Investigating the role of iASPP in normal prostate development and prostate tumourigenesis

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Declaration

All the work presented in this thesis is the result of my own work unless otherwise stated and does not constitute part of any other thesis. The work herein described was carried out while I was a graduate student of the Nuffield Department of Surgical Sciences working at the Ludwig Institute for Cancer Research, under the supervision of Professor Freddie Hamdy and Professor Xin Lu.

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Abstract

Loss of TP63 expression in the prostate epithelium is a hallmark of invasive prostate cancer. An inability of prostate epithelial cells to undergo apoptosis, a process regulated by both TP63 and TP53, is a feature of their malignant transformation. *p53* mutations in prostate cancer are uncommon, being mainly associated with advanced metastatic disease. Therefore it is likely that the inability of wild-type *p53* to initiate and execute apoptosis is due to molecular alterations elsewhere in the apoptotic pathway. iASPP is an inhibitory member of the ASPP family of proteins and is known to inhibit p53-mediated apoptosis and regulate *p63* function.

In this work we have investigated how iASPP can affect normal prostate development and prostate tumourigenesis. By utilising an iASPP-deficient mouse model, we show that iASPP plays a key role in normal prostate development and homeostasis by maintaining the TP63-positive basal cell lineage of the prostate epithelium. The loss of iASPP is associated with alterations in differentiation, proliferation and apoptosis, resulting in a smaller organ size. Furthermore, by using both benign and malignant human prostate tissue we show that changes in iASPP expression have prognostic value. Nuclear and cytoplasmic iASPP expression are significantly increased in prostate cancer and associate with poor clinical outcome. Increased nuclear iASPP associates with cells that express high levels of *TP53*, suggesting that iASPP cooperates with mutant *p53* to enhance its gain-of-function activity. Analysis of iASPP expression in both the mouse and human prostate epithelium led to the identification of iASPP as a novel junctional protein. *In vitro* studies suggest that membranous iASPP functions to add strength and stability to cell-to-cell contacts, and its' reduction increases cellular invasion and motility. Finally, iASPP significantly reduces chemosensitivity of prostate cancer cells, given the evident dual functionality of iASPP as both an oncoprotein and a tumour suppressor, depending on cellular localisation, targeting this protein to improve therapy efficacy may be challenging.

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Abbreviations

AMACR	α-methylacyl coenzyme A racemase
ΔN	Delta N
AR	Androgen receptor
ASPP1	Apoptotic stimulating protein of p53 1
ASPP2	Apoptotic stimulating protein of p53 2
BPH	Benign prostatic hyerplasia
CARN	Castrate-resistant Nkx3.1 expressing cells
Ck	Cytokeratin
CRPC	Castrate-resistant prostate cancer
DBD	DNA binding domain
DHT	Dihydrotestosterone
EMT	Epithelial-to-mesenchymal transition
H&E	Heamotoxylin and Eosin
iASPP	Inhibitory apoptotic stimulating protein of p53
LOH	Loss of heterozygosity
MMP	Matrix metalloproteinase
NAP	Normal adjacent prostate
NE	Neuroendocrine
NED	Neuroendocrine differentiation
OD	Oligomerisation domain
OS	Overall survival
PAP	Prostatic acid phosphatase
PCSS	Prostate cancer-specific survival

PI	Propidium iodide
PIN	Prostate intraepithelial neoplasia
PSA	Prostate specific antigen
RAI	RELA/p65 associated inhibitor
SASP	Senescence associated secretory phenotype
siRNA	Small interfering RNA
TA	Transactivation domain
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UGM	Urogential sinus mesenchyme
UGS	Urogential sinus
UV	Ultraviolet

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Chapter 1: Introduction

1.1 A brief introduction to tumourigenesis

Tumourigenesis is the process by which normal cells transform into tumour cells, resulting in a group of heterogeneous pathologies known as cancer. Tumour cells are somatic cells that have acquired mutations in genes that encode for proteins that are normally responsible for regulating cell division, differentiation and survival. Dysregulation of these systems causes cells to proliferate uncontrollably resulting in the formation of tumours (Hanahan and Weinberg, 2000). Tumour progression is a multi-step process which begins with the acquisition of behavioural changes by a single cell (Nowell, 1976), or two or more different progenitors or clones of cells cooperating in the genesis of the tumour resulting in growth advantages compared to the normal cell population (Parsons, 2008). These properties are transmitted to the descendent cells which can then undergo further rounds of genetic changes. The tumour cells are then able to prevail over those cells that do not carry an advantageous mutation leading to rapid clonal expansion into the host tissue. As well as mutations that enhance cell survival and proliferation, mutations can arise that increase a tumour cells' ability to invade surrounding tissue, reaching the circulatory and lymphatic system, where they can be transported to and proliferate in different tissues around the body, allowing the formation of secondary tumour foci; this process is known as metastasis. It is this presence of distant metastases that accounts for 90% of human cancer deaths (Sporn, 1996, Mehlen and Puisieux, 2006). In order for normal cells to progress to invasive, metastatic tumour cells certain genetic characteristics must be acquired; these are referred to as the hallmarks of tumourigenesis which have been classified in six main categories. These hallmarks are self-sufficiency in growth signals, insensitivity to anti-growth signals,

limitless replicative potential, evasion of apoptosis, sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg, 2000). Recently, two additional hallmarks have been added to the list namely the capacity of tumour cells to reprogram their energy metabolism and to avoid immune destruction. To facilitate the acquisition of these hallmarks the tumour has to possess enabling traits such as intrinsic genomic instability and the ability to promote inflammation (Hanahan and Weinberg, 2011).

Although all cancers are a product of an accumulation of genetic alterations, these DNA mutations can be inherited through parental genes (familial or hereditary cancer) or can arise during life (non-inherited cancer). Often, cancer can be attributed to a combination of both inherited and non-inherited mutations.

Genes that are affected by mutation in the context of cancer development are known as cancer-critical genes. These can be divided into two main classes; proto-oncogenes and tumour suppressors. Proto-oncogenes are genes that normally participate in cell proliferation, differentiation and survival pathways, these mutated genes are known as “oncogenes” and are characterised by a “gain of function” dominant phenotype (Monier, 1990, Karayi and Markham, 2004). This means that mutations in only one of the two alleles can result in alterations of cellular behaviour (Croce, 2008). Conversely, tumour suppressor genes usually have “loss of function” and display a recessive phenotype (Karayi and Markham, 2004). Unlike oncogenes both alleles usually need to be affected for there to be a marked change in cellular behaviour (Stanbridge, 1990). However, some tumour suppressor genes have been shown to require the inactivation of only one allele to confer a selective advantage towards tumour growth. This condition is called haploinsufficiency and can be attributed to a reduction in the gene dosage of the tumour suppressor (Cook and McCaw, 2000).

The molecular pathways by which normal cells acquire a tumorigenic phenotype are extremely diverse. Even within an individual case of cancer, different clonal populations of cells may show different mutations. Moreover, the gain of mutations in specific oncogenes and/or tumour suppressor genes, whether early in some tumours or late in others, may determine the stages at which other tumorigenic hallmarks are acquired. The one common factor is that all human cancers with progressing degrees of malignancy share these biological endpoints, regardless of how they are ultimately reached.

1.2 The Prostate

1.2.1 Prostate function

The normal adult prostate is a walnut-sized exocrine gland that is a component of the male reproductive tract. It is located just below the bladder and surrounds the urethra. The function of the prostate is to store and secrete a slightly acidic fluid that is mixed with the alkaline secretions from the seminal vesicles to give an overall alkaline pH of around 7.2-8.0. Secretions from the prostate usually constitute 25-30% of the total volume of semen. Seminal fluid is generally composed of simple sugars, calcium, zinc, citric acid and proteolytic enzymes such as prostatic acid phosphatase (PAP) and prostate specific antigen (PSA). PSA is an enzyme which is produced primarily by cells lining the acini and ducts of the prostate gland and functions to liquify semen. The function of the seminal fluid is to protect the genetic material (DNA) of the spermatozoa by aiding sperm motility and promoting their survival within the acidic vaginal tract. The prostate also contains a system of complex valves which, during ejaculation, direct semen into the urethra, while the urethral sphincter contracts and seals off the bladder to prevent semen from retrogradely entering the bladder. Some prostatic secretions also protect the urinary tract and reproductive system from harmful bacteria that may enter the urethra. This protective element of prostate function may be why all mammals have a prostate gland owing to the fact that mammals have a long reproductive period. The prostate gland also contains the crucial enzyme 5-alpha-reductase. This enzyme converts testosterone in to its more potent form dihydrotestosterone (DHT). DHT is the primary driver of androgen receptor (AR) activation and transcriptional activity, which is essential for normal growth, development and maintenance of the prostate. Dysregulation of AR signalling is a critical step in the progression of prostate tumourigenesis.

1.2.2 Prostate Structure

The adult prostate is composed of glandular epithelial and fibromuscular stroma compartments. Both components turn over slowly with balanced levels of cell proliferation and cell death. It has been demonstrated that normal prostatic epithelial cells, in a steady-state self-renewing condition in which there is undetectable net prostatic growth, have a low apoptotic index (1.9%) which is coupled to a relatively low proliferative index (1.1%) (Tu et al., 1996). These levels maintain a tissue homeostatic equilibrium so that neither over-growth nor involution of the gland normally occurs.

In 1981 McNeal developed an anatomical model to define the different areas of the prostate; by dividing the glandular component of the prostate into four anatomically distinct zones; termed the peripheral, transitional, central and anterior (figure 1.1A). The peripheral zone contains nearly 75% of the prostate glands. The transitional zone contains the majority of the remaining glands. These zones can be distinguished only by their anatomical relationship and the composition of their stromal elements, as they share a similar acinar structure reflecting the common origin from the urogenital sinus. The central zone consists of morphologically distinct glands which are far smaller than the glands of the peripheral and transitional zones (McNeal, 1981) this is possibly due to their embryonic origin from the Wolffian duct which is of mesodermal origin (Schulz et al., 2003). The anterior zone contains no glands and is composed of fibromuscular stroma. It forms the entire anterior surface of the prostate as a thick covering, shielding from view the other three glandular regions. Around 60-70% of prostate cancers occur in the peripheral zone, 10-20% in the transitional zone and 5-10% in the central zone.

Another condition that is thought to have no malignant potential is benign prostatic hyperplasia (BPH) which occurs mainly in the transitional zone (McNeal, 1981, Schulz et

al., 2003). BPH causes an enlargement of the prostate owing to continuous slow prostate growth; as the prostate grows a layer of tissue surrounding it stops it from expanding, causing the gland to press against the urethra, like a clamp on a garden hose, impacting on normal urination. There are a number of growth phases in the natural history of the prostate. The first occurs during fetal development, the second induced at puberty, and the third begins at around 25 years of age and can continue indefinitely. It has been hypothesised that the signalling pathways that are activated during fetal prostate development are re-awakened causing induction of these further phases of growth and can result in the development of prostatic diseases such as BPH (Schaeffer et al., 2008, Pritchard and Nelson, 2008).

Unlike the human prostate the mouse prostate consists of four distinct lobes termed anterior, dorsal, lateral and ventral lobes (figure 1.1B). Each lobe was named to reflect their anatomical aspect in regards to the prostatic urethra, from which they branch during development. They all have distinct patterns of ductal branching which appear histologically different. They also have differences in cell morphology, gene expression and secretory protein expression (Cunha et al., 1987). The dorsal and lateral lobes are often analysed as one entity owing to their common origin from the dorsolateral aspect of the endodermal urogenital sinus (UGS). An analysis of gene expression profiling data has shown that the dorsolateral lobe of the mouse prostate is the most analogous to the human peripheral zone (Berquin et al., 2005).

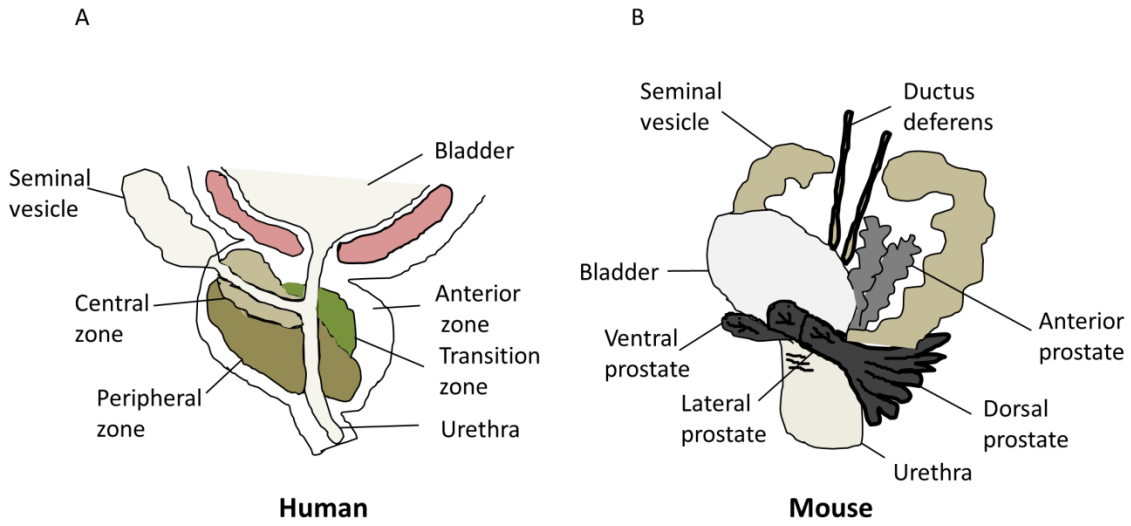

Figure 1.1 The anatomy of the human and mouse prostate

(**A)** The human prostate consists of four anatomically different zones. The peripheral zone contains 75% of the prostate glands, transition zone contains the majority of the remaining glands and the central zone contains a number of smaller glands surrounded by a compartment of fibromuscula stroma. (**B)** The mouse prostate consists of four distinct lobes termed the anterior lobe and the ventral, lateral and dorsal lobes which surround the urethra

At the histological level the human and mouse prostate glands are composed of ducts which are comprised primarily of: luminal and basal cells along with a third smaller subpopulation of neuroendocrine cells (NE) (figure 2). The luminal cells are characterised by the expression of cytokeratins (Ck) 8 and 18 and are androgen receptor (AR) positive. The basal cells are characterised by the expression of Ck5 and 14 and are p63 positive with low or undetectable levels of AR. There are two types of NE; open and closed which refers to their contact with the glandular lumen. They are both characterised by the expression of synaptophysin and Chromogranin A and are located scattered within the luminal layer. Although there are three distinct cell types within prostatic epithelium, there are also cells that are in a transitional state known as intermediate cells. These cells are basal cells that have entered into a program of differentiation and are phenotypically between basal and luminal cells. They are characterised by the expression of both luminal and basal cell markers as well as their own marker, Ck19. All three cell types are thought to be derived from a common pluripotent progenitor/stem cell in the basal compartment. (Wang et al., 2001a, Xue et al., 1997). The differentiation process of the prostate epithelium is illustrated in (figure 1.3). It is postulated that all three cell types arise from a population of intermediate stem cells. These cells give rise to NE cells and basal cells. Basal cells may then move along the differentiation program to an intermediate cell phenotype and finally to a terminally differentiated luminal cell.

