study. It was observed that over expression of gp96 in the 1542 CP3TX cells, could be decreased significantly by treatment with IFNγ. An opposite pattern of gp96 expression was observed in 1542 NPX cells, as in this cell line, stimulation with IFNγ appeared to increase the expression level of gp96 and its isoforms. These results support the previous finding that gp96 is upregulated in the cancer cell line and add a novel dimension in the discovery of production of previously unreported gp96 isoforms in normal prostate cells following stimulation with IFNγ. In their normal resting state, the prostate cancer cells appear to over express a single form of gp96, a feature known to be associated with avoidance of apoptosis (Chen et al., 2002, Liu et al., 1998). In these cancer cells, on stimulation with IFNγ, there is an apparent decrease of the expression of this single gp96 isoform and a failure to produce the multiple isoforms of gp96 observed in immunoblotting of normal cells. Although the immunologic activity of gp96 has yet to be characterized, gp96-associated peptides constitute the antigenic repertoire of the source tissue. As such the multiple isoforms may be required as part of the functional role of

gp96 as a molecular chaperone involved in the transport of antigenic peptides to the MHC class I proteins on the cell surface. gp96 mediated antigen display has been demonstrated to elicit a CD8-specific T cell response and therefore possible anti-tumour immunity (Blachere et al., 1997). The multiple isoforms of gp96 produced in response to IFNγ in the normal cells could represent the result of glycosylation events, as a recent study observed differences in the gp96 monosaccharide composition between normal and cancer cells from rat and cell line prostate cancer models. Additionally a further decrease in gp96 glycosylation was observed in cells with more aggressive cell phenotypes (Suriano et al., 2005). Down regulation of gp96 glycosylation may be selectively advantageous for the cancer cells in avoidance of the immune response, a mechanism also observed in bacterial infection by *Orientia tsutsugamushi* (Cho et al., 2004).

Suppression of gp96 is a potential negative effect of IFNγ treatment, as it is possible that MHCI display of tumour-associated antigens may be adversely affected. To further investigate the expression of gp96 in cancer cells and its regulation by IFNγ, additional research is required. The potential anti-apoptotic effect of over expression of gp96 could be investigated through apoptosis assays comparing rates in the normal and cancer cell line, transfected cells over expressing the protein and in gp96 knockout cells. In addition, further characterisation of the gp96 isoforms in normal cells would be required as confirmation that the proteins observed represent variants of the gp96 protein and are not due to a non-specific effect of the antibody. This could be achieved by using immunodetection with antibody specific to a unique part of the protein. The use of specific antibodies, for example to the amino or carboxyl terminus, would also aid in the identification of whether variants are produced through cleavage of the full length protein and if so, possibly suggest the site of this modification. 2DGE followed by MS analysis to identify the isoforms via peptide sequencing, or by lectin overlays to detect glycosylation could also be used to further characterise gp96 isoforms. The effect of differences in gp96 expression on MHC presentation could be analysed by observation of MHC presentation in cells under/over expressing gp96 and in normal and cancer prostate cells stimulated with IFNγ. The subsequent effect on cellular interaction with the host immune system could be examined by xenograft transplant into animal models. However,

anti-sense mediated down regulation of gp96 has previously been reported as having no effect on MHCI presentation in P13.1 cells (Lammert et al., 1996)

### 6.4 Characterisation of Annexin II

Other cell surface proteins found to be modulated by IFNγ were identified as Selectin, IFN induced protein 53 and Annexin II. AII was chosen for further study as it has been reported to have a potential role in cell invasion (Falcone et al., 2001, Emoto et al., 2001, Mai et al., 2000c, Wu et al., 2002, Brownstein et al., 2003, Mai et al., 2000b) and it is at the later stages of PCa where invasion often leads to incurable metastases, that interventions are needed. AII was found to be expressed at the same level in both normal and cancer prostate cell lines. However, cell surface expression of AII in 1542 CP3TX and NPX appears to be suppressed by IFNγ in a surface specific manner, this was also observed in a second isogenic pair of cell lines 1532 NPX and CP3TX. The apparent surface specific suppression of AII by IFNγ also appeared to result in a decrease in the invasive capacity of cells expressing AII. This finding is in accordance with the research of Falcone et al, 2001, which demonstrated that matrix invasion and degradation was dependant on cell surface AII expression in macrophages (Falcone et al., 2001). The role of AII in invasion was further suggested by transfection of an AII expression vector into LNCaP cells, which appeared increase in invasive capability. However, the vector was a dual construct also encoding GFP, which could interfere with the expression and normal biological function of AII. This study suggests that AII may have a significant role in prostate epithelial cell invasion, possibly through interaction with endopeptidases and extra cellular matrix components (Kirshner et al., 2003, Chung and Erickson, 1994, Choi et al., 2000). The novel finding in this study is that IFNγ down regulates AII expression in a cell surface specific fashion. This finding has not been previously reported.

As the transport pathway of AII is unknown, the involvement of ABCA1, a protein known to transport the structurally similar AI (Chapman et al., 2003), was investigated as a potential candidate transporter. Treatment of cells with glyburide, a potent inhibitor of ABC transporters, appeared to decrease expression of AII on the cell surface, whereas dexamethasone a promoter of ABC transporter activity appeared to increase expression.

This finding suggested that the transport of AII to the cell surface was influenced by the activity of an ABC transporter. ABCA1 has been shown to be transcriptionally regulated by IFNγ (Wang et al., 2002b), however western blotting followed by immune detection showed that ABCA1 expression was apparently unaffected by IFNγ treatment. This suggested that any effect on ABCA1 activity was post translational. Experiments conducted by Dr Naaby-Hansen contradicted this result by demonstrating that levels of ABCA1 did decrease, although data for ABCA1 expression were not available to be quantified or verified by statistical analysis and no loading controls were provided. This result therefore requires repeated demonstration and quantitative analysis. Dr Naaby-Hansen's experiments did recognise the existence of low molecular weight proteins detected by the ABCA1 antibody. Radio labelling experiments indicated that ABCA1 and the low molecular weight forms became phosphorylated on exposure to IFNγ. Once phosphorylated the PEST sequence of ABCA1 may act as a target for degradation by calpains (Wang et al., 2003). It is possible that the low molecular weight proteins recognised by the ABCA1 antibody may represent the degradation products of this process.

This theory was explored further by the inhibition of calpain, which appeared to maintain the expression of AII in the presence of IFNγ, indicating that IFNγ may regulate the expression of AII on the cell surface by a calpain-mediated process. The model proposed is that cell surface expressed AII is regulated by IFNγ via calpain-mediated inactivation of ABCA1 or a similar transporter. Further investigation of this mechanism is required to isolate the exact protein involved in the transport of AII, as the inhibitors and promoters of activity used in these experiments have common effects in all ABC transporters. The results of a recent study suggest that treatment with the drug Probucol protects ABCA1 from calpain-mediated degradation (Wu et al., 2004a), observation of cell surface AII in cells exposed to this drug may further implicate ABCA1 involvement in the transport of AII to the cell surface. Isolation of the exact ABC transporter responsible for the expression and localisation of AII could also be examined through the use of ABC transporter knockout cells.

IFNγ has been shown to directly and indirectly regulate the expression of calpains causing effects such as down (Gollob et al., 2005, Shiraha et al., 2002) and up regulation (Gallardo et al., 2001, Deshpande et al., 1995). The latter findings concur with those of this study, which found that calpain II expression was apparently induced following one hour of IFNγ stimulation in both normal and cancer isogenic cell lines. Due to the non-specificity of the calpain inhibitor used in this study, further analysis is required to isolate the calpain implicated in AII transport. This could be achieved by observing the cell surface expression of AII in response to IFNγ in cells lacking different calpain isoforms. The mechanism of action of IFNγ on calpain could be further investigated at the transcriptional level by expression analysis of calpain mRNA via QPCR and by protein immunodetection at the translational level, by 2DGE and analysis of the phosphorylation status of the protein by radiolabelling and/or detection by phosphorylation specific antibodies.

Immunofluorescence analysis indicated that AII expression in the 1542 cell lines was similar to that described in other studies (Zobiack et al., 2002, Deora et al., 2004). However the presence of AII expression in the cancer cell line is contradictory with recent research that indicated that AII expression, although expressed in benign prostate epithelium (BPE), benign prostate hyperplasia (BPH) and prostatic intra-epithelial neoplasia (PIN), is lost in prostate cancer (Lehnigk et al., 2005). Similarly, it was found that AII expression was reduced in prostate cancer cell lines and clinical samples (Chetcuti et al., 2001a). However, in this study a panel of prostate cell lines all, with the exception of LNCaP, expressed AII. This concurs with the findings of Bannerjee et al who also noted the expression of AII in high grade PCa from American but not Indian patients (Banerjee et al., 2003). The discrepancy between the results of this study and that of cited literature indicate that several factors may influence the detection of AII expression in prostate cancer cells. In PCa cell lines it appears that differences in cell culture methods or the specificity of the anti-AII antibody may affect detection of the protein, this could be tested by mimicking the culture and assay conditions used in previous studies. In immunohistochemical analyses it appears that racial factors and stage of disease may influence the detection of AII in prostate sections, this may reflect true

variation or an inability of the antibody used to detect variant AII forms. This could be investigated via a large cohort study including specimens from various racial backgrounds and varying grade of disease. Initially an immunohistochemical trial of different AII antibodies on a subset of specimens representative of the apparent racial and disease grade variation should be performed alongside immunodetection of AII in cell lysates to test the efficacy of the antibodies in detecting AII and any variant forms. This could be further confirmed at the molecular level by QPCR analysis of AII expression in a large population cohort.

The apparent ability of IFNγ to decrease AII expression on the cell surface demonstrated in this study has a potentially therapeutic application, as it may decrease the invasive potential of prostate cancer cells. This could be further examined in the mouse model of prostate cancer, where TRAMP mice treated with IFNγ could be monitored for time to metastasis and compared to untreated controls. In a study using prostate cancer xenografts in mice, Shou et al found that with interferon treatment the rate of progression to metastasis was decreased (Shou et al., 2002). In the present study however, it appears that the loss of surface expressed AII is accompanied by a reduction in lipid raft structures in the cancer cells. These lipid raft structures are known to contain many of the signalling factors, receptors and MHC molecules required for interaction with the host immune system (Sehgal et al., 2002). In normal cells the response to IFNγ treatment has been shown to involve the formation of signalosomes, as was seen in this study. In cancer cells, however, this cytokine appeared to decrease the quantity of lipid rafts and no signalsome formation was noted. Exogenous interferon treatment could therefore be potentially detrimental as it may impede detection of cancer cells by the host immune system. Further investigation into the formation of lipid rafts and the response to interferons in normal and cancer prostate cell lines is warranted. This could include co-localisation of lipid raft structures with other molecules involved in interferon signalling such as the IFNGR and STAT proteins and molecules involved in immune detection e.g. MHCI. It would also be of benefit to confirm that the structures observed in this study are lipid rafts and not random collections of the GM1 ganglioside. This could be done be co-staining with fillipin, an auto-fluorescent molecule known to bind to cholesterol.