The stromal compartment is the final element that contributes to the composition of the prostate gland. It is composed of fibroblasts, smooth muscle cells, endothelial cells, dendritic cells, nerves and some infiltrating cells such as mast cells and lymphocytes. Some stromal cells are androgen responsive and produce growth factors that act in a

paracrine fashion on the epithelial cells. This stromal–epithelial crosstalk is an important regulator of the growth, development and hormonal responses of the prostate.

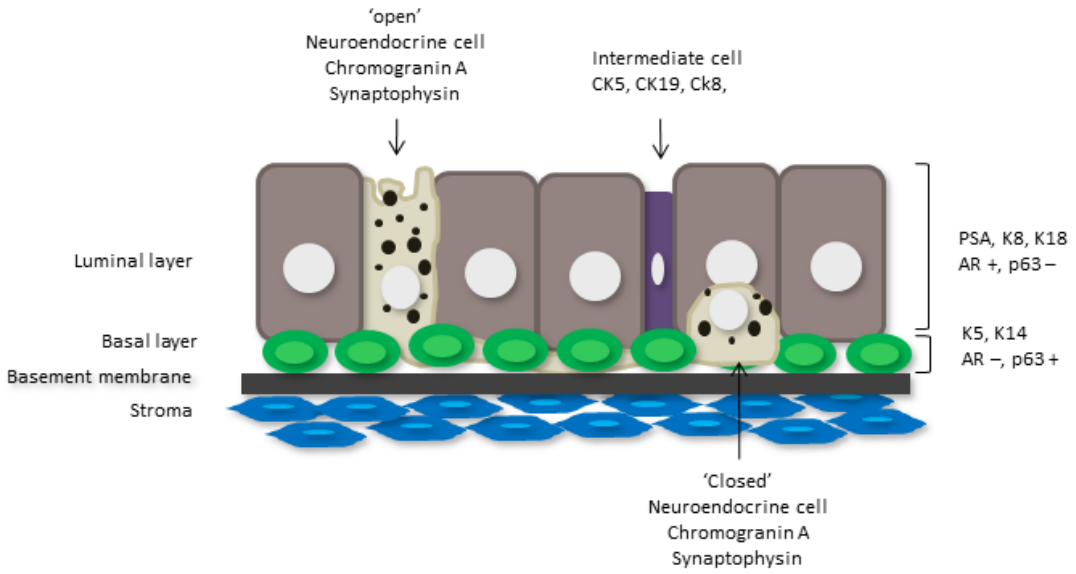

Figure 1.2 A schematic representation of the different cells of the human prostate gland

The luminal layer shown in brown is characterised by the expression of the androgen receptor (AR), Ck8 and 18, and the secretion of prostate-specific antigen (PSA) and it is p63-negative. The basal layer shown in green is characterised by the expression of Ck5 and 14 and is p63-positive. The intermediate cell shown in purple between the luminal cells is characterised by the expression of both luminal and basal cell markers as well as Ck19. The NE cells shown in cream secrete a number of hormones and neurotransmitters such as Serotonin, Chromogranin A and Synaptophysin, NE cells can be characterised as either 'open' which is depicted on the left hand side and 'closed' as depicted on the right hand side.

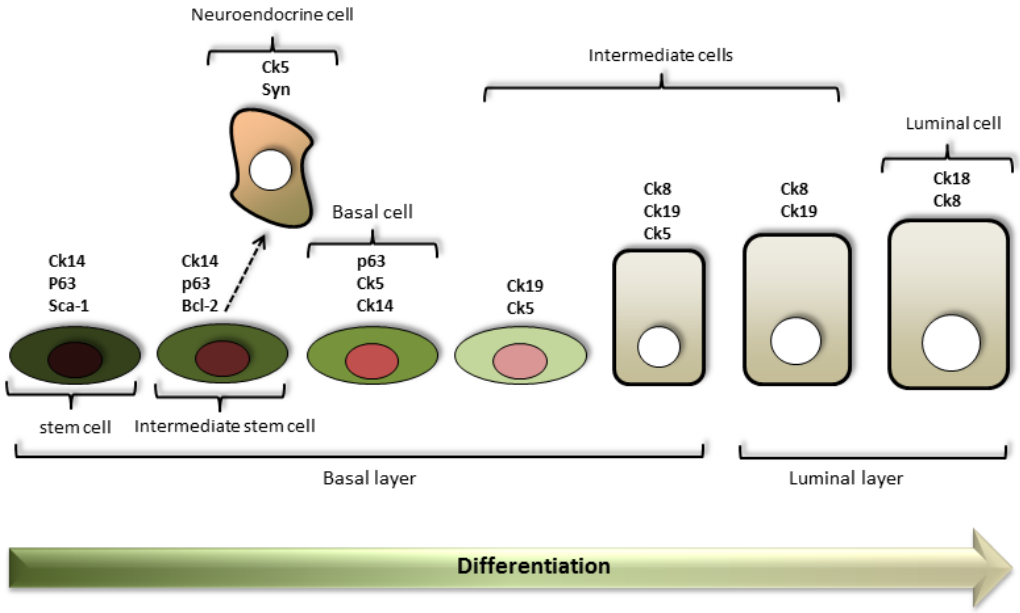

Figure 1.3 The differentiation process in the developing prostate

All three cell types found in the developing prostate; basal, luminal and NE cells are thought to arise from a common progenitor, most probably an intermediate stem cell in the basal layer. As cells move along the differentiation program they express different cytokeratin markers. The parental stem cell is characterised by expression of Ck14 and p63 as well as the cell surface marker stem cell antigen 1 (Sca-1). The intermediate stem cells also express Ck14 and p63 and these cells may give rise to a highly proliferative basal cell that expresses Ck5 and Ck14 as well as p63, or a NE cell that also expresses markers of basal cells such as Ck5 as well as NE markers such as synaptophysin. The basal cells can then progress along the differentiation program becoming intermediate cells, losing the expression of Ck14 and p63, and instead beginning to express Ck19. As they move towards a more luminal cell phenotype they lose the expression of Ck5 and start to express Ck8. Finally terminally differentiated luminal cells lose the expression of Ck19 and express both Ck8 and Ck18.

One fundamental difference between human and mice with regards to the architecture of the prostate is the ratio of basal to luminal cells. In humans the ratio is approximately 1:1 whereas in the mouse it is around 1:7. In the human the basal cells form a continuous layer along the basement membrane separating the luminal cells from any interaction with the stroma. However, in the mouse the basal cells are more scattered allowing the luminal cells and stroma to interact. It has been demonstrated that the epithelial-stromal interactions are a crucial part of prostate development, growth and homeostasis, and this raises the intriguing possibility that this difference influences the biology and cellular cross-talk in the mouse prostate compared to the human (El-Alfy et al., 2000). Another observed difference is the ratio of epithelial to stromal cells; in the human the ratio is around 1:5 whereas in the adult mouse there are approximately equal numbers. There is evidence that when these ratios are met during development, prostate growth ceases preventing overgrowth, even in the presence of abnormally high levels of testosterone. While there are some key differences between the prostate of mice and humans, the mouse prostate is nevertheless thought to represent a useful model for the study of human prostate development and carcinogenesis (Roy-Burman et al., 2004).

1.2.3 Prostate Development

The prostate is an endodermal tissue that begins to develop from the UGS at about 10 weeks of gestation in humans and the 17th day of gestation in mice. In humans the ducts grow rapidly in length, branch and canalise; by 13 weeks there are approximately 70 primary ducts. However in the mouse, branching of the prostatic ducts occurs mainly postnatally, essentially continuing until sexual maturity at around 6 weeks of age (Cunha et al., 1987). Prostatic development is dependent upon reciprocal mesenchymal-epithelial interactions (Cunha, 1994). AR in the urogenital sinus mesenchyme (UGM) is stimulated by testicular androgens to initiate epithelial budding, proliferation, and differentiation to form ductal structures (Cunha et al., 1987, Hayward and Cunha, 2000). Testosterone stimulates the budding of the prostate epithelium, and in turn, causes the developing epithelium to produce growth factors such as sonic hedgehog which activate the underlying mesenchyme (Podlasek et al., 1999). During branching morphogenesis the UGM expresses high levels of AR. At this point in development the epithelium has almost undetectable levels of AR. Thus androgens are required to act on mesenchyme cells suggesting paracrine signalling is necessary for prostate induction. However, during maturation of the gland, the AR appears in the luminal secretory cells. Therefore, in the adult, androgens act on stromal and secretory epithelial cells (Cunha et al., 1987). The prostate has also been shown to regress in response to androgen withdrawal, while subsequent restoration of systemic androgen promotes regeneration. Androgen receptors in the stroma regulate these processes (Kurita et al., 2001). Interestingly, in male seasonal breeders such as the ram, the prostate undergoes a cycle of regression followed by regeneration in response to seasonal changes in systemic androgens, suggesting the presence of a stem cell population within the epithelium.

1.3 Prostate Cancer

1.3.1 Overview

Prostate cancer is the second most common male cancer worldwide, and the most common in the UK. In 2009 there were 40,841 men with prostate cancer and over 10,000 prostate cancer-related deaths in the UK. Statistics show however that the five year survival rate is high at around 81% and this has been attributed to early detection methods, notably prostate specific antigen (PSA) screening (www.cancerresearchuk.org). PSA is a kallikrein-related serine protease that is produced in normal prostate secretions. An elevated PSA level can indicate that the numbers of cells that produce PSA have increased, or that there has been a change in prostate architecture allowing PSA to escape into the bloodstream (Lilja et al., 2008). Men who have elevated PSA levels are typically assessed for prostate cancer by histopathological examination of prostate tissue taken by biopsy. Prostate cancer tissue is classified by Gleason Grade. The tumour is scored 1 to 5 (most to least differentiated) based on its most prevalent architecture, it is then given a second score of 1 to 5 for its second most common pattern. The sum of these two scores gives the Gleason Sum Score (Gleason, 1966). Patients with prostate cancer are also staged with the primary tumour ranging from organ-confined to fully invasive (T1-4), with or without lymph node involvement (N0 or 1) and with or without distant metastases (M0 – M1a-c) (Ohori et al., 1994). If at detection the disease is completely localised within the capsule of the prostate gland it can be cured by definitive local therapy such as radical prostatectomy or radiotherapy. If the cancer is non-organ confined, extending outside of the prostate capsule into adjacent tissue the disease is more difficult to treat. If the cancer has metastasised to distant sites e.g. bone then it is not curable, and treatment aims to control the malignancy and palliate any symptoms.

The transition from organ confined to non-organ confined disease is thought to be partly due to a process of epithelial-to-mesenchymal transition (EMT). EMT is a normal physiologic process during embryogenesis by which cells of epithelial origin convert into cells bearing mesenchymal characteristics; these include the reactivation of developmental genes and the down regulation of a number of junctional proteins. These changes in gene expression allows the cells to invade through the surrounding stroma, in a similar fashion to cells during development, as they organise themselves into different parts of the body to form the correct anatomical arrangement for each particular organ. It has been proposed that EMT is co-opted by cancer cells during their metastatic dissemination from the primary site in the prostate to secondary sites around the body (Nauseef and Henry, 2011). The extent at which this recapitulates physiological EMT remains unclear.

Along with surgery and radiotherapy, androgen deprivation therapy is one of the main treatment options for patients with prostate cancer. Prostate cancer cells are initially dependent on androgens for growth and survival through their interactions with the AR. In 1941, Huggins and Hodges demonstrated that by removing androgen stimulation by surgical castration resulted in tumour regression (Huggins and Hodges, 1941). However, androgen depletion is not a cure and recurrence is usually inevitable. Patients with metastatic disease have a median progression-free survival of around 12 to 30 months after treatment is initiated (Hellerstedt and Pienta, 2002, Diaz and Patterson, 2004). For those cells that are sensitive to androgens, testosterone depletion causes the expression of death-signalling genes that are repressed by activated AR, therefore androgen ablation allows these genes to be expressed leading to apoptosis (Denmeade et al., 1996). Androgen ablation therapy results in the eradication of a large fraction of androgen-

dependent prostate cancer cells (Kyprianou et al., 1990), however, this treatment does not eradicate androgen-independent cells, and it is the outgrowth of this population that is responsible for the lethality of advanced "castration resistant" disease (Isaacs, 1999). Metastatic prostate cancers are lethal due to tumour heterogeneity as they are composed of both androgen-dependent and independent tumour cells.

Interestingly NE cells are also affected during androgen ablation therapy. These cells are not androgen responsive and so do not undergo apoptosis in response to androgen ablation. Interestingly, they have been shown to do the opposite and increase in number in response to androgen withdrawal. A number of studies have shown this affect, highlighting the fact that an increased numbers of NE cells correlates with more aggressive disease and poor clinical outcome (Grobholz et al., 2000, Hirano et al., 2005, Yuan et al., 2007) It is conceivable that after hormonal therapy these cells will be enriched and therefore may establish paracrine networks to stimulate androgen-independent proliferation of the remaining prostate cancer cells (Grobholz et al., 2005, Vashchenko and Abrahamsson, 2005). This would lead to tumour recurrence which would be androgen independent.

Some prostatic tumours are composed entirely or almost entirely of cancer cells with neuroendocrine differentiation (NED). The origin of these cancer NE cells remains controversial and unclear. Normal NE cells express Ck5, (Schalken and van Leenders, 2003) a basal cell marker, whereas prostate cancer NE cells exhibit characteristics of luminal secretory cells, expressing prostatic acid phosphatases (Huang et al., 2006) and/or PSA alongside their NE markers (Vashchenko and Abrahamsson, 2005). There are two theories, the first being that cancer NE cells share the same origin as normal NE cells originating from intermediate stem cells (Bonkhoff, 1998) A second theory is that these

cancer cells can undergo a transdifferentiation process to become NE cells, acquiring a similar phenotype to normal NE cells and expressing NE markers. In support of the latter model a recent genetic analysis of clinical archival specimens revealed that prostate cancer NE cells share essentially the same allelic profile as non-NE prostate cancer cells, but not with normal prostatic epithelium or normal NE cells (Sauer et al., 2006). Another question that remains unanswered is whether NE cells are considered to be terminally differentiated post mitotic cells and if so how do these cells gain proliferative potential in prostate cancer?

As well as the use of androgen deprivation therapy, other effective treatments are needed to address the problem of castrate-resistant prostate cancer (CRPC). In 2004, the chemotherapy drug docetaxel was introduced; clinical trials demonstrated an increase in median overall survival (OS). However, it was showed to have a number of adverse effects such as hepatotoxicity and neutropenia although its toxicity was deemed acceptable. The results of these clinical trials lead to docetaxel becoming a standard of care in the first-line treatment of CRPC. Unfortunately, a number of patients have shown resistance to docetaxel treatment, the reasons for this remains uncertain. However, it does highlight the need for new therapies, or for drugs that can be used in combination with the current treatments to enhance the level of apoptosis induced in the tumours of these patients, to further target the heterogeneous behaviour of this disease.