### 6.5 Appraisal of the therapeutic benefit of IFNγ

The results of this study suggest that IFNγ treatment may prove beneficial as an adjuvant pre-treatment before exposure to further cytokine-based therapy, to limit proliferation and retard the invasive potential of cells in prostate cancer. However, it has also been demonstrated that this cytokine may affect proteins, i.e. gp96 and structures, i.e. lipid rafts, involved in cell-immune system interactions. Therefore, the potential benefits of IFNγ treatment may be negated by the effect of the cytokine on immune system interactions. As such, treatment with IFNγ may cause a lack of detection of cancer cells and potentially increase the disease load. However, as a result of examining the response of cells to IFNγ this study has identified proteins that show apparent expression differences between normal and cancer cells from an isogenic pair of cell lines. These proteins could therefore become targets for therapeutic intervention in their own right. gp96, for example, has been used previously as an anti-tumour vaccine in a rat model of prostate cancer. The results of this study suggested that administration of host derived gp96 limits both the occurrence and growth of prostate tumours indicating treatment with this vaccine could be of prophylactic or therapeutic use in prostate cancer patients (Yedavelli et al., 1999b). The Yedavelli study could be repeated in TRAMP mice to assess whether administration of host-derived gp96 would lead to regression of prostate tumours and evaluate the longevity of the effect. Clinical trials would be required before this could be considered a viable treatment option. It may also be of interest to observe the effect of inducing the expression gp96 isoforms in cancer cells in a xenograft transplant model, as it is possible that this may increase MHC class I antigen presentation and the subsequent immune mediated destruction of cancer cells.

AII expression could provide a potential drug target to limit remodelling of the basement membrane by cancer cells. This would require the design of a pharmacological agent, possibly mimicking the calpain-mediated regulation of AII by IFNγ, without causing the additional detrimental effects on lipid raft structures and gp96 expression. Evaluation of such an agent would require extensive trials in vivo and in vitro, before any assumptions could be made regarding its therapeutic potential. If effective, such treatment may be of

particular therapeutic use in mid to late stage prostate cancer in limiting further spread of the disease.

### 6.6 Conclusion

In conclusion, this study has led to the demonstration of a novel procedure capable of enriching membrane proteins in cell extract, which has subsequently led to the identification of proteins with apparent differential expression in normal and cancer cells and those exhibiting altered expression in response to IFNγ in the cancer cell line. The outcomes of this study are primarily that proteins with the potential utility in the treatment of prostate cancer have been identified, though further characterisation of these proteins required before their clinical application can be realised. Secondly, this study has shown that IFNγ may be of therapeutic benefit in the suppression of prostate cancer, but that the effects of this cytokine on cell-host immune system interactions may limit its clinical application. Finally, this study has uncovered several indicators that, in the case of 1542 NPX and CP3TX, the cancer cell line has acquired several features which could be selectively advantageous in avoidance of immune system detection. Further research of these mechanisms may indicate the molecular basis for the evasion of host responses necessary for tumourigenesis.

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## Appendix 1

## The TNM grading system for prostate cancer (Alliance, 2007)

**T Stages** *(Tumor)*

**T1**
The tumor cannot be felt or seen with imaging such as transrectal ultrasound.

**T1a**
The cancer is found incidentally during a transurethral resection (TURP) for benign prostatic enlargement and is present in less than 5 percent of the tissue removed.

**T1b**
The cancer is found through TURP and is present in more than 5 percent of the tissue removed.

**T1c**
The cancer is found by needle biopsy done because of an elevated PSA level.

**T2**
The cancer can be felt through a digital rectal exam (DRE).

**T2a**
The cancer is in one side of the prostate.

**T2b**
The cancer is in both sides of the prostate.

**T3**
The cancer has begun to spread outside the prostate and may involve the seminal vesicles.

**T3a**
The cancer extends outside the prostate but not to the seminal vesicles.

**T3b**
The cancer has spread to the seminal vesicles.

**T4**
The cancer has spread to tissues next to the prostate (other than the seminal vesicles), such as the bladder's external sphincter (muscles that help control urination), the rectum and/or the wall of the pelvis.

**Appendix 1 contd.**

**N Stages** *(Lymph nodes)*

**N0**
The cancer has not spread to any lymph nodes.

**N1**
The cancer has spread to one or more regional (nearby) lymph nodes in the pelvis.

**M stages** *(Metastasis)*

**M0**
The cancer has not spread beyond the regional nodes.

**M1**
The cancer has spread to distant (outside of the pelvis) lymph nodes, bones or other organs such as the lungs, liver or brain.

**Stage I**

T1a, N0, M0, low grade or score

**Stage II**

T1a, N0, M0, intermediate or high grade or score
T1b, N0, M0, any grade or score
T1c, N0, M0, any grade or score
T1, N0, M0, any grade or score
T2, N0, M0, any grade or score

**Stage III**

T3, N0, M0, any grade or score

**Stage IV**

T4, N0, M0, any grade or score
Any T, N1, M0, any grade or score
Any T, any N, M1, any grade or score

## Appendix 2

### Bradford protein concentration assay (Pierce Biotechnology, 2004a)

**Test tube procedure**

1. Pipette 0.03ml of each standard or sample into labeled test tubes.
2. Add 1.5ml Coomassie reagent and mix well.
3. Incubate sample for 10mins at room temperature.
4. Set spectrophotometer to 595nm and zero the instrument using water only blank.
5. Prepare a calibration curve by plotting standard concentration against absorbance, use this to determine the protein concentration of the unknown sample.

### Lowry protein concentration assay (Bio-rad, 2007a)

**Standard Assay Protocol**

1. Prepare working reagent by adding 20µl of reagent S to each ml of reagent A required. (stable for 1 week, although precipitate may be present following 24hrs which can be dissipated by mixing)
2. Prepare dilutions of a protein standard from 0.2mg/ml to 1.5mg/ml.
3. Pipette 100µl of standards and samples into clean, dry test tubes.
4. Add 500µl of working reagent A and vortex.
5. Add 4ml of reagent B and vortex.
6. Incubate at room temperature for 15mins and read absorbance at 750nm.

## Appendix 3

## Procedure for packing avidin columns (Pierce_Biotechnology, 2004b)

1. Equilibrate column, 50% gel slurry and buffer solution to room temperature
2. Secure bottom cap on column tip and secure column upright.
3. Add sufficient buffer to the column to fill up to the reservoir portion, tap to dislodge air bubbles.
4. Float porous disc of correct diameter on the buffer, then push to the bottom of the column using the open end of a serum separator.
5. Decant the column, return bottom cap.
6. Add a sufficient volume of gel slurry to obtain desired bed volume.
7. Allow gel to settle for 30mins.
8. Position a second porous disc on the settled gel bed using the serum separator, leaving a gap of 1-2mm from top of column bed.
9. Wash the inner top of the column with buffer, the column is now ready for use or storage.

**Appendix 4**

**Biosafe Coomassie staining (Bio-rad, 2007b)**

SDS-PAGE Gels:

1) Wash gel 3 times for 5 minutes each in 200ml of $ddH_20$ per gel.
2) Remove all water from the container and add 50ml Bio-Safe Coomassie Stain (or enough to completely cover gel).
3) Gently shake for 1hr.
4) Protein bands will be visible within 20 minutes and reach maximum intensity within 1hr.
5) Rinse gel in 200ml of ddH20 for at least 30 minutes.
6) Store gel in water.

Note: Residual SDS in the gel may cause background staining and interfere with band intensity. Rinsing the gel extensively after staining will decrease background.

**Appendix 5**

**Silver stain protocol adapted from (Shevchenko et al., 1996).**

1. After electrophoresis, fix gel in 50% methanol, 5% acetic acid in water for 20min.
2. Wash gel for 10min with 50% methanol in water and additionally for 10min with water to remove the remaining acid.
3. Sensitized gel by 1min incubation in 0.02% sodium thiosulfate.
4. Rinse with two changes of distilled water for 1min each.
5. Submerge gel in chilled 0.1% silver nitrate solution and incubate for 20min at 4°C.
6. After incubation, discard the silver nitrate and rinse gel twice with water for 1min.
7. Develop the gel in 0.04% formalin [35% formaldehyde in water (Merck, Darmstadt)] in 2% sodium carbonate with intensive shaking.
8. After the developer turns yellow, discard and replace with a fresh solution.
9. After the desired intensity of staining is achieved, terminate development by discarding the reagent and washing the gel slab with 5% acetic acid.
10. Silver-stained gels can be stored in a solution of 1% acetic acid at 4°C until analyzed.

**Appendix 6**

**Protocol for isolation of plasmid DNA (Qiagen, 2005b)**

1. Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 2-5ml LB medium containing the appropriate selective antibiotic. Use a tube or flask with a volume of at least 4 times the volume of the culture. Incubate for ~8 h at 37°C with vigorous shaking (~300 rpm).

2. Dilute the starter culture 1/500 to 1/1000 into selective LB medium. For high-copy plasmids, inoculate 25ml or 100ml medium. Use a flask or vessel with a volume of at least 4 times the volume of the culture. For low-copy plasmids, inoculate 100ml or 500ml medium. Grow at 37°C for 12-16hrs with vigorous shaking (~300 rpm). The culture should reach a cell density of approximately 3-4 x $10^9$ cells per ml, which typically corresponds to a pellet wet weight of approximately 3g/litre medium

3. Harvest the bacterial cells by centrifugation at 6000 x g for 15min at 4°C. Remove all traces of supernatant by inverting the open centrifuge tube until all medium has been drained. Pellets can be stored at -20°C for later processing.