One area of prostate cancer biology that remains controversial is the cell type that gives rise to and maintains prostate carcinoma (Goldstein et al., 2010) Luminal cells were previously thought to be the cells of origin of prostate cancer, as the malignancy is characterised by a loss of basal cell markers such as p63, and the expansion of cells with a luminal phenotype expressing AR and PSA. However, there is considerable evidence

that the histological hallmarks of the disease do not necessarily correlate with its cellular origin, and that it is the basal cells that contain the prostate cancer-initiating cells (Schalken and van Leenders, 2003, Maitland et al., 2011). In the last few years this issue has been highly studied and, as a result of this, a minor luminal stem cell population was shown to be an efficient target for oncogenic transformation in mice. These rare luminal cells have been termed castrate-resistant Nkx3.1 expressing (CARN) cells. These cells were shown to have the potential to repopulate the prostate giving rise to both luminal and basal cells during prostate tissue regeneration induced by androgen depletion (Wang et al., 2009b). In the light of these findings it has been argued that using these studies to elucidate the cell of origin of prostate cancer may result in bias, owing to the fact that mouse models of prostate cancer are commonly induced by luminal-specific promoters such as probasin, and so their effects are concentrated only on the luminal cell population. Moreover, to date the majority of studies have implicated the basal cell population as the cell of origin of prostate cancer. Many studies have shown that injecting the basal cell population together with testosterone under the kidney capsule of nude mice enables prostate structures containing all the prostate lineages to develop (Wang et al., 2001b, Goldstein et al., 2010, Leong et al., 2008, Lukacs et al., 2010, Barclay et al., 2008). Moreover, this affect is not seen when the luminal cells are injected alone. A number of studies have also shown the presence of a transit-amplifying intermediate cell population (Hudson, 2004, Long et al., 2005). It has been postulated that it is this cell population that undergoes malignant transformation, and that the expression of luminal cell markers in these cancer cells is due to aberrant differentiation of transformed intermediate cells (Walker et al., 2008). The cell of origin of prostate cancer has evoked much debate and controversy. One possibility that has been put forward is that there could be different

subtypes of cancer that arise from different cell types within the prostate akin to observations in breast cancer (Visvader, 2009, Wang et al., 2013c). A study carried out by Wang et al., revealed the inherent plasticity of basal cells, and supported the theory that different cells of origin could generate distinct molecular subtypes of prostate cancer (Wang et al., 2013c, Choi et al., 2012). In these studies lineage tracing techniques were used to follow the proliferation and differentiation of prostate cells within transgenic mice. It was demonstrated that cells within the luminal layer also have proliferative potential, suggesting that although the basal cells appear to be the most likely candidates for malignant transformation owing to their ability to self-renew, it is conceivable that tumour initiation from distinct cell types in the lineage hierarchy can occur. If this were to be the case, different tumour subtypes with different prognosis and/or treatment might arise, thereby potentially explaining tumour heterogeneity.

Prostate cancer progression is a multistep process with the single most significant risk factor being advanced age. Its progression is related to a number of genetic abnormalities that affect the AR, along with mechanisms involved in cell survival, proliferation and apoptosis. It is postulated that the progressive accumulation of genetic alterations facilitate the transformation of normal prostate epithelial cells to prostate intraepithelial neoplasia (PIN); a precursor of prostate cancer characterised by thickening of the epithelial layer and loss of basal cell layers, followed by the development of invasive prostate cancer. One family of proteins that play a fundamental role in protecting the genome against mutations and thus cancer formation, are the p53 family. Prostate cancer is thought to be more of a disease driven by the inability of cells to undergo apoptosis rather than a disease driven by uncontrollable proliferation (Gurumurthy et al., 2001).

Therefore an understanding of how prostate cancer cells evade the apoptotic machinery is vital in order to develop potential new therapies to target the disease.

1.4 The p53 family of proteins

1.4.1 Overview

The *p53* gene is one of the most extensively studied human genes with over 60,000 publications on Pub Med. TP53 was discovered in 1979 as an interacting protein of the large T-antigen in SV40-transformed cells (DeLeo et al., 1979, Lane and Crawford, 1979, Linzer and Levine, 1979). Initially *p53* was categorised as an oncogene (Eliyahu et al., 1984, Jenkins et al., 1984, Parada et al., 1984). However, in 1989, the simultaneous publication of the anti-proliferative property of wild-type *p53* and the demonstration of inactivating mutations in human cancers eventually changed the status of *p53* to a tumour suppressor (Finlay et al., 1989, Baker et al., 1989, Takahashi et al., 1989). Since then the true importance of the *p53* gene in human cancer has been uncovered. It has been demonstrated that the *p53* pathway is involved in most, cases of human cancer. In approximately 50% of cancer cases, *p53* is directly targeted for mutations, deletions or interaction with viral proteins. In the remaining instances *p53* signalling is defective, most frequently due to the up-regulation of *p53* inhibitors or the inactivation of *p53* activators. These alterations in general impair the efficient accumulation of TP53 in response to stress signals, allowing cells with damaged DNA to replicate. Consequently these cells have a higher probability of accumulating additional cancer promoting events, which in turn drives cancer progression. *p53* can respond to different stress signals in a number of ways ranging from transient cell-cycle arrest, the facilitation of DNA repair (Kuerbitz et al., 1992), and the more irreversible state of senescence or apoptosis (Levine,

1997, Lowe, 1999). These functions make *p53* potentially the most important controller of genomic integrity leading to its popular name 'the guardian of the genome'.

Since its discovery over 30 years ago *p53* has been identified as having numerous functions. As well as being crucial for genomic integrity it has also been implicated in processes such as autophagy, oxidative stress, regulation of metabolism and female fertility, (Crighton et al., 2006, Teodoro et al., 2006, Bensaad and Vousden, 2007, Hu et al., 2007). In the late 1990's almost twenty years after the discovery of *p53* two other family members named *p63* and *p73* were identified (Kaghad et al., 1997, Schmale and Bamberger, 1997, Osada et al., 1998, Yang et al., 1998). These two homologues share sequence similarities with *p53* and are able to transactivate a number of TP53-mediated genes in response to DNA damage such as *BAX*, resulting in apoptosis (Yang et al., 1998). This initial observation suggested a co-operative role in *p53*-mediated cell damage response. However, despite some similarities with *p53* the precise roles of *p63* and *p73* in cancer have not yet been elucidated (Crum and McKeon, 2010). Furthermore, the study of germ line deletions of these genes has revealed a much more complex relationship between these genes and development (Melino et al., 2003).

1.4.2 Members and structure

The p53 family of genes has a high degree of sequence similarity and have three evolutionary conserved structural domains comprising an amino-terminal acidic transactivation domain (TA), a central core DNA-binding domain (DBD) and a multifunctional carboxyl-terminal oligomerisation domain (OD). The highest sequence similarity between the family members resides in the DBD (around 60%) suggesting that TP53, TP63 and TP73 are able to bind many of the same DNA sequences resulting in the transcription of the same set of genes. They have also been shown to drive the

transcription of genes specific to each family member. The DBD is the region where 95% of all cancer-associated mutations occur. The OD domain has around 35% sequence identity between family members and is subjected to a number of post translational modifications including phosphorylation, acetylation, methylation and ubiquitylation. These modifications are likely to contribute to the tight regulation of all three members of the family. Unlike TP53, both TP63 and TP73 have an additional domain at the C-terminal containing a sterile alpha motif which is important in protein-protein interactions and is often found in proteins that have a functional role in development (Schultz et al., 1997).

All three p53 family genes can encode two transcripts owing to the presence of two distinct promoters; P1 and P2. The full length transcripts of *p53*, *p63* and *p73* are TP53, TAp63 and TAp73 and are derived from the P1 promoter upstream of exon 1. Alternatively, the P2 promoter gives rise to a set of proteins that have a truncated N-terminus and so lack the transactivation domain; Δ40p53, Δ133p53, ΔNp63 and ΔNp73 (where the N denotes dominant-negative). These dominant negatives have been shown to play an important role in autoregulating the full length proteins. To add further to the complexity of all three family members, additional splicing events at the C-terminus give rise to a number of different variants. *p53* has two [β, γ] *p63* has three [α, β, γ] and *p73* at least seven different variants [α, β, γ, δ, ε, ζ, and η]. The different variants of the three p53 family members are shown in (figure 1.4).The ΔNp63 and ΔNp73 isoforms were first thought to be functionally inactive as they were shown to be unable to transactivate a promoter that contains a p53 response element and normally induces cell death. They were shown to bind to TP53 and promote proliferation and block apoptosis, resulting in an initial belief that these proteins were purely dominant-negatives (Yang et al., 1998).

Further studies however have shown that the roles of these variants are far more complex. In the case of TP63 it has been shown that although ΔNp63 variants lack the p53-like transactivation domain they instead contain a unique 14 amino acid sequence that contributes to the formation of an alternative transactivation domain (Duijf et al., 2002). The ΔNp63α variant also contains a transactivation inhibitory domain that can bind directly to the TA domain, thereby exerting an inhibitory effect (Sayan et al., 2007). It is now clear that these isoforms can both activate and repress gene transcription resulting in a functionally the complex set of proteins.

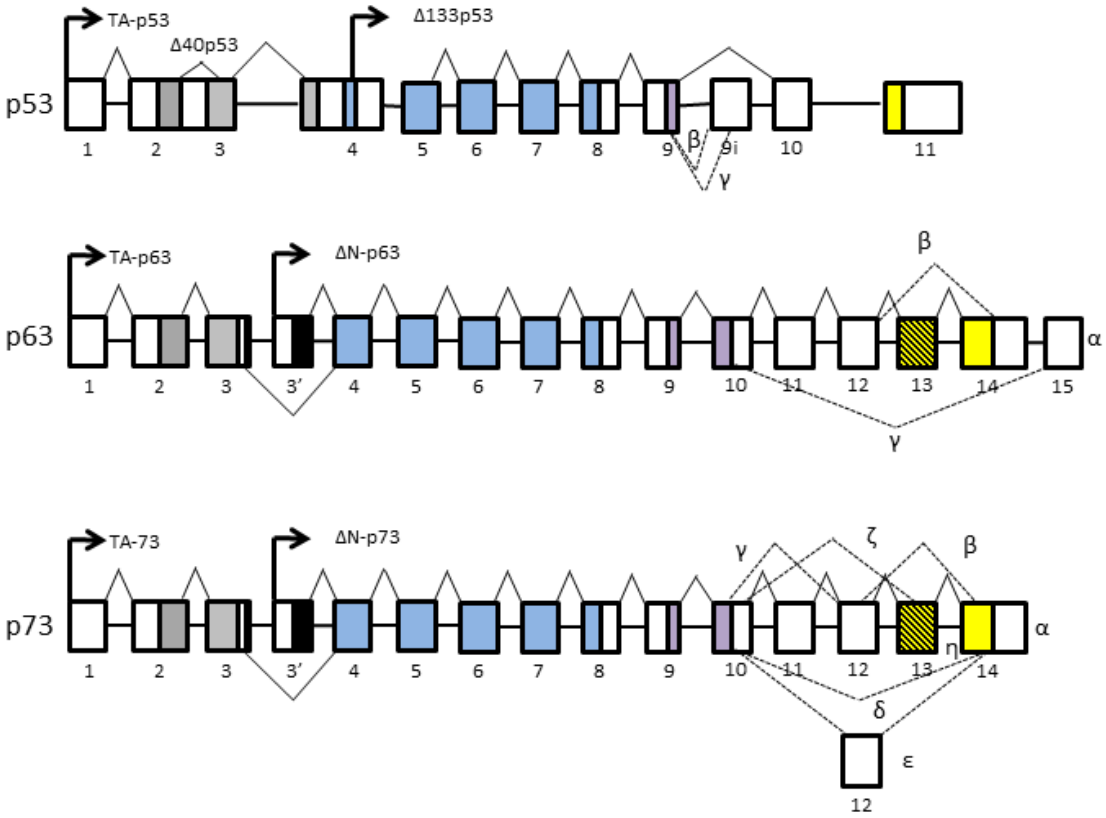

Figure 1.4 Structure of the p53 family of proteins

Genomic organisation of *p53*, *p63* and *p73*, showing the modular organisation of the resultant proteins from the N-terminal to the C-terminal. Transcriptional start sites are indicated by arrows Grey = transactivation domain (TA); blue = DNA-binding domain (DBD); purple = oligomerisation domain (OD); yellow hatched = part of the SAM domain, transactivation inhibitory domain (TI); yellow = sterile alpha motif domain (SAM). In addition to the three full length proteins there are a number of resultant truncated N-terminal proteins termed Δ40p53, Δ133p53, ΔNp63 and ΔNp73. Alternative splicing at the C-terminus generates different isoforms for TP63 and TP73 denoted by the Greek letters. There are two for TP53 (β,γ) three for TP63 (α,β,γ) and seven for TP73 (α,β,γ,δ,ε,ζ,η). The ΔN isoforms have been shown to act as dominant-negatives although there is evidence to suggest they are able to activate as well as repress.

1.4.3 The p53 response

TP53 can be activated by a broad range of cytotoxic stress signals, including DNA damage, hypoxia and activation of particular oncogenes such as *c-myc* (Vousden and Lane, 2007). In the majority of unstressed cells p53 mRNA is constitutively expressed. However, the level of protein expression is low due to the action of the E3-ligases Mdm2 and Mdm4, which target TP53 for ubiquitination and proteasomal degradation (Haupt et al., 1997, Kubbutat et al., 1997, Francoz et al., 2006). Stress induced *p53* activation induces protein stabilisation and this is mediated primarily through inhibition of Mdm2 by the tumour suppressor ARF. In addition, multiple post-translational modifications affect p53 stability, its ability to bind to target genes, and its transcriptional activity (Vousden and Lane, 2007). Upon activation TP53 forms a homotetramer and translocates to the nucleus where it binds to specific sequences within target genes triggering a multitude of effector pathways which are illustrated in (figure 1.5). These responses include cell cycle arrest, coordinated DNA repair, cellular senescence and apoptosis. The transcription-activating powers of the TP53 tetramer depend on more than simply its recognition of sequence motifs within a specific promoter. For example it also depends on the addition of an array of covalent modifications many of which affect the C-terminal domain, such as acetylation, glycosylation and phosphorylation. It is likely that these modifications affect the ability of TP53 to determine the identities of the specific target genes that are activated in response to various stress signals (Beckerman and Prives, 2010). TP53 activation also triggers the direct transcription of *Mdm2* as a fail-safe mechanism to ensure that TP53 levels return to basal levels as soon as the stress response is alleviated (Vousden and Lane, 2007). To induce *p53*-mediated apoptosis TP53 directly up-regulates the transcription of several pro-apoptotic genes such as *PUMA*, *NOXA* and

BAX (Nakano and Vousden, 2001, Yu et al., 2001, Oda et al., 2000, Miyashita and Reed, 1995) and actively represses the transcription of pro-apoptotic genes such as *Bcl-2* and *Bcl-X_L*. Furthermore interactions with transcriptional co-regulators such as the ASPP family of proteins may contribute to TP53's preference for particular target genes and may play an important role in *p53*-mediated induction of either cell cycle arrest or apoptosis.