4. Resuspend the bacterial pellet in 4ml or 10ml Buffer P 1. For efficient lysis, it is important to use a vessel that is large enough to allow complete mixing of the lysis buffers. Ensure that RNase A has been added to Buffer Pl. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

5. Add 4ml or 10ml Buffer P2, mix gently but thoroughly by inverting 4-6 times and incubate at room temperature for 5min. Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Do not allow the lysis reaction to proceed for more than 5min. After use, the bottle containing

**Appendix 6 contd.**

Buffer P2 should be closed immediately to avoid acidification from $CO_2$ in the air.

6. Add 4ml or 10ml of chilled Buffer P3, mix immediately but gently by inverting 4-6 times and incubate on ice for 15min or 20min. Precipitation is enhanced by using chilled Buffer P3 and incubating on ice. After addition of Buffer P3, a fluffy white material forms and the lysate becomes less viscous. The precipitated material contains genomic DNA, proteins, cell debris and SOS. The lysate should be mixed thoroughly to ensure even potassium dodecyl sulfate precipitation. If the mixture still appears viscous and brownish, more mixing is required to completely neutralize the solution.

7. Centrifuge at >20,000 x g for 30min at 4 °C. Remove supernatant containing plasmid DNA promptly. Before loading the centrifuge, the sample should be mixed again. Centrifugation should be performed in non-glass tubes (e.g., polypropylene). After centrifugation, the supernatant should be clear. Centrifuge the supernatant again at > 20,000 x g for 15min at 4°C. Remove super-natant containing plasmid DNA promptly. Remove a 240µl or 120µl sample from the cleared lysate supernatant and save for an analytical gel (sample 1) in order to determine whether growth and lysis conditions were optimal.

8. Equilibrate a QIAGEN-tip 100 or QIAGEN-tip 500 by applying 4ml or 10ml Buffer QBT and allow the column to empty by gravity flow. Allow the QIAGEN-tip to drain completely. QIAGEN-tips can be left unattended, since the flow of buffer will stop when the meniscus reaches the upper frit in the column.

9. Apply the supernatant from step 8 to the QIAGEN-tip and allow it to enter the resin by gravity flow. The supernatant should be loaded onto the QIAGEN-tip promptly. If it is left too long and becomes cloudy due to further precipitation of

protein, it must be centrifuged again or filtered before loading to prevent clogging of the QIAGEN-tip. Remove a 240µl or 120µl sample from the cleared lysate supernatant and save for an analytical gel (sample 2) in order to determine the efficiency of DNA binding to the QIAGEN Resin.

10. Wash the QIAGEN-tip with 2 x 10ml or 2 x 30ml Buffer QC. Allow Buffer QC to move through the QIAGEN-tip by gravity flow. The first wash is sufficient to remove all contaminants in the majority of plasmid DNA preparations. The second wash is especially necessary when large culture volumes or bacterial strains producing large amounts of carbohydrates are used. Remove a 400µl or 240µl sample from the combined wash fractions and save for an analytical gel (sample 3).

11. Elute DNA with 5ml or 15ml Buffer QF. Collect the eluate in a 10ml or 30ml tube. Use of polycarbonate centrifuge tubes is not recommended, as polycarbonate is not resistant to the alcohol used in subsequent steps. Remove a 100µl or 60µl sample of the eluate and save for an analytical gel (sample 4).The protocol can be stopped here and continued later, store the eluate at 4°C. Storage periods longer than overnight are not recommended.

12. Precipitate DNA by adding 3.5ml or 10.5ml (0.7 volumes) room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at ~ 15,000 x 9 for 30 min at 4°C. Carefully decant the supernatant. All solutions should be at room temperature in order to minimize salt precipitation, although centrifugation is carried out at 4°C to prevent overheating of the sample. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be more easily located.

**Appendix 6 contd.**

Isopropanol pellets are also more loosely attached to the side of the tube and care should be taken when removing the supernatant.

13. Wash DNA pellet with 2ml or 5ml of room-temperature 70% ethanol and centrifuge at > 15,000 x g for 10 min. Carefully decant the supernatant without disturbing the pellet. The 70% ethanol removes precipitated salt and replaces isopropanol with the more volatile ethanol, making the DNA easier to re-dissolve.

14. Air-dry the pellet for 5-10 min and re-dissolve the DNA in a suitable volume of buffer (e.g., TE buffer, pH 8.0, or 10 mM TrisCl, pH 8.5). Re-dissolve the DNA pellet by rinsing the walls to recover the entire DNA, especially if glass tubes have been used. Pipetting the DNA up and down to promote resuspension may cause shearing and should be avoided. Over drying the pellet will make the DNA difficult to re-dissolve. DNA dissolves best under slightly alkaline conditions; it does not easily dissolve in acidic buffers.

## Appendix 7

### Protocol for transfection of cells using FuGENE 6 reagent (Roche, 2005a).

1. Prepare the cells the day before transfection.
2. Ensure the cells are growing well and are in log phase. Occasionally cells slow down when they reach confluence and it can take a day or two to recover.
3. For monolayers, plate the cells so they will only be 50-80% confluent the following day.
4. Plate the cells in medium that does not contain antibiotics.
5. For most adherent cell lines, plate 100,000-300,000 cells in 2 ml medium (50,000 to 150,000 cells/ml) in one well of a 6-well plate Plan to test at least 3 ratios of reagent to DNA See the table below for a suggested layout of a 6-well plate

| **Cell control** | **Reagent control** | **DNA control** | **3:1 ratio** | **3:2 ratio** | **6:1 ratio** |
|---|---|---|---|---|---|
| No reagent<br>No DNA | 6µl reagent<br>No DNA | No reagent<br>1µg DNA | 3µl reagent<br>1µg DNA | 3µl reagent<br>2µg DNA | 6µl reagent<br>1µg DNA |

6. Select a plasmid that you know transfects your cell line and make sure you are able to measure the expression of reporter gene or transfected protein
7. Handle the DNA as aseptically as possible to avoid contaminating your cell cultures during the transfection.
8. Determine the concentration of the DNA with a spectrophotometer. Transfection-grade DNA should have a 260/280 ratio of 1.8.
9. Check the stock DNA concentration, if you need to dilute the DNA, use sterile DNase-free water or sterile DNase-free TE buffer as the diluent
10. Calculate accordingly for your DNA concentrations.
11. Prepare the FuGENE 6 Transfection Reagent and allow it to warm to room temperature.
12. Meanwhile, ensure cells look healthy and are 50-80% confluent.

**Appendix 7 contd.**

13. Warm serum-free DMEM (no additives) to room temperature and aliquot 94-100 µl into each of 6 tubes as indicated below. Use sterile Eppendorf tubes or small sterile polystyrene tubes labelled;

- A (100µl)
- B (94µl)
- C (100µl)
- D (97µl)
- E (97µl)
- F (94µl), corresponding to the plate layout above.

14. Mix the room-temperature FuGENE 6 Reagent by tapping or briefly vortexing (1 sec).
15. Carefully add the FuGENE 6 Reagent to the serum-free medium as follows;

- 6µl toTube B
- 3µl to Tube D (3:1 ratio)
- 3µl to Tube E (3:2 ratio)
- 6µl to Tube F (6:1 ratio)

16. Ensure the entire amount of reagent is delivered directly from the pipette tip into the serum-free medium. Avoid contact of the FuGENE 6 Transfection Reagent with the walls of the tube containing the serum-free medium. To avoid a FuGENE 6 Reagent layer on top of the serum-free medium as soon as the FuGENE 6 Reagent is added, immediately tap, flick, or vortex for 1 second to ensure adequate mixing of the components.
17. Incubate at room temperature for 5 minutes.
18. Add the DNA to each tube (see table above) the amount added will depend on the starting concentration of DNA.
19. Mix by tapping or vortexing the tube for 1 second

**Appendix 7 contd.**

20. Incubate for at least 15 minutes at room temperature. Longer complex incubation times of 30-60 minutes may enhance transfection efficacy.
21. Add 30µl FuGENE 6 Reagent to 970µl serum-free medium. Mix, then incubate for 5 minutes.
22. Add 10µg DNA to the diluted FuGENE 6 reagent mix, incubate for 15 minutes and then start adding 100µl aliquots to cells at 15-minute intervals.
23. A slight haze may be visible if the transfection complex is in clear tubes
24. Remove the cells from the incubator.
25. Add 100µl of complex drop-wise to its designated well as in the plate outline above swirl the plate to ensure mixing of the complex in all of the wells.
26. Return the cells to the incubator.
27. Incubate the cells for 1-3 days prior to measuring gene expression the incubation period is dependent on the reporter gene in
28. The plasmid if the plasmid contains a selectable marker, do not add the selection antibiotic until the first post-transfection passage.

## Appendix 8

## Immunoprecipitation With Soluble Antibodies (BD Biosciences, 2004)

PREPARATION OF THE CELL LYSATE

**Denaturing Conditions**

1. Rinse a 60mm culture dish of confluent cells with 1x phosphate-buffered saline (PBS).
2. Lyse the cells with 0.5ml boiling lysis buffer (1% SDS, 1.0mM sodium ortho-vanadate, 10mM Tris pH 7.4).
3. Scrape the cells from the dish, transfer lysate to a 1.5ml microcentrifuge tube, and boil for an additional 5 minutes.
4. Pass several times through a 26 gauge needle; centrifuge (16,000 x g) for 15 minutes. The supernatant is the total cell lysate (denatured).

**Native Conditions**

1. Rinse a 60mm culture dish of confluent cells with PBS.
2. Lyse the cells with 0.5ml cold immunoprecipitation buffer (1% Triton X-100, 150mM NaCl, 10mM Tris pH 7.4, 1mM EDTA, 1mM EGTA pH 8.0, 0.2mM sodium ortho-vanadate, protease inhibitor cocktail (Boehringer Mannheim), 0.5% IGEPAL CA-630).
3. Maintain constant agitation for 30 minutes at 4°C.
4. Scrape the cells from the dish and pass several times through a 26 gauge needle to disperse any large aggregates. Centrifuge (16,000 x g, 4°C). for 15 minutes; keep on ice. The supernatant is the "total cell lysate (native).

**Pre-clearing**

It is important to pre-clear the lysate immediately before immunoprecipitation.

1. Centrifuge the cell lysates (16,000 x g, 4°C) for 15 minutes. Remove the supernatant.
2. For the denatured lysates, boil the supernatant for 5 minutes.
3. To 750-1000µl of supernatant, add 5µg of rabbit anti-mouse IgG antibody , vortex, then add 75-100µl of Protein A:Agarose (Life Technologies). Incubate at 4°C for 30 minutes with agitation.
4. Centrifuge lysate (9000 x g, 4°C). for 2 minutes to pellet the agarose beads. The supernatant is the "total cell lysate".