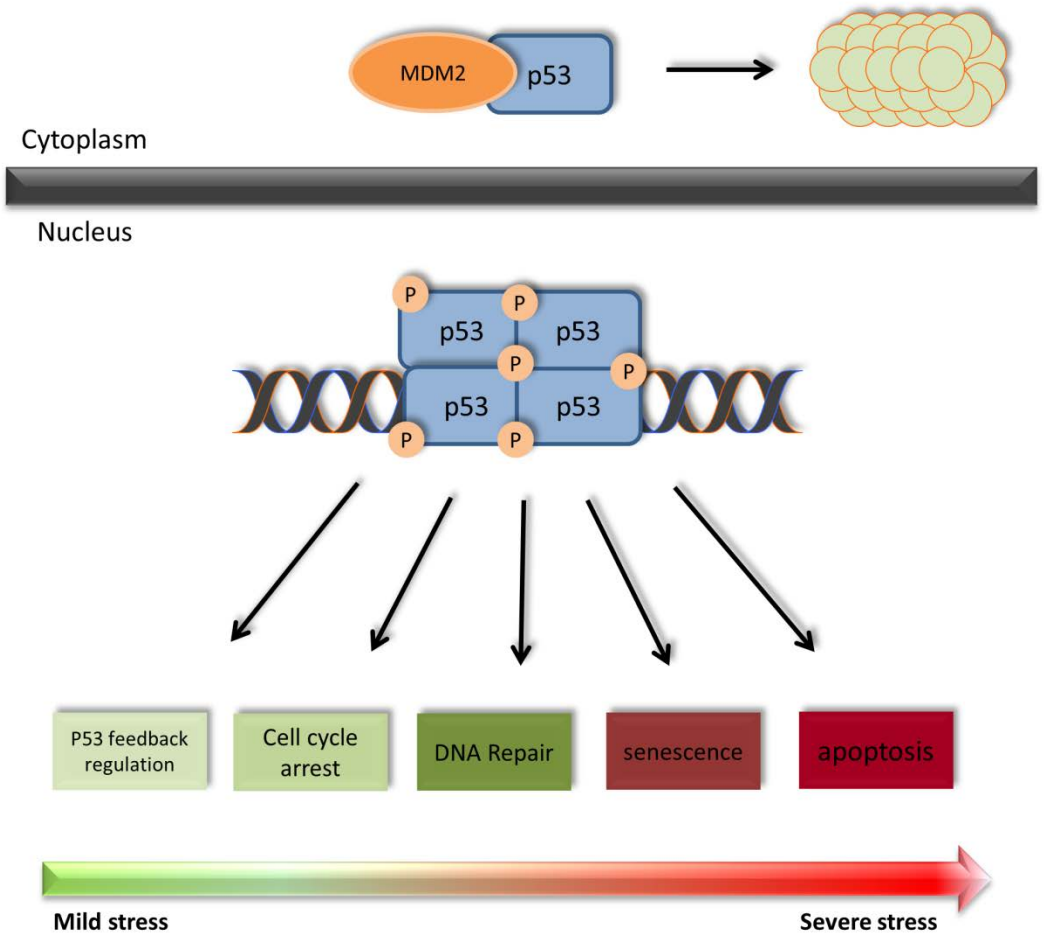

Figure 1.5 Simplified diagram of the p53 response

In unstressed conditions the levels of TP53 are maintained by its negative regulator Mdm2 which targets TP53 for ubiquitination by the proteasome. In the presence of stress signals TP53 undergoes post-translational modification preventing its degradation and resulting in increased TP53 levels. TP53 tetramers bind to the promoters of a large array of target genes. The nature and severity of the stress signal dictates the precise set of genes that are activated. Under conditions of mild stress cells undergo cell cycle arrest in order to allow the cell time to repair. In more severe stress conditions cells are directed to irreversible senescence or apoptosis.

1.4.3.1 Cell cycle arrest

In the event of DNA damage TP53 is activated and induces a program of temporary cell cycle arrest at both G1 and G2 checkpoints. This process allows time for DNA repair to take place and provides an opportunity for the cell to re-enter the cell cycle if repair is successful. All three members of the p53 family are able to induce this program of cell cycle arrest thereby preventing the propagation of cells carrying oncogenic mutations. Upon activation TP53 transcriptionally induces $p21^{cip1}$ which is a crucial inhibitor of two cyclin-dependent kinases (CDKs). $p21^{cip1}$ subsequently halts cell proliferation by inhibiting both CDK2 and CDC2 at G1. Two other genes activated by TP53 encode the 14-3-3σ and Reprimo proteins which govern G2/M transition. These proteins sequestered cyclin B-CDC2 complex into the cytoplasm thereby preventing its nuclear translocation which would ordinarily drive the cell into mitosis (Hermeking et al., 1997, Ohki et al., 2000). It has been shown that GADD45 is another TP53 target and that this can directly interact with CDC2 thereby disrupting its binding with cyclin B, again preventing mitosis (Jin et al., 2000).

1.4.3.2 Apoptosis

In the event of severe irreparable genomic damage TP53 can provoke a process of programmed cell death known as apoptosis. There are two main apoptotic pathways. Firstly, the extrinsic pathway that involves the ligation of cell surface death receptors such as Fas and DR5 by the corresponding ligand, resulting in the binding and activation of the death domain protein FADD and the caspase-8 cascade. Secondly, the intrinsic pathway which is triggered by cell stress induced by DNA damaging agents such as UV radiation, causing an alteration in the mitochondrial membrane, leading to the release of

cytochrome C into the cytoplasm which associates with Apaf-1 and pro-caspases-9 to form the apoptosome which, in turn, activates the downstream effector caspase-3.

TP53 is able to activate the extrinsic pathway by transcriptionally regulating the expression of death receptors (Wu et al., 1997, Muller et al., 1998) in response to certain stimuli such as anticancer drugs. In the context of the extrinsic pathway TP53 is able to affect the transcription of many of the components of the Bcl-2 family. The Bcl-2 family of proteins are localised to the outer membrane of the mitochondria and act to regulate mitochondrial membrane potential and volume (Harris and Thompson, 2000). They can be categorised as either anti-apoptotic proteins, such as Bcl-2 itself and Bcl-$X_{L,}$ and pro-apoptotic proteins, such as Bax, Bak, NOXA and PUMA. TP53 has been shown to repress the anti-apoptotic genes while activating the transcription of the pro-apoptotic Bcl-2 members (Basu and Haldar, 1998) tipping the balance in favour of apoptosis. Additionally, cytoplasmic TP53 can promote apoptosis in a transcriptional-independent manner by directly interacting with Bcl-2, Bcl-X_L and Bak at the mitochondria membrane resulting in permeabilization of the outer membrane of the mitochondria (Green and Kroemer, 2009). *Bcl-2* is a known proto-oncogene and its overexpression results in malignant transformation in several cancers (Reed, 1998) including prostate cancer. *Bcl-2* has been established as a major player in prostate cancer progression, particularly during the development of androgen-independent and metastatic disease (Furuya et al., 1996), these observations demonstrate the importance of the correct balance of the Bcl-2 family members.

Owing to their similarities in DNA-binding sequences, TP63 and TP73 can also form oligomers and bind to DNA and transactivate TP53 responsive anti- and pro-apoptotic genes, although there are some differences in selectivity and degree of activation between

the family members (Jost et al., 1997, Yang et al., 1998). Moreover it has been shown that the combined loss of *p63* and *p73* results in the failure of cells to undergo apoptosis in response to DNA damage (Flores et al., 2002), indicating that both *p63* and *p73* are required for p53-dependent apoptosis. Although TP63 and TP73 share many of the functional properties of TP53, and cooperate with TP53 in the regulation of tumourigenesis as demonstrated by Flores et al in 2005, in the absence of stress the most important role of TP63 and TP73 is regulation of differentiation and development (Flores et al., 2005).

1.4.3.3 Senescence

Cellular senescence was first described over 40 years ago as a process that limits the proliferation of normal human cells in culture (Hayflick, 1965). This pathway is known to be regulated by *p53* transcription. Cellular senescence refers to the irreversible cell cycle arrest activated in response to stress signals such as oncogenic activation and DNA damage. Senescent cells in culture are characterised by an enlarged cell size, flattened morphology, inability to synthesise DNA and the expression of senescence-associated β-galactosidase (SA-β-gal) which is detected by a colorimetric assay using X-gal as a substrate at pH 6.0 (Dimri et al., 1995). The activation of senescence prevents cells that carry mutations from expanding, without immediate cell eradication. Interestingly senescent cells are only present in premalignant lesions and are not present in malignant tumours (Collado and Serrano, 2005). This may be due to the loss of p53-function early on in disease progression. For senescence to be bypassed in human cells TP53 needs to be inactivated, coupled with the inactivation of pRB, the latter often being mediated by the inhibition of the cyclin-dependent kinase inhibitor p16, a known regulator of the cell cycle (Ben-Porath and Weinberg, 2005).

p63 has been implicated in the activation of senescence. Mice deficient in *p63* were shown to have a shortened life span and displayed features of accelerated aging, both of which are associated with premature cellular senescence. Both germline and somatically-induced *p63* deficiency were shown to activate widespread cellular senescence with enhanced expression of the senescence markers SA-β-gal and p16 (Keyes et al., 2005). The role of TP63 in cellular processes such as senescence is further complicated by its many isoforms. ΔNp63α, the predominant isoform expressed in stratified epithelia, promotes proliferation and extends cellular lifespan by inhibiting senescence. Loss of ΔNp63 isoforms triggers senescence, whereas gain of ΔNp63α enhances proliferation, allowing senescence to be bypassed by cells with augmented $p19^{Arf}$/p53 (Papazoglu-Statescu). Furthermore, TAp63 isoforms positively modulate the senescence process and inhibit cellular proliferation by enhancing TP53's tumour suppressing activity, as well as providing tumour suppression independently of TP53. Enhanced expression of TAp63 correlates with decreased proliferation, and loss of TAp63 isoforms bypasses $p19^{Arf}$/p53 cellular senescence and sensitizes cells to oncogenic transformation (Guo et al., 2009). The involvement of *p73* in regulating senescence remains unclear.

1.5. The p53 family and development

1.5.1 p53

TP53 has been implicated as having an important role in embryonic development. In 1991 Schmid et al described the *p53* expression levels in different organs during embryogenesis using *in situ* hybridisation studies of mouse embryos. He showed high expression of *p53* mRNA in all tissues until midgestation, followed by a shift to a more heterogeneous pattern during organogenesis which is characterised by complex differentiation events. In this phase of development dynamic and tissue-specific *p53* expression patterns were observed. Meanwhile in terminally differentiated tissues *p53* mRNA expression declined to almost undetectable levels. These shifts in *p53* expression implied that TP53 was necessary for normal mouse development. It was therefore a surprise that when the first *p53*-null mice were generated they appeared to develop normally, suggesting that TP53 is dispensable for proper development. These mice did however confirm the role of *p53* as a tumour suppressor gene as they were found to be prone to the spontaneous development of a variety of neoplasms by 6 months of age (Donehower et al., 1992). The importance of *p53* during development seems more pronounced in other species, such as *Xenopus laevis* embryos. These exhibit inhibited mesodermal differentiation and severe gastrulation defects. One possibility for these observed differences between species may be that *p53* is required for a developmental process in *Xenopus* that is not utilized during murine development. The mostly likely candidate process would be the mid-blastula transition, which is critical to frog development but has no true equivalent in mice (Wallingford et al., 1997). Alternatively the other p53 family members, *p63* and *p73*, which are also expressed during

embryogenesis, may compensate for the absence of TP53 in the mouse, but not the frog, during early developmental stages.

It has been shown that the over-expression of wild-type *p53* may disrupt normal embryogenesis, as *Mdm2* knock-out mice die during early embryogenesis. This death is attributed to a failure of TP53 inhibition resulting in accelerated cell-cycle arrest and apoptosis during a stage in which rapid cell division is required. The direct contribution of *p53* to this phenotype was clarified by the fact that the lethal phenotype could be rescued in a *p53*-null background (Jones et al., 1995, Montes de Oca Luna et al., 1995). The evidence collected over the last 25 years suggests that *p53* plays a role in development by inhibiting differentiation and thereby maintaining a proper cellular state and preventing improper maturation. In the absence of *p53* however the other members of the p53 family appear to be able to offer a level of compensation. A role, if any, for *p53* in prostate development has, to date, not been identified.

1.5.2 p73

TP73 has distinct developmental roles and *p73* expression is required for neurogenesis in specific neural structures such as the hippocampus. Mice deficient for all p73 isoforms have been reported to suffer from hydrocephalus, chronic infections and inflammation (Yang et al., 2000).The most predominant transcript of *p73* is ΔNp73; its expression has been shown to be instrumental in neuronal survival during development (Pozniak et al., 2000, Pozniak et al., 2002). The mechanism by which ΔNp73 mediates its 'antiapoptotic' or 'survival' function in neurons appears to be, at least in part, similar to how it works in tumor cells. It is likely that ΔNp73 blocks p53-dependent transcriptional activation of pro-apoptotic genes, potentially by preventing the binding of TP53 to the *BAX* promoter, which is required for sympathetic neuron apoptosis (Deckwerth et al., 1996). The

developmental role of *p73* appears to be mainly neurological and has not been implicated in normal prostate development.

1.5.3 p63

TP63 is phylogenetically the oldest protein in the p53-family and has the potential to bind to over 5800 target sites (Yang et al., 2006) making it also the most complex. It is known to play a crucial role in stem cell viability and renewal, apoptosis, specification of cell lineage and cell fate. *p63* is also known to play a substantial role in normal mouse embryonic development. *p63*-null mice demonstrate gross abnormalities at birth, including a lack of ectodermal covering leading to death due to dehydration (Mills et al., 1999, Yang et al., 1999a). They also have severe craniofacial, limb and epithelial developmental defects and lack several epithelial organs including the epidermis, salivary, lachrymal and mammary glands, and have severe defects in the development of the UGS, (Grisanzio and Signoretti, 2008). The prostate gland normally develops by epithelial budding of the intermediate region of the primitive UGS. The UGS consists exclusively of p63-positive cells that subsequently differentiate into basal, luminal and NE cells, and these function as the progenitor/stem cell population (Crum and McKeon, 2010, Signoretti and Loda, 2006). *p63*-null mice show no evidence of prostate development; this could be a direct result of the severe defects in the UGS. However, these mice die hours after birth, whereas the majority of prostate development takes place in the last days of gestation and beyond. The absence of *p63* may therefore simply cause a developmental delay rather than complete developmental arrest, and so the assessment of prostate development in these mice may not be fully conclusive. To investigate the role of TP63 expression in prostate development Kurita and colleagues isolated cells from the UGS of $p63^{+/+}$ and $p63^{-/-}$ embryos and grafted them under the renal capsule of adult male

nude mice. They found that *p63*$^{-/-}$ UGS developed ductal structures resembling normal prostate however it lacked morphologically definable basal epithelial cells as assessed by a lack of expression of the prostate basal cell markers Ck14 and Maspin. Interestingly, goblet cell-like cells were detected in the *p63*$^{-/-}$ mice using Alcian blue staining for mucinous secretions. Signoretti and colleagues also observed crypt-like structures in 5% of the mucinous epithelium demonstrating the presence of goblet cells and lysosome-positive Paneth cells (Signoretti et al., 2005). These findings suggest that although *p63* is not necessary for prostate secretory cell differentiation, its expression is required in multipotent UGS endodermal cells to restrict development to the prostate cell lineage, and thus prevent the expression of intestinal cell markers (Kurita et al., 2004, Signoretti et al., 2005).