IMMUNOPRECIPITATION

1. To a microcentrifuge tube, add 1–5µg of antibody, 400µl of water, 200-500µg of total lysate and 500µl of 2X immunoprecipitation buffer (2% Triton X-100, 300mM NaCl, 20mM Tris pH 7.4, 2mM EDTA, 2mM EGTA pH 8.0, 0.4mM

sodium ortho-vanadate, protease inhibitor cocktail (Boehringer Mannheim), 1.0% IGEPAL CA-630).

2. Vortex and incubate for one hour with agitation at 4°C. If monoclonal antibodies are used, add 5µg rabbit anti-mouse IgG antibody, vortex, and continue the incubation for an additional 30 minutes at 4°C.
3. Add 50µl of 50% Protein A:Agarose (Life Technologies). Vortex and incubate for 30 minutes with agitation at 4°C.
4. Wash with cold 1X immunoprecipitation buffer (1% Triton X-100, 150mM NaCl, 10mM Tris pH 7.4, 1mM EDTA, 1mM EGTA pH 8.0, 0.2mM sodium ortho-vanadate, protease inhibitor cocktail, 0.5% IGEPAL CA-630). by centrifuging 2 minutes (8000 x g, 4°C). Decant supernatant and repeat wash twice.
5. Resuspend pellet in 50µl of 0.1 Glycine pH 2.5 vortex and incubate with agitation for 10 minutes at 4°C.
6. Centrifuge (9000 x g, 4°C) for 2 minutes. Remove supernatant, this is your IP sample.
7. Add 5µl of 1M Tris pH 8.0 to each tube to neutralize the pH. Add approximately 10µl of 5X concentrated electrophoresis sample buffer (125mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% ß-mercaptoethanol) to each sample, and boil for 5 minutes.
8. Load the supernatant onto an SDS-PAGE gel and electrophorese.
9. Transfer to PVDF and probe with appropriate antibodies.

## Appendix 9

**Manufacturers protocol for the running of Immobiline IEF gels** (Amersham, 2005a)

Typical running condition for a whole Ampholine PAGplate pH 3.5-9.5 at 7°C:

| | | | |
|---|---|---|---|
| Max. 1700 V | 50 mA | 30 W | 2 hours 15 min |

For narrower gradients, e.g. pH 4.0 to 6.5, the separation time must be prolonged, since the proteins with a low net charge must migrate long distances. Hydrophobic proteins need the presence of 8M urea to stay in solution. Because of the buffering capacity of urea, there is a light increase in the pH in the acid part of the gel. High urea contents in the gel lead to structural changes in many proteins and disruption of the quaternary structure. The solubility of very hydrophobic proteins, such as membrane proteins for example, can be increased by the addition of non-ionic detergents (e.g. Nonidet NP-40, Triton X-100) or zwitterionic detergents (e.g. CHAPS, Zwittergent). Because the gels do not co-polymerize with the support films in the presence of non-ionic detergents, it is recommended to rehydrate a pre-polymerized, washed and dried gel in the relevant solution (see below).

Electrode solutions for IEF in polyacrylamide gels:

| pH Gradient | Anode | Cathode |
|---|---|---|
| 3.5-9.5 | 0.5 mol/L H3PO4 | 0.5 mol/L NaOH |
| 2.5-4.5 | 0.5 mol/L H3PO4 | 2% Ampholine pH 5-7 |
| 2.5-4.5 | 0.5 mol/L H3PO4 | 0.4 mol/L HEPES |
| 3.5-5.0 | 0.5 mol/L H3PO4 | 2% Ampholine pH 6-8 |
| 4.0-5.0 | 0.5 mol/L H3PO4 | 1 mol/L glycine |
| 4.0-6.5 | 0.5 mol/L acetic acid | 0.5 mol/L NaOH |
| 4.5-7.0 | 0.5 mol/L acetic acid | 0.5 mol/L NaOH |

## List of publications resulting from this thesis

**Differential protein synthesis and expression levels in normal and neoplastic human prostate cells and their regulation by type I and II interferons.**

**Nagano, K., Masters, J. R., Akpan, A., Yang, A., Corless, S., Wood, C., Hastie, C., Zvelebil, M., Cramer, R. and Naaby-Hansen, S. (2003) *Oncogene.***

**Abstract**

Protein expression and de novo synthesis in normal and prostate cancer cell lines derived from the same patient were compared by proteomic analysis and the effects of IFN alpha and IFN gamma (IFN=interferon) determined. The expressions of several IFN-inducible proteins, including MxA, Nmi, PA28a and IFP53, were downregulated in the cancer cells. IFN gamma induced a more than twofold increase or decrease in the synthesis rates of almost twice as many proteins in the cancer cell line. The positive regulator of IFN-induced transcription ISGF3gamma was upregulated in the cancer cells and inversely regulated by IFN alpha and IFN gamma in the normal and cancer cells. Moreover, ISGF3gamma's induction by IFN gamma in the cancer cells was more enhanced by simultaneous stimulation with EGF, than its induction in the normal cells. In all, 31 differentially regulated proteins were identified by mass spectrometry analysis, several of which are involved in chaperone-assisted protein folding in the endoplasmic reticulum (ER) or in regulated protein degradation. Our results suggest that the exclusion of proteins by the ER quality control system, crosstalk between the EGF- and IFN-induced signalling pathways and the regulation of IFN-inducible genes are all altered in the prostate cancer cells. The combination of upregulated activity in the growth-promoting PI3K/Akt pathway, suppression of Nmi and overexpression of hnRNP-K and c-myc proteins may explain why the prostate cancer cells were found to be more resistant to the growth inhibitory effects of IFNgamma.