Although luminal prostate epithelial cells have been shown to have the ability to differentiate from *p63*$^{-/-}$ UGS these results were gained in a non-physiological setting. When the prostate buds were analyzed from *p63*$^{-/-}$ chimeric mice they were found to be exclusively *p63*$^{+/+}$ whereas UGS epithelium was populated by a mixed population of both *p63*$^{+/+}$ and *p63*$^{-/-}$ cells. The *p63*$^{+/+}$ cells were found to line the basement membrane whereas the *p63*$^{-/-}$ cells were found to line the lumen. Taken together these results support the theory that *p63* is essential for normal prostate bud formation and that at an early stage in prostate development all the cells are required to express *p63* (Signoretti et al., 2005). Furthermore, even though *p63*$^{-/-}$ UGS has been shown to differentiate into secretory cells it has not been found to differentiate into basal cells. Moreover, during normal prostate organogenesis the prostate cells are exclusively TP63-positive implying that secretory cells are derived at this stage in development from TP63-positive basal cells. However, these findings cannot rule out the hypothesis that once developed the

secretory cell population may be maintained by a process of self-duplication (Ousset et al., 2012). A model of the prostate cellular hierarchy and TP63 expression during postnatal development, homeostasis and regeneration after castration, is illustrated in figure 1.6.

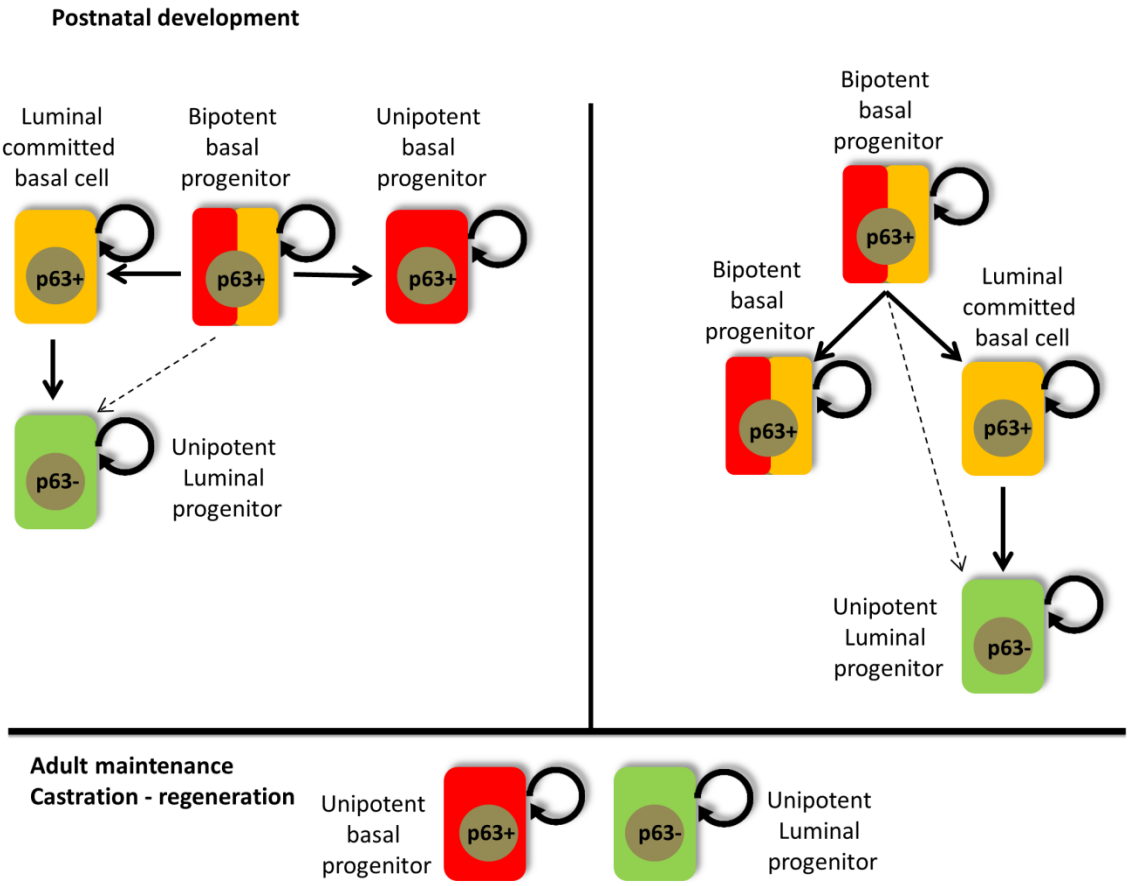

Figure 1.6 A model of the prostate cellular hierarchy and *p63* expression during postnatal development, homeostasis and regeneration after castration (Adapted from Ousset M et al. 2012, Nature Cell Biology).

Postnatal prostate development is ensured by the presence of TP63-positive bipotent basal progenitors and TP63-positive unipotent basal cells as well as TP63-negative luminal progenitors. There are two models to describe the presence of TP63-positive basal bipotent and unipotent progenitors. In the left-hand model, basal cells contain pre-existing basal bipotent and basal unipotent progenitors, whereas in the right-hand model, one single bipotent basal progenitor can divide symmetrically, giving rise to two bipotent basal progenitors, or asymmetrically giving rise to one bipotent basal and one luminal committed basal progenitor with equal probability. During adult homeostasis and androgen-induced prostate regeneration, the luminal and basal lineages are replenished by unipotent progenitor cells of their own lineage. The dashed arrows represent other transitions that may occur.

The most abundant TP63 isoform in the prostate is ΔNp63α and this lacks the transactivating domain (Signoretti et al., 2000). This isotype has been shown to act as a dominant-negative over both TAp63 and TP53 (Yang et al., 1998). It has previously been shown that keratinocyte differentiation *in vitro* is associated with down-regulation of the ΔNp63 transcripts (Parsa et al., 1999) due to the fact that secretory cells do not express TP63. Removal of the dominant-negative function of ΔNp63 in basal cells may be necessary in order to activate the TP53-dependent gene transcription machinery required for differentiation of these cells into secretory cells.

TP63-positive basal cells have also been shown to play key roles in maintaining the ductal structure of the prostate in the absence of androgen. In response to castration normal prostatic ducts lose their luminal secretion content and a substantial portion of luminal cells undergo apoptosis. This is a highly coordinated process, allowing normal prostatic ducts to shrink while maintaining their morphologic integrity. *p63*$^{-/-}$ prostate glands were shown to have a complete loss of ducts following castration, resulting in a total loss of ductal integrity (Kurita et al., 2004). In normal prostate epithelium, a substantial portion of luminal cells undergo apoptosis in response to castration, but a subset of luminal cells always survive in order to maintain ductal structure and permit regeneration when androgens are restored. The mechanism controlling which luminal cells die and which ones survive following castration is not known, however it has been postulated that the surviving luminal cells are those that are in direct contact with basal cells (Rouleau et al., 1990). These observations suggest that TP63-positive basal cells play an important role in the control of luminal cell survival/apoptosis in the prostate in response to changes in androgen levels. Furthermore, TP63 is expressed at sites of epithelial-mesenchymal interaction. It is known that normal prostate organogenesis can

only take place with normal cross-talk between stromal cells and epithelial cells. It is possible that TP63 plays a role in maintaining the proliferative potential of progenitor cells through ensuring proper cell fate specification. TP63 may define the difference between stromal and epithelial cells at the interface between these two populations.

The role of the *p63* gene appears to be the most crucial of the three p53 family members in both normal prostate development and maintenance; however the mechanisms governing its regulation are still poorly understood.

1.6. The p53 family and prostate cancer

1.6.1 p53 and prostate cancer

The role of TP53 in preventing tumour formation is clearly demonstrable in the *p53*-null mouse model which has normal embryonic development but is highly susceptible to spontaneous tumour formation in early life (Donehower et al., 1992). However, these mice do not spontaneously form prostate tumours. This may be a consequence of their shortened lifespan, and they may have developed prostate cancer if they had lived longer. *p53* is thought to play a role in prostate cancer progression although the extent of its contribution is poorly understood. *p53* is the only p53 family member known to be commonly mutated in the majority of human malignancies but, intriguingly, this event is not as common in prostate cancer. Within the 10-30% of prostate cancer cases that harbour a *p53* mutation the most prevalent changes are missense single base substitutions at codons 273, 245 and 248 which affecting the central DBD of the protein. A recent study of 156 prostate cancers found only 2 cases with missense *p53* mutations but, interestingly, 24% of samples contained hetero- or homozygous copy-number loss (Taylor et al., 2010). These differences may provide some insight into whether the loss of a *p53* allele rather than a mutation in *p53* has an impact on the aetiology and clinical outcome of this malignancy.

An underlying role for *p53* in prostate cancer progression was found by Thompson and colleagues in 1995 using the mouse prostate reconstitution (MPR) model. Prostate cancer was identified in 100% of heterozygous and homozygous *p53* mutant MPRs with signs of metastasis in 95% of the mice; however no metastasis were found in wild-type *p53* mice (Thompson et al., 1995). These findings support the theory that mutant *p53* function plays a fundamental role in the development of invasive prostate cancer and may potentially be

one of the primary drivers of metastatic disease. Another study convincingly showed that a reduced *p53* expression or function in LNCaP cells confers the ability of these cells to form tumours in castrated male nude mice compared to parental LNCaP cells that do not have an invasive phenotype (Burchardt et al., 2001). In addition, TP53 and AR have been shown to mutually regulate one another (Alimirah et al., 2007, Rokhlin et al., 2005), and AR signalling has been shown to be compromised by the loss of *p53* (Guseva et al., 2012). This may explain why prostate cancer cells retain wild-type *p53*, thereby ensuring that the full complement of androgen-responsive genes can still be activated until the transition from androgen-dependent to androgen independent disease occurs and wild-type *p53* expression is no longer needed. Interestingly, it has also been shown that in the context of androgen deprivation the loss of *p53* promotes prostate cancer cell proliferation. Therefore it would be intriguing to understand whether the rates of *p53* mutation are increased after androgen deprivation therapy as it would appear that the loss of *p53* expression bestows a growth advantage in these particular conditions. The rising levels of PSA in advanced prostate cancer may also be due to changes in TP53 function as it has been shown that wild-type *p53* negatively regulates PSA. In the presence of a *p53* mutation introduced into LNCaP cells, PSA mRNA levels have been shown to increase fourfold, with a 4-8 fold increase in the amount of PSA secreted by these cells. Any sharp rise in PSA levels detected in patients with prostate cancer may therefore potentially be used as an indicator of aberrant *p53* function (Gurova et al., 2002).

Given that *p53* mutation is associated with castrate-resistant disease it was historically thought that mutation of this gene is a late event in the development of this disease. However, recently this theory has been challenged by the finding that transgenic mice expressing $TP53^{R270H}$ (equivalent to the human hotspot mutation R273H) combined with

a loss of *Nkx3.1*, develop PIN and prostate lesions more readily than mice with loss of *Nkx3.1* alone. This suggests that the R273H mutation may play a role in disease initiation (Vinall et al., 2012). However in this particular mouse model AKT activation is increased suggesting that PTEN inactivation may have occurred. It is well documented that loss of PTEN function combined with *p53* mutation results in prostate cancer (Chen et al., 2005b). However, the precise kinetics of these events and their potential impacts on disease progression are still unknown. These observations demonstrate however that there are key events, in the progression of prostate cancer, one of which is the dysregulation of *p53*. How these events might be interpreted in terms of treatment strategies remains unclear. Another question that remains unanswered is how p53-signalling is evaded in the cancer cases that still appear to express wild-type *p53*. One hypothesis is that the aberrant expression of TP53 co-regulators leads to loss of function of TP53. It may therefore be possible to inhibit these co-regulators and restore normal TP53 activity.

1.5.3 p73 and prostate cancer

Following the discovery of the *p73* gene it was first thought that, in contrast to *p53*-deficient mice, those lacking *p73* shown no increased susceptibility to spontaneous tumourigenesis. However, the generation of isoform specific *p73*-knockout mice has shown that *p73* does indeed play a role in cancer. TAp73$^{-/-}$ mice show an increased susceptibility to spontaneous and induced carcinogenesis, with more than 70% of the knockout animals developing tumors. In contrast, there is no evidence of tumor development in ΔNp73$^{-/-}$ mice. These findings support a tumour suppressive role for TAp73 and an oncogenic role for ΔNp73. Unlike *p53*, *p73* is rarely mutated in human cancer, however its expression has been found to be frequently dysregulated. Over expression of p73 has been found in a number of different cancers including prostate

cancer (Rufini et al., 2011). It has been postulated that the ratio between the different p73 isoforms is pivotal in maintaining a normal apoptotic response and preventing the development of malignant cells.

1.6.4 p63 and prostate cancer

The question as to whether *p63* is an oncogene or a tumour suppressor has been greatly debated throughout recent years. The role of *p63* in tumourigenesis remains controversial, this is mainly due to the fact that susceptibility to cancer has been shown to be substantially different in the two existent *p63*-deficient mouse models (Flores et al., 2005, Keyes et al., 2006). The *p63* heterozygous mice constructed by Yang et al. are prone to spontaneous tumours while the ones generated by Mills and colleagues are not. The differences observed may be due to the methods used by each of the groups to inactivate *p63* function. Yang et al deleted exons 6, 7 and 8 which are part of the DNA-binding domain (Yang et al., 1999a), whereas Mills et al. inserted two different target vectors which integrated into different regions of the *p63* locus thereby causing disruption of the *p63* gene (Mills et al., 1999). Both groups claimed that their resultant mutant alleles caused a deficiency of all functioning isoforms of TP63. However, it could be possible that deletion of a number of exons could lead to the production of truncated protein products, and this might explain the differences observed between the mutant mice. Another fundamental difference between these studies was the genetic background of the mice. Keyes et al. used a mixed background of C57BL/6J with 129S5 while Flores et al used a pure C57BL/6 background. This alone has been shown to influence tumour spectra. Thus, the presence of modifier genes on the strains used in both studies could account for the differences in tumour incidence.

p63 has been shown to have very different expression patterns in different tumour types. In squamous cell carcinoma of the head and neck *p63* is up-regulated which would suggest an oncogenic function. However, in contrast, *p63* has been shown to be lost or reduced in others cancers such as mammary and prostate adenocarcinomas which would point to more of a tumour suppressive role. The elucidation of *p63* function was for many years further complicated by the lack of anti-TP63 antibodies against the different isoforms. Many recent studies using antibodies that distinguish between TA and ΔN isoforms have demonstrated that the "oncogenic versus tumour suppressor" argument may be even more complicated, with tumour cells exhibiting an imbalance between the two transcripts in different tissues. One study examined *p63* expression in bladder carcinomas using quantitative RT PCR. Over 50% of tumours exhibited marked reduction in TAp63 and/or over-expression of ΔNp63, indicating that loss of this isoform may contribute to the progression of bladder carcinomas (Park et al., 2000).

One of the hallmarks of a tumour suppressor gene is its loss of heterozygosity (LOH) in a tumour. To determine whether *p63* can behave as a classical tumour suppressor, PCR analysis was performed on the heterozygous mice that developed tumours. They showed that the majority of the animals exhibited LOH. Interestingly, Yang et al. also examined $p53^{+/-}$, $p63^{+/-}$ mice and found that loss of the remaining *p63* allele in squamous cell carcinoma cells was independent of *p53* loss, indicating that loss of *p63* is an important event leading to the formation of this tumour type. Further elucidation of the complex role of *p63* in the initiation of carcinogenesis will no doubt require more refined mouse models that allow manipulation of individual p63 isoforms. Furthermore, to study its role in prostate tissue would require a mouse model with prostate specific gene manipulation to ensure the mice do not die of other causes before the prostate is affected.

p63 deficiency has been implicated in the activation of cellular senescence and accelerated aging (Keyes et al., 2005). Although senescence is often portrayed as a suppressor of cancer, as it prevents the division of cells that may have aberrant gene expression (Hayflick, 1965), it has recently been shown that senescent cells can also be cancer promoting, as they develop a senescence associated secretory phenotype (SASP) that can affect the behaviour of neighbouring cells. Many SASP factors have been implicated in the stimulation of phenotypes associated with aggressive cancer cells. For example, senescent cells have been shown to secrete high levels of interleukin-6 and -8, which stimulate premalignant and weakly malignant cells to invade the basement membrane (Coppe et al., 2008). Furthermore, senescent fibroblasts and keratinocytes have been shown to secrete matrix metalloproteinase (MMPs) which facilitate tumour cell invasiveness (Millis et al., 1992, Kang et al., 2003, Rodier and Campisi, 2011). Therefore the loss of *p63* in the prostate may result in the activation of cellular senescence which could have a detrimental effect on remaining neighbouring cells.

Unlike *p53, p63* is rarely mutated in human cancer. Even though its expression pattern has been highly studied in different cancers the mechanism of its regulation is still poorly understood. However, *p63* appears to play a tumour suppressive role in prostate tissue. One of the main hallmarks of invasive prostate cancer is loss of TP63-positive basal epithelial cells. *p63* has also been shown to act as a suppressor of metastasis, thus the loss of TP63 expression is associated with more aggressive tumours suggesting that the reduction of p63 accelerates tumourigenesis and metastatic spread (Melino, 2011). The consequence of losing the basal layer and the loss of *p63* expression has not been extensively studied separately, and so the details of the molecular advantages bestowed on these cells are not fully known. It would be intriguing to understand whether the loss

of *p63* expression results in the destruction of the basal cells or whether the loss of TP63 is purely a consequence of the loss of the basal cell population. One hypothesis is that the basal cells undergo malignant transformation resulting in the loss of basal markers and the exhibition of a more luminal phenotype (Goldstein et al., 2010). The model of regeneration shown in (figure 1.6) could explain the continuing rapid proliferation of the luminal layer observed in prostate cancer in the absence of the basal layer, which in normal tissue acts as the proliferative compartment. It may be that the TP63-positive cells act as controllers of cellular organization, almost in a "cage-like" manner. They keep the luminal cells away from interacting with the stromal compartment thereby preventing any interaction with stromal derived growth factors. Once this cage is destroyed the cells are able to spread and divide in an uncontrolled manner.

TP63 has been shown to physically interact with mutant TP53 (Melino, 2011). Lang et al. generated a mouse with a *p53* point mutation in the DNA-binding domain $p53^{R172H}$ corresponding to a hot spot mutation found in human cancer at amino acid 175. These mice exhibited a pronounced metastatic phenotype which was later accredited to the interaction between mutant TP53 and TP63. This interaction resulted in the inhibition of transcription by TP63 due to its sequestration in transcriptionally inactive complexes with mutant TP53. (Lang et al., 2004). This is an intriguing finding as many studies in prostate tissue use normal adjacent tissue as a control. The finding that mutant *p53* suppresses the anti-metastatic properties of TP63 poses the possibility that even in benign-looking glands, with normal structural architecture, many proteins including TP63 may already have attenuated function owing to other protein alterations within the tissue. Therefore, even before TP63 loss occurs, its' function may already be compromised.

The observation that basal cells are invariably absent from the malignant glands of prostatic adenocarcinoma, alongside the ability of immunohistochemical staining for high molecular weight cytokeratin to detect basal cells, has proven to be diagnostically invaluable (Brawer et al., 1985). However, due to the fact that the diagnostic utility of cytokeratins to define malignant areas from benign was based on negative staining for Ck5 and Ck14, there has been concern over the possibility of false-negatives. The fact that TP63 was observed to be expressed only in the basal cells of the prostate fuelled a number of studies into its usefulness as a diagnostic marker (Signoretti et al., 2000, Parsons et al., 2001). It was shown that immunostaining for TP63 was in fact a useful tool in differentiating benign prostatic epithelial structures from prostate cancer and so it is now routinely used clinically by pathologists diagnosing prostate cancer histologically. It is also used in antibody cocktails in combination with positive markers such as α-methylacyl coenzyme A racemase (AMACR or P504S) which is positively expressed in luminal cells in 80%-100% of prostatic adenocarcinomas (Dabir et al., 2012). AMACR is known to play a role in the body's metabolism of fatty acids. Using AMACR alone is of little diagnostic use as it is also expressed in high grade PIN. However, in combination with antibodies against TP63 and cytokeratins it is possible to characterise benign and malignant glands, as well as glands that exhibit PIN which stain positive for both markers, making this antibody cocktail an invaluable diagnostic tool.

There are still many unanswered questions surrounding the role and regulation of *p63* in prostate tissue. The apoptotic stimulating proteins of p53 (ASPP), are a family of proteins which have recently been identified as new regulators of all three members of the p53 family. These proteins may play a key role in the p53 family's regulation of normal development and cancer initiation and/or progression.

1.7 The ASPP family of proteins: regulators of p53, p63 and p73

1.7.1 Overview

The decision-making abilities of *p53* have been the subject of intensive study over past decades. The factors determining whether TP53 will push cells towards programmed cell death or cell cycle arrest are still poorly understood. A better understanding of how *p53*-dependent apoptosis can be reactivated in cancer cells that have retained wild-type *p53* would have important implications for the development of new anti-cancer therapies (Murray-Zmijewski et al., 2008). The *p53* response is governed by many factors, such as the nature, dose or severity of the stress and the cell type exposed to that stress, as well as the presence or absence of cofactors. The combination of these factors ultimately predicts cellular fate. Moreover, it has been proposed that various post-translational modifications of TP53 can influence it's ability to initiate either cycle arrest or apoptosis (Murray-Zmijewski et al., 2008). It is thought that these modifications can influence which proteins can interact with TP53, thereby modulating its overall response. A number of proteins have been identified to cooperate directly with TP53 and influence the expression of its target genes and regulate cell fate. One family of proteins that has been identified to do just this is the ASPP family (Samuels-Lev et al., 2001, Bergamaschi et al., 2003). ASPP proteins have been demonstrated to specifically regulate the apoptotic, but not cell cycle function, of all three members of the p53 family. A detailed understanding of the ASPP proteins may offer new insights into how *p53* discriminates between different cellular pathways, providing another level of regulation for its activity and selectivity (Sullivan and Lu, 2007).

1.7.2 The identification and structure of the ASPP family

The Apoptotic Stimulating Proteins of p53 (ASPP) family was first discovered in 2001 in Prof Lu's laboratory. The family consists of three members termed ASPP1, ASPP2 and iASPP. The most evolutionary conserved member is iASPP which is an inhibitory member and the only one to be identified in *Caenorhabditis elegans (Bergamaschi et al., 2003)*. However, mammals have evolved to express the two additional members ASPP1 and ASPP2 (Samuels-Lev et al., 2001) All three members of the family share sequence similarities in their C-termini (figure 1.7) which contains their signature sequences of Ankyrin repeats, an SH3 domain and a Proline-rich region hence an alternative reason for the name 'ASPP' protein. The C-terminus of ASPP proteins is the preferred binding site of ASPP binding partners which include TP53 and its family members, (Gorina and Pavletich, 1996, Robinson et al., 2008), RelA/p65 (Yang et al., 1999c), Bcl-2 (Naumovski and Cleary, 1996), adenomatous polyposis coli-like (APCL) (Nakagawa et al., 2000), Hepatitis-C core protein (Cao et al., 2004), amyloid beta precursor protein binding protein 1 (APP-BP1) (Chen et al., 2003), PP1 (Helps et al., 1995) and YES-associated protein 1 (YAP1) (Espanel and Sudol, 2001). The N-terminus is only conserved in the evolutionarily younger members ASPP1 and ASPP2.

Figure 1.7 The structure of the three human ASPP proteins

The modular domains are highlighted with different colours, and the amino acid length of the peptide is indicated on the right of each family member. Abbreviation: α, helical domain; Pro, proline rich region; Ank, ankyrin repeats; SH3, Src homology 3 domain.

ASPP2 was the first ASPP protein to be identified, and it remains the best characterised family member to date. It was discovered using a fragment of murine TP53 as bait (aa73-390), in a yeast two-hybrid screen of a library of cDNA sequences generated from a transformed human B-cell line and fused to DNA sequences encoding theGal4 activation domain. The screen identified two novel TP53 binding partners termed 53BP1 and 53BP2 (Iwabuchi et al., 1994). Interestingly, even though both proteins were able to bind to the DNA-binding domain of wild-type TP53, they were shown to be incapable of binding to TP53 mutants R175H and R273H. This was a worthy observation as both of these mutations are common hotspots in human cancer. Although these proteins were shown to interact with the same domain of TP53 they did not themselves show sequence homology. Only 53BP2 contained the signature sequence now associated with the ASPP family. Further studies of 53BP1 revealed that it was a protein involved in the DNA repair process and completely unrelated to 53BP2 (Joo et al., 2002).

53BP2 was initially named p53-binding protein 2 however subsequent studies went on to identify it as a small fragment of ASPP2 (600-1128). Two years after the discovery of 53BP2 a longer fragment (123-1128) was identified in another yeast two-hybrid screen using the N-terminal fragment of Bcl-2 (aa1-188) as bait. This longer fragment was named Bcl-2 binding protein (Bbp). In 2001, the full length protein; ASPP2 was finally identified to be a protein 1128 amino acids long (figure 1.7) (Samuels-Lev et al., 2001). However, it was not until 2004 that it was finally confirmed that 53BP2 was in fact a shorter form of ASPP2. Takahashi and colleagues showed that ASPP2 was a longer version of 53BP2 with an additional 123 amino acids located at the N-terminus. They showed that the gene *TP53BP2* encoded both 53BP2 and ASPP2 and that they were products of alternative splicing at exon 3 (Takahashi et al., 2004).

The first paper describing the sequence of ASPP1 was published in 1998 (Nagase et al., 1998). Nagase et al reported the identification of a new protein named KIAA0771 which was 948 amino acids in length with high sequence homology to 53BP2/Bbp. Later studies revealed that KIAA0771 was a truncated N-terminal form of the longer protein ASPP1, which was finally determined to be 1091 amino acids in length (Samuels-Lev et al., 2001). Moreover, it was in Samuels-Lev et al. publication that ASPP1 was identified as a member of the ASPP family, showing that it had high homology with ASPP2 in its N- and C-terminal regions. The contact residues for TP53 were also found to be conserved between ASPP1 and ASPP2 confirming that ASPP1 could also interact with TP53. In contrast to ASPP2 which has a known splice variant 53BP2, no splice variants have been reported for ASPP1.

The final member of the ASPP family to be identified was the inhibitory member iASPP. As was the case for ASPP2 and ASPP1, it was first discovered as a shorter form, being reported as only 351 amino acids in length and functioning as a RELA/p65 associated inhibitor (RAI) (Yang et al., 1999b). RAI was initially isolated in a yeast two-hybrid screen of human cDNA libraries generated from placenta and brain using p65 (amino acid 176-405) as bait. A partial cDNA isolated in the screen was used to isolate the full length RAI cDNA from a human placenta library. This yielded two cDNAs, one of 2.1kb and one of 2.6kb in length. Both contained the open reading frame (ORF) for RAI and only differed in their 5'-untranslated region. In 2004 a publication by Slee et al. confirmed that the longer transcript detected by Yang in 1998 was in fact the full length protein; iASPP (Slee et al., 2004). Slee and colleagues carried out a BLAST search using the National Centre for biotechnology Information (NCBI) database using the *RAI* sequence and uncovered three sequences with high homology to *RAI*. One of these

sequences encoded for a protein 828 amino acids long. Further comparison between this protein's DNA sequence and the one revealed in the originally study demonstrated complete overlap. Finally, intron-exon mapping showed that the 2.6kb cDNA sequence and the 828 amino acid sequences were derived from the same gene; *PPP1R13L*, and that the 2.1kb shorter ORF encoded RAI. This confirmed that RAI, which was a product of a single base pair insertion that introduced a new stop codon, was in fact a shorter form of the newly identified protein iASPP (Slee et al., 2004).

1.7.3 The function of the ASPP proteins

The most well-documented function of the ASPP family is their ability to influence the apoptotic but not cell cycle arrest function of *p53*, (Trigiante and Lu, 2006). ASPP1 and ASPP2 enhance TP53 binding to the promoters of pro-apoptotic genes such as *BAX*, *PIG3* (*p53-induced gene 3*) and *PUMA* (Slee and Lu, 2003, Bergamaschi et al., 2004) (figure 1.8). iASPP has an opposing effect by inhibiting *p53*-mediated apoptosis and thereby favouring cell proliferation (Bergamaschi et al., 2003) (fig 1.8). The precise mechanism behind ASPP's selectivity towards apoptosis rather than cell cycle arrest has not yet been clarified, however it has been clearly demonstrated using CHIP analysis whereby ASPP1 and ASPP2 have been found to be in complex with TP53 on the promoters of pro-apoptotic genes (Samuels-Lev et al., 2001).

The ASPP family's influence on the apoptotic pathway not only applies to TP53 but extends to all three members of the p53 family (Trigiante and Lu, 2006). Crystal structure analysis showed that the C-terminus of all three ASPP proteins complex with the DNA-binding domain of TP53, TP63 and TP73. However the affinities with which the ASPP's bind to the p53 family of proteins are variable. ASPP2 and iASPP bind TP53 with a similar affinity, however iASPP was found to bind TP63 and TP73 with a 3-fold higher

affinity than ASPP2. These variations may be explained by differences in the eight residues involved in the DNA binding domain of TP53, as five residues are identical and three are different between ASPP2 and iASPP (Robinson et al., 2008). Intriguingly many *p53* mutations have been demonstrated to disrupt the binding of ASPP2 and TP53 *in vitro* (Gorina and Pavletich, 1996) supporting the tumour suppressive role of ASPP2 in promoting apoptosis.

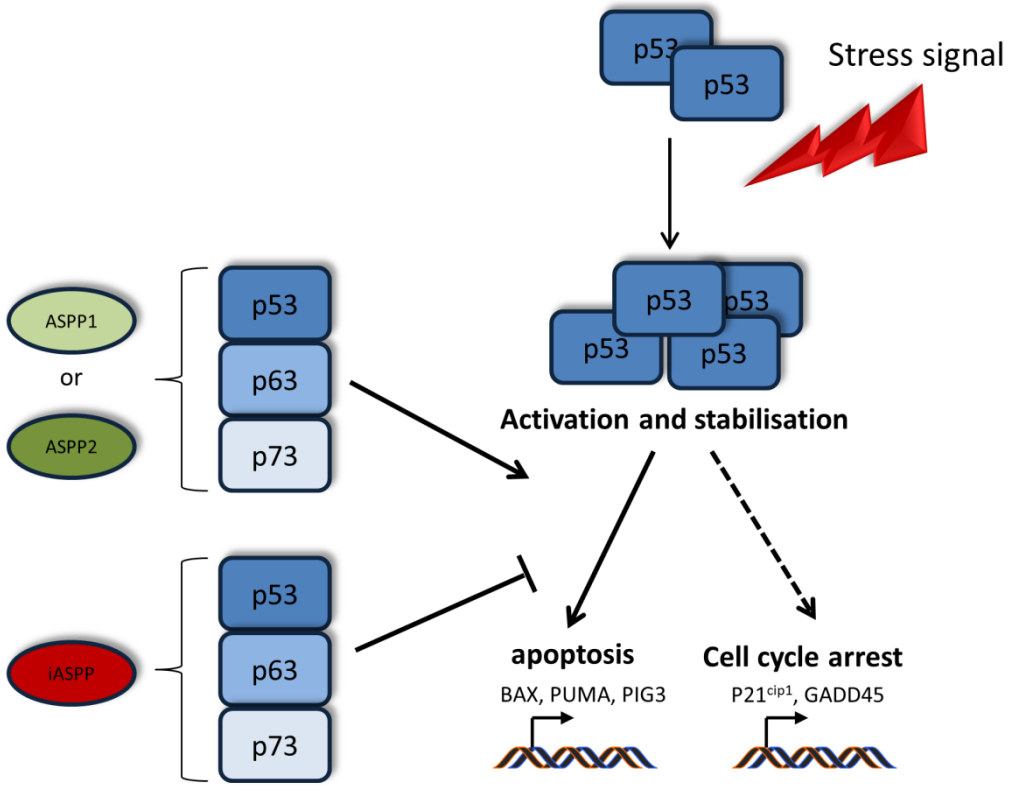

Figure 1.8 Schematic model of ASPP function

Stress signals lead to the activation and stabilisation of TP53. In response to a particular stress TP53 transactivates a variety of genes involved in apoptosis such as *BAX*, *PUMA*, *PIG3*, or cell cycle *GADD45* and *p21*^{cip1}. The ASPP proteins mediate the apoptotic function of TP53 with ASPP1 and ASPP2 enhancing the binding of TP53 to the promoters of pro-apoptotic genes, whereas iASPP inhibits this interaction. The ASPP family also bind TP63 and TP73 and modulate their apoptotic function.

Although the C-terminus of the ASPP proteins appears to be the only region involved in direct binding to TP53, the N-terminus of ASPP1 and ASPP2 has been shown to stimulate *p53*-dependent apoptosis and has been shown to be required for maximal transactivation activity (Samuels-Lev et al., 2001). Furthermore, structural studies of ASPP1 and ASPP2 revealed a small G protein Ras-association domain in the N-terminus that was shown to directly interact with Ras-GTP to stimulate Ras-induced senescence in nontransformed human cells (Wang et al., 2013b, Wang et al., 2013a). This study highlights the multi-functionality of ASPP2, providing evidence that ASPP2 plays an important role as an inducer of p53-independent cellular senescence as well as p53-mediated apoptosis. Additionally, a study carried out by Tidow et al. revealed that ASPP2 has a N-terminal ubiquitin-like fold which may mediate its interaction with other components of the apoptotic network, providing further mechanisms by which ASPP2 could regulate apoptosis (Tidow et al., 2007). As the N-terminal domain is the main differentiator between pro-apoptotic (ASPP1 and ASPP2) and anti-apoptotic (iASPP) members of the ASPP family, it could be postulated that it plays a crucial role in promoting apoptosis, and iASPP may simply function as a competitive inhibitor of ASPP1/2 in the context of p53-mediated apoptosis. However recent studies have demonstrated that the interaction between ASPP2-p53 and iASPP-p53 are slightly different, in that ASPP2 binds with high affinity to the DNA-binding domain of TP53 and weakly to the proline-rich region of TP53, whereas iASPP behaves in the opposite manner. This would imply that iASPP may not be a simple competitor of ASPP2 as one might expect them to bind preferentially to the same region on the TP53 protein. This second binding region could suggest a mechanism whereby iASPP can inhibit ASPP2-p53 interaction. iASPP is able to engage the proline-rich region of TP53 even in the

presence of ASPP2. This may interfere with the pro-apoptotic activity of TP53 by altering the precise positioning of the DNA binding domain at the promoters of apoptotic genes (Ahn et al., 2009, Bergamaschi et al., 2006).

As well as influencing the apoptotic activity of the p53 family iASPP has been implicated as a regulator of *p63* in the prevention of cellular senescence. A recent study using transgenic mice, in which iASPP expression was controlled by the Cre/LoxP system, showed that iASPP was able to prevent premature cellular senescence, and that loss of iASPP resulted in increased differentiation of primary keratinocytes both *in vitro* and *in vivo*. This study also showed that iASPP is able to bind to TP63 and inhibit the transcriptional activity of both TAp63 and ΔNp63 *in vitro,* influencing the expression levels of TP63-regulated genes (Notari et al., 2011). These data were further confirmed by Chikh and colleagues who showed that silencing iASPP in keratinocytes using RNA interference promotes and accelerates a differentiation pathway (Chikh et al., 2011). These studies suggest that iASPP is not just a regulator of apoptosis but also a mediator of epithelial homeostasis in stratified epithelia.

The ASPP family of proteins has been identified as binding partners to a long list of interacting proteins with diverse cellular functions, and it is reasonable to conceive that ASPPs have multiple roles in the cell, above and beyond mediating apoptosis and cellular senescence. Some of the new functions have already been characterised, providing intriguing new perspectives in the study of this family of proteins. ASPP2 has been identified as a binding partner of Par-3 and plays a key role in the maintenance of apical-basal cell polarity *in vitro* and *in vivo* (Sottocornola et al., 2010, Cong et al., 2010). ASPP2 has also been shown to act as a negative regulator of Drosophila *dsrc*, which is an important kinase involved in the establishment of cell-cell junctions in epithelial cells

(Langton et al., 2007). Taken together these findings suggest that ASPP2 plays a functional role at the cell junctions as well as in the nucleus. iASPP has also been implicated as a junctional protein through its binding to desmoplakin, a protein involved in the integrity of desmosomes (personal communication, Mario Notari) However, despite these recent findings, for many of the other interactions, other possible apoptotic-independent functions through other protein: protein interactions remain to be elucidated.

1.7.4 Regulation of the ASPP proteins

There are currently three identified ways in which the ASPP proteins can be regulated. Firstly, the transcriptional factor E2F-1 has been shown to bind to the promoters of ASPP1 and ASPP2 resulting in an increase of both mRNA and protein levels. E2F-1 expression is induced in response to various DNA damaging agents, and once activated it can induce apoptosis in both a p53-dependent and independent manner. E2F-1 is therefore able to cooperate with TP53 to promote apoptosis through the transcriptional regulation of the pro-apoptotic ASPP family members (Chen et al., 2005a, Fogal et al., 2005, Hershko et al., 2005). Secondly *ASPP1* and *ASPP2* have been shown to be silenced by promoter hypermethylation in a number of different studies, suggesting that epigenetic modification is another way in which the ASPP proteins can be regulated (Liu et al., 2005, Zhao et al., 2010). Finally ASPP2 has been shown to be a target for proteasomal degradation following ubiquitylation of its' central core. Treatment of cells with the proteasomal inhibitor bortezomib, or anthracycline-based chemotherapeutic agents, increased ASPP2 protein levels but not RNA levels. (Zhu et al., 2005). Despite the knowledge that has been gathered concerning ASPP1 and ASPP2, little is known about the transcriptional or translational regulation of iASPP. Due to the fact that there are different splice variants it is reasonable to hypothesise that there may be some level of

transcriptional regulation via alternative splicing. However, the mechanisms involved in the regulation of the ASPP family remain poorly understood.

One aspect of ASPP regulation that will be important to elucidate is how the cellular localisation of the proteins is regulated. Some regulation is due to alternative splicing, for example RAI, the shorter form of iASPP, has been shown to have a stronger inhibitory effect on p53-mediated apoptosis compared to full-length iASPP (Bergamaschi et al., 2003). This is due to it being almost exclusively localised to the nucleus. Full length iASPP, in contrast, is predominantly cytoplasmic, suggesting that the additional N-terminal portion of the protein might represent a regulatory domain involved in its retention in the cytoplasm. This is also true for ASPP1/2 as they are both predominantly cytoplasmic and removal of the N-terminal half of ASPP2 causes its C-terminal part, which contains a nuclear localization signal (Sachdev et al., 1998), to localize in the nucleus (Samuels-Lev et al., 2001). Owing to the fact that ASPP proteins have distinct functions depending on their precise cellular location it would be of great value to uncover which external stimuli and post-translational modifications can trigger the shuttling of the ASPPs to different sub-cellular compartments.

1.7.5 The ASPP proteins and development

Studies conducted in genetically modified mice show that the ASPP proteins play a role in normal development independently of TP53's apoptotic activity. All three ASPP-deficient mice display developmental defects not ascribable to functions of the p53 family of proteins. Their role in development must therefore be due to interaction with other, binding partners. Interestingly, the individual ASPP mutant mice have very distinct phenotypes, suggesting that in particular tissues they have a tissue specific non-redundant function during development.

ASPP1-deficient mice have a lymphatic vasculature phenotype. ASPP2-knockout mice have been shown to have severe defects of the central nervous system. ASPP2-deficient pups in a mixed 129SvxC57BL/6J background died around the time of birth due to hydrocephalus and neural tube closure defects (Sottocornola et al., 2010, Vives et al., 2006). These mice also showed extensive loss of cell polarity in the neuroepithelium, and abnormal expansion of neuroprogenitor cells within the brain and retina. Consequently ASPP2 has been implicated in maintaining the integrity of tight junctions through its influence on the cellular localisation of Par3, a component of the Par3/aPKC/Par6 polarity complex (Sottocornola et al., 2010).

iASPP-deficient mice suffer from sudden death due to cardiomyopathy. They also have a defect in eyelid closure and skin abnormalities, due to the effects on the expression of several differentiation markers together with changes in both the number and orientation of hair follicles (Notari et al., 2011). These defects in skin result in a wavy hair phenotype, and these observations were also reported in Waved 3 (Wa3) mice which were documented as having a spontaneous mutation in the *NFkB interacting protein 1* (*Nkip1*) gene. This gene was subsequently identified as being the full length *iASPP* gene. These mice were shown to have reduced postnatal viability and failed to thrive due to defects in the shape and size of the heart (Herron et al., 2005). Further research showed that iASPP is important for the integrity of cardiomyocytes, and iASPP may play a structural role in coping with mechanical stress by mediating contacts between the cytoskeleton and desmosomal components (personal communication, Mario Notari). In the skin the defect was ascribable to the fact that iASPP cooperates with *p63* in order to maintain homeostasis of the stratified epithelium, and loss of iASPP results in a decrease in cell proliferation coupled with an increase in differentiation (Notari et al., 2011).

The recent generation of these mouse models has given great insight into the role of the ASPP family of proteins in normal embryonic development. The immediately obvious phenotypes associated with these mouse models have already been described, however there may still be more subtle differences caused by the loss of these individual proteins that have not yet been uncovered.

1.7.6 The ASPP proteins and cancer

Given the fundamental role of the ASPP proteins in the regulation of apoptosis, it is logical to expect them to have a central role in tumourigenesis. Consistent with a role for ASPP1/2 in inducing apoptosis, and for iASPP in inhibiting this process, several lines of evidence have accumulated over the past decade to support the hypothesis that *ASPP1/2* are tumour suppressor genes and *iASPP* is a proto-oncogene. One of the major pieces of evidence to suggest that ASPP2 has a tumour suppressive role came from a study carried out by Vives et al. ASPP2-deficient mice in the mixed 129SvJ x C57BL/6 background were shown to exhibit postnatal lethality. ASPP2 heterozygous mice were viable but had a high incidence of spontaneous and radiation-induced tumour formation, compared with wild-type mice (Vives et al., 2006). Additionally, they showed that spontaneous tumour formation in the heterozygous mice was not accompanied by a loss of heterozygosity for *ASPP2*, suggesting that *ASPP2* is a haploinsufficient tumour suppressor gene. Moreover, a number of studies have reported that ASPP1 and ASPP2 may be down-regulated in human tumours such as breast cancer (Samuels-Lev et al., 2001), hepatitis B virus positive hepatocellular carcinomas (Zhao et al., 2010) and endometrial endometrioid adenocarcinoma (Liu et al., 2010). Furthermore, lower levels of ASPP1 and ASPP2 expression have also been linked to poor clinical outcome in leukaemia (Roman-Gomez et al., 2005) and diffuse large B-cell lymphomas (Lossos et al., 2002).

In contrast to ASPP1/2's tumour suppressive function, iASPP is believed to have oncogenic properties as significantly higher levels of iASPP have been detected in a number of human tumours including breast carcinoma (Bergamaschi et al., 2003), acute leukaemia (Zhang et al., 2005), non-small cell lung cancer (Chen et al., 2010) head and neck squamous cell carcinoma (HNSCC) (Liu et al., 2012) and prostate cancer (Zhang et al., 2011). In addition, iASPP has been shown to cooperate with Ras, E1A and E7, but not mutant *p53* to transform cells *in vitro* due to its ability to inactivate *p53*. In contrast to this ASPP1 and ASPP2 suppressed the transforming activity of Ras and E1A (Bergamaschi et al., 2003). Furthermore, increased expression of iASPP also conferred resistance to ultraviolet radiation and to cisplatin-induced apoptosis in U2OS and MCF7 cell lines, due to its influences over TP53 function. Consistent with its role as an inhibitor of TP53 and as a potential oncogene, iASPP over-expression was tolerated in U2OS cells expressing wild type *p53*, whereas ectopic expression of ASPP1 and ASPP2 resulted in apoptosis (Bergamaschi et al., 2003).

In addition to iASPP's role in p53-mediated apoptosis there is emerging evidence to suggest that it also plays a role in cell proliferation and tumourigenesis, even in cells lacking *p53* function. This was demonstrated in a study carried out using the prostate cancer cell lines PC3 and DU145 (Zhang et al., 2011). The ability of iASPP to continue to influence apoptosis, even in a *p63* and *p53* null setting, may be due to the fact that iASPP regulates all three members of the p53 family. Hence iASPP may still be able to promote tumour growth by influencing p73-dependent apoptosis (Cai et al., 2012). The targeting of iASPP may therefore have great therapeutic value in all cancers that demonstrate an increase in expression, regardless of *p53* status.

The Aim of the study

The aim of this work was to investigate the role of iASPP, a member of the evolutionarily conserved ASPP (ankyrin repeats, SH3 domain and proline-rich region containing protein) family of proteins, in prostate gland development and prostate cancer progression. Firstly, the question of iASPP's potential role in the development and homeostasis of the prostate gland was be addressed. This was done by characterising the prostate gland of iASPP-deficient mice, taking advantage of histological techniques in order to analyse prostate epithelial architecture and cell lineage markers. Aspects of prostate epithelial cell proliferation, identity and cell death were also investigated.

Secondly, the expression pattern of iASPP in both benign and malignant human prostate tissue was explored. This was carried out by analysing tissue microarrays containing prostate tissue taken from patients with benign prostatic hyperplasia and from patients with varying degrees of prostate cancer. Additional tissue sections taken from patients with invasive tumours were also analysed in order to investigate iASPP expression in relation to cellular invasiveness. By analysing the expression pattern in these various tissue samples, and by analysing the available clinical data, the role of iASPP in disease progression was investigated.

Finally, aspects of prostate cancer cellular behaviour were examined in prostate cancer cell lines that have been subjected to siRNA-mediated knockdown of iASPP. Questions regarding iASPP's potential role in invasion, motility and drug resistance were addressed, as well as its functional relationship with mutant *p53*. Taken together these results provide new insights into the biological function of iASPP in prostate cancer progression.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Reagents

All chemicals, unless otherwise stated, were obtained from Sigma (Sigma, MO, USA) or BDH Chemicals (UK). Autoradiography films (Hyperfilm) and ECL (Enhanced Chemi-Luminescence) reagents were purchased from Amersham Pharmacia Biotech (UK). Nitrocellulose membrane was purchased from Whatman, Germany. All tissue culture dishes and flasks were obtained from Corning Incorporated (Corning, NY, USA). The avidin/biotin peroxidase kit and DAB (3,3'diaminobenzidine) substrate were obtained from Vector Labs

Alcian Blue

1% (W/V) final solution of Alcian blue 8G was prepared in 3% glacial acetic acid pH was adjusted to 2.5

Ammonium Persulphate (APS)

10% (w/v) stock solution was prepared in water and stored at -20˚C in single-use aliquots.

Bicalutamide Stock Solution

8.61mg of bicalutamide was dissolved in 1ml of ethanol to make up a 20mM stock and stored in aliquots at -20˚C

Blocking Solution

10% (w/v) fat-free milk (Marvel, UK) was prepared in 1X TBS-T.

Citric Acid Buffer

10mM citric acid solution was prepared in water and the pH adjusted to 6.0 with NaOH

cOmplete™ protease inhibitor cocktail

cOmplete™ protease inhibitor (Roche, Boehringer Mannheim, Germany) tablet was dissolved in 2.0ml of sterilised distilled water as a 25x stock solution that is stable at -20˚C for 12 weeks.

DAPI staining solution

Powdered DAPI was dissolved at 1mg/ml and stored at -20°C in aliquots.

Dihydrotestosterone Stock Solution

5.81 mg of DHT was dissolved in 1ml ethanol to make up a 20mM stock and stored in aliquots at -20˚C

Docetaxel Stock Solution

5mg of Docetaxel was dissolved in 6.2ml DMSO to make a 1mM stock and stored in aliquots at -20˚C

EDTA Buffer

0.37g Ethylenediamine Tetraacetic Acid (EDTA), Disodium salt Dihydrate

1.21g Tris (hydroxymethyl) aminomethane 99 +% for biochemistry

The solution was dissolved in 800ml of distilled water and the pH was adjusted to pH 9. Finally, 500ul of Tween-20 was added and the solution was made up to 1L with distilled water.

EDTA Solution

A 0.5M $C_{10}H_{14}N_2O_8Na_2.2H_2O$ (EDTA) stock solution was made by dissolving 18.6 g of EDTA in 70ml distilled water. The pH was adjusted to pH 8.0 with NaOH and the volume made up to 100ml with distilled water.

Freezing medium

10% (v/v) DMSO

40% (v/v) FBS

50% (v/v) Complete medium (RPMI 1640, keratinocyte-SPF or DMEM)

Freezing medium was prepared fresh each time.

IP Lysis Buffer

50mM Tris pH 7.4

0.15M NaCl

2mM EDTA

1% NP-40

The final volume was made up with dH_20

5x Laemmli Sample Buffer

10% SDS

250mM 1M Tris pH 6.8

0.1% Bromophenol blue

50% Glycerol

The final volume was made up with dH_20

Mowiol Solution

6ml Glycerol

2.4g Mowiol 4-88 (Calbiochem)

12ml Tris-HCL 200mM (pH8.5)

6ml distilled water

The solution was made in a 50ml falcon tube and heated to 60˚C with constant agitation until the mowiol was completely dissolved. The solution was then passed through a 0.45µm filter and stored in aliquots at -20˚C. Before use 2.5% w/v 1,4-diazabicylo[2.2.2]octane (DABCO) was added.

Paraformaldehyde Solution (4%)

The solution was made by adding 100µl of 10N NaOH and 4g of paraformaldehyde into 98ml of RNAse free PBS. The solution was then heated by placing the bottle of solution into a beaker containing distilled water that had been per heated to 62-64˚C. The solution was kept at this temperature until fully dissolved. The solution was then filtered and stored in aliquots at -20˚C.

Ponceau S Staining Solution (10 X)

5% (v/v) Acetic acid

2% (v/v) Ponceau S (sodium salt)

30% (w/v) Trichloroacetic acid $CCl_3.COOH$

30% (w/v) 5-sulfosalicyclic acid $C_7H_6O_6S.2H_2O$ (Sigma, MO, USA)

The solution was dissolved in water to a 1x dilution before use.

Protein G Sepharose

Stored in 20% ethanol at 4˚C (Pharmacia Biotech). Protein G Sepharose beads were washed 3 times with cold PBS and resuspended in one volume of PBS before use.

Protein Molecular weight markers

Prestained protein markers were purchased from New England Biolabs (UK) and were used as a size standard for SDS-polyacrylamide and tris-acetate gel electrophoresis.

Phosphate Buffered Saline (PBS)

12.5mM NaCl

1mM Sodium dihydrogen phosphate, NaH_2PO_4

1.6mM Disodium dihydrogen phosphate, Na_2HPO_4

The pH was adjusted to 7.0 and autoclaved.

Permabilisation solution for prostate spheroids

The solution was freshly prepared each time by dissolving 10% (v/v) FBS and 0.5% (v/v) Triton X-100 in PBS. The solution was kept at 4˚C until required.

SDS Solution

A 10% (w/v) solution of sodium dodecyl sulphate (SDS) was dissolved in water and stored at room temperature.

10x SDS-PAGE Running Buffer

720g Glycine

150g Tris

50g SDS

Final volume adjusted to 5L with distilled water

10x SDS-PAGE Transfer Buffer

725g Glycine

145g Tris

Final volume adjusted to 5L with distilled water. The buffer was then used with 20% ethanol as 1x solution.

Stripping Buffer

62.5mM 15.5 ml of 1 M Tris-HCl, pH 6.7

100mM 1.75 ml of β-mercaptoethanol

2% 5g SDS

Make up to 250 ml in distilled water.

Tris Stock solutions

Tris base was dissolved in water to provide 1M and 1.5M solutions which were pH adjusted with concentrated HCl.

Tris Buffered Saline Tween (TBS-T)

8g of NaCl

0.2g of KCl

3g of Tris base

500µl Tween-20

The above were dissolved in 800ml of dH_20 pH adjusted to pH 7.4 with concentrated HCl and made up to a final volume of 1L.

Urea Buffer

8M Urea

1M Thiourea

0.5% CHAPS

50mM DTT

24mM Spermine

Once buffer was prepared it was store at -20˚C in single-use aliquots.

Water

Nanopure water (type I) generated from MilliQ water system was used for all procedures

2.1.2 SDS-polyacrylamide Gels

	Resolving Gels					Stacking
	6%	**8%**	**10%**	**12%**	**15%**	**4 %**
Acryl/Bis	2 ml	2.7 ml	3.3 ml	4.0 ml	5.0 ml	1.3 ml
1.5 M Tris-HCl pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml	--
1.0 M Tris-HCl pH 6.8	--	--	--	--	--	2.5 ml
10% SDS	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl
10% APS	100 µl	100 µl	100 µl	100 µl	100 µl	50 µl
TEMED	10 µl	8 µl	5 µl	5 µl	4 µl	10 µl
Distilled Water	5.3 ml	4.6 ml	4.0 ml	3.3 ml	2.3 ml	6.1 ml
Total volume	10 ml	10 ml	10 ml	10 ml	10 ml	10 ml

Table 2.1 Preparation of SDS-polyacrylamide gels

All resolving and stacking gels were prepared using 30% acrylamide/bis-acrylamide (Acryl/Bis) 29:1 (NBL, UK or BioRad, UK). Values given are per 10ml of gel required. Abbreviations: APS: Ammonium Persulphate, TEMED: N,N,N',N',-tetramethyethylenediamine, Tris: (Tris(hydroxymethyl)aminomethane), sodium dodecyl sulphate (SDS).

2.1.3 Antibodies

Antigen (Name)	Host	Type	Source (catalogue #)	Application
BrdU	rat	monoclonal	Abcam (ab6326)	IF 1:200
Claudin-1	rabbit	polyclonal	Abcam (ab15098)	IF 1:200
Connexin-43	rabbit	polyclonal	Abcam (ab11370)	IF 1:500
Cytokeratin 19	rabbit	polyclonal	Abcam (ab53119)	WB 1:500
Cytokeratin 5	rabbit	polyclonal	Abcam (ab53121)	WB 1:750, IF 1:3000
Cytokeratin 8	rabbit	polyclonal	Abcam (ab59400)	WB 1:500, IF 1:200
Desmin	rabbit	polyclonal	Abcam (ab15200)	IF 1:250
Desmonplakin	mouse	monoclonal	Progen (65146)	IF 1:4 of working solution
E-cadherin	rabbit	polyclonal	Abcam (ab53033)	IF 1:1000
iASPP (LX049.3 purified)	mouse	monoclonal	Ascite	IF 1:250, IHC 1:250
iASPP (LX049.3)	mouse	monoclonal	Ascite	WB 1:1000
iASPP (N1)	rabbit	polyclonal	Ascites	WB 1:1000
ki67	rabbit	polyclonal	Vector Labs	IHC 1:200
Ku80	mouse	monoclonal	Thermo Fisher	WB 1:2000
p53 (CM1)	rabbit	polyclonal	Ascites	WB 1:1000
p53 (DO-1)	mouse	monoclonal	Ascites	WB 1:1000
p53 (DO-7)	mouse	monoclonal	Santa-cruz (sc-47698)	IHC 1:10
p63 (4A4)	mouse	monoclonal	Santa Cruz (sc-8431)	WB 1:1000, IF 1:200, IHC 1:200
PSA	rabbit	polyclonal	Abcam (ab537774)	WB 1:1000
Synaptophysin	mouse	monoclonal	Millipore MAB5258-20UG	WB 1:1000, IF 1:750
α- Smooth Muscle Actin	rabbit	polyclonal	Abcam (ab5694)	IF 1:250
β-Actin	mouse	monoclonal	Santa Cruz (sc-47778)	WB 1:1000
β-catenin	mouse	monoclonal	BD (610154)	IF 1:250
β-tubulin	mouse	monoclonal	Abcam (ab11308)	WB 1:5000

Table 2.2 List of primary antibodies

Abbreviations – WB; western blot, IF; immunoflourscence, IHC; immunohistochemistry

Antibody Name	Host	Source	Application
Anti-Mouse immunoglobulins/HRP	Rabbit	Dako	WB 1:2000
Anti-Rabbit immunoglobulins/HRP	Swine	Dako	WB 1:2000
Alexa Fluor® 488 F(ab')2 fragment Anti-Rabbit	Goat	Invitrogen	IF 1:400
Alexa Fluor® 488 F(ab')2 fragment Anti-Mouse	Goat	Invitrogen	IF 1:400
Alexa Fluor® 488 Anti-Mouse IgG (H+L)	Donkey	Invitrogen	IF 1:400
Alexa Fluor® 647 Anti-Mouse IgG (H+L)	Donkey	Invitrogen	IF 1:400
Alexa Fluor® 488 Anti-Rabbit IgG (H+L)	Donkey	Invitrogen	IF 1:400
Alexa Fluor® 647 Anti-Rabbit IgG (H+L)	Donkey	Invitrogen	IF 1:400
Alexa Fluor® 647 Anti-Rat IgG (H+L)	Goat	Invitrogen	IF 1:400
Biotinylated Anti-Mouse IgG	Goat	Vector Labs	IHC 1:250
Biotinylated Anti-Rabbit IgG	Goat	Vector Labs	IHC 1:250

Table 2.3 List of secondary antibodies

Abbreviations – WB; western blot, IF; immunofluorescence, IHC; immunohistochemistry

2.1.4 Cell lines

Cell Line	Origin	Source	Description
PC3	Prostate	American Type Culture Collection (Manassas)	Low levels of AR expression, rapidly proliferating and highly invasive, $p53^{-/-}$ no protein detected
DU145	Prostate	American Type Culture Collection (Manassas)	Low levels of AR expression, rapidly proliferating and highly invasive. $p53^{-/-}$ both alleles are mutated P223L exon 6, V274F exon 8
LNCaP	Prostate	American Type Culture Collection (Manassas)	High levels of AR expression, slowly proliferating and relatively non-invasive, $p53^{+/+}$
LNCaP-LN3	Prostate	Gifted by Curtis Pettaway, M.D.Anderson, Houston.	Reduced Androgen sensitivity, serially passaged in mice, slowly proliferating and highly invasive $p53^{+/+}$
RWPE-1	Prostate	Gifted by Mercedes Rodriguez, Hammersmith Hospitial	HPV18 immortalised histologically normal prostate epithelial cells (peripheral zone) p53 function impaired
NHF (Normal human fibroblast)	Foreskin	Ludwig Institute for Cancer Research (Oxford)	Immortalised cells $p53^{+/+}$

Table 2.4 List of cell lines and relevant information

2.2 Methods

2.2.1 Tissue Culture

Basic media

RPMI 1640, keratinocyte serum-free medium, Dulbecco's Modified Eagle Medium (DMEM) and Nutrient Mixture F12 (DMEM/F12) was purchased from Gibco (UK). Prostacult^{TM} Basal Medium was purchased from Stem Cell Technologies. All culture medium was stored at 4˚C.

Media supplements

Fetal calf serum was purchased from PAA laboratories and tested for its ability to support the growth of various cell lines. FCS was heat inactivated for 30 minutes at 55˚C and stored at -20˚C in 50ml aliquots. Penicillin/Streptomycin was purchased from Gibco-BRL at 10000 units/ml and stored at -20˚C in 5ml aliquots. A final concentration of 200 units/ml was used. The following supplements were purchased from Invitrogen and stored at -20˚C in 5ml aliquots; 100x sodium pyruvate, 100x MEM vitamins and 100x MEM non-essential amino acids.

Maintenance of Cell Lines

Human prostate cancer cell lines PC3, DU145 and LNCaP and LNCaP-LN3 were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1x sodium pyruvate, 1x MEM vitamins, and 1x MEM non-essential amino acids. RWPE-1 cells were grown in keratinocyte serum-free medium with the addition of 2.5µg rh EGF and 25mg Bovine Pituitary Extract. NHF were maintained in DMEM supplemented with 10% fetal calf serum (FCS). All cell lines were supplemented with100 units/ml penicillin and

100 units/ml streptomycin and incubated in T75 flasks (Corning) in a Heraeus incubator at 37°C in a 5% CO_2 atmosphere. Medium was changed every 2 – 4 days depending on cell line requirements. On reaching confluency cells were washed with 1x PBS and incubated with 2ml of pre-warmed 0.05% trypsin-EDTA (Gibco) at 37˚C until the cells detach from the flask. The trypsin was neutralised by the addition of 8ml of pre-warmed appropriate growth media. Cells were then seeded in to new flasks at the desired cell density.

Mouse primary prostate cells were cultured in Prostacult medium supplemented with 10ng/ml of rh EGF and rh bFGF and 4µg/ml of Heparin in a 12.5cm^2 flask purchased from Santa Cruz. Media was changed every 48hrs.

Freezing/thawing of cells

Cells were grown to about 80% confluency and collected by trypsinization (as described above). The cell pellet was resuspended in the appropriate amount of freezing medium and aliquoted in cryovials (Corning). The vials were then labeled and cooled at the rate of 1˚C per minute in a -80˚C freezer (New Brunswick Scientific) for at least 24hrs, before being transferred to a liquid nitrogen tank for long term storage.

To thaw cells from liquid nitrogen stock, vials were placed