**Combined affinity labelling and mass spectrometry analysis of differential cell surface protein expression in normal and prostate cancer cells**

**Hastie, C., Saxton, M., Akpan, A., Cramer, R., Masters, J. R., Naaby-Hansen, S. (2005)** ***Oncogene.***

**Abstract**

Differences in the expression of cell surface proteins between a normal prostate epithelial (1542-NP2TX) and a prostate cancer cell line (1542-CP3TX) derived from the same patient were investigated. A combination of affinity chromatographic purification of biotin-tagged surface proteins with mass spectrometry analysis identified 26 integral membrane proteins and 14 peripheral surface proteins. The findings confirm earlier reports of altered expression in prostate cancer for several cell surface proteins, including ALCAM/CD166, the Ephrin type A receptor, EGFR and the prostaglandin F2 receptor regulatory protein. In addition, several novel findings of differential expression were made, including the voltage-dependent anion selective channel proteins Porin 1 and 2, ecto-5'-nucleotidase (CD73) and Scavenger receptor B1. Cell surface protein expression changed both qualitatively and quantitatively when the cells were grown in the presence of either or both interferon IFN alpha and IFN gamma. Co stimulation with type I and II interferons had additive or synergistic effects on the membrane density of several, mainly peripherally attached surface proteins. Concerted upregulation of surface exposed antigens may be of benefit in immuno-adjuvant-based treatment of interferon-responsive prostate cancer. In conclusion, this study demonstrates that differences in the expression of membrane proteins between normal and prostate cancer cells are reproducibly detectable following vectorial labelling with biotin and that detailed analysis of extracellular-induced surface changes can be achieved by combining surface-specific labelling with high-resolution two-dimensional gel electrophoresis and mass spectrometry.

**IFNgamma reduces cell surface expression of annexin 2 and suppresses the invasive capacity of prostate cancer cells**

**Hastie, C., Masters, J. R., Moss, S.E., Naaby-Hansen, S. (2006) Submitted *JBC*.**

**Abstract**

The effect of interferon-γ (IFNγ) treatment on cell surface protein expression was compared in a benign epithelial (1542-NP2TX) and prostate cancer (1542-CP3TX) cell line derived from the same patient. While IFNγ increased both the number and abundance of proteins in membrane fractions, the expression of annexin 2 and its binding partner p11 decreased. After 24 h exposure to IFNγ, the surface density of the annexin 2 / p11 complex was reduced four-fold and the remaining proteins localized to lipid rafts. The cytoplasmic expression of annexin 2 was unaffected by cytokine treatment. In the same time scale, IFNγ reduced the abundance of the peripherally attached proteases procathepsin B and plasminogen. The invasiveness of the cancer cells was strongly reduced both following IFNγ treatment and after exposure to an antibody against annexin 2. In contrast, the invasiveness of the annexin 2 negative prostate cancer cells LNCaP was unaffected by IFNγ treatment and up-regulation of annexin 2 expression by transfection increased the invasiveness of LNCaP cells. IFNγ induced calpain expression and activation and increased the phosphorylation and degradation of ABCA1 in 1542-CP3TX cancer cells. Surface expression of annexin 2 was reduced in cells treated with glyburide, an ABCA1 inhibitor, while inhibition of calpain abrogated the IFNγ-induced annexin 2 down-regulation. These data indicate that IFNγ reduces invasiveness by suppressing annexin 2 / p11 -associated pericellular protease activity via down-regulation of lipid transporter activity and that this effect is facilitated by calpain-dependent inactivation of ABCA1 in the 1542-CP3TX cells. The study identifies a new mode-of-action which enables IFNγ to control the activity of cell surface associated hydrolases by regulating the peripheral membrane density and localization of the heterotetrameric annexin 2 / p11 complex.

## List of presentations of the work contained in this thesis

Invited speaker and author at the 2004 Entente Cordiale Proteomics Conference, Paris, on the topic of "*Surface specific downregulation of annexin II by interferon γ*". 5th May 2004.

Invited speaker at the London Prostate Interest Group, Institute of Cancer Research, Fulham, London, on the topic of "*Differential cell surface protein expression on normal and neoplastic human prostate cells and their regulation by interferons*". 7th September 2004.

Invited speaker at the Institute of Biomedical and Biomolecular Sciences, Portsmouth, on the topic of "*Surface specific downregulation of annexin II by interferon γ*". 20th April 2005.

Invited speaker at the NHS liason meeting, University of Portsmouth, on the topic of "*Surface specific downregulation of annexin II by interferon γ*". 16th June 2005.

Invited speaker at the Androgens Conference 2006, University of Cambridge, on the topic of "*INF, a therapeutic option for late stage prostate cancer?*". 26th September 2006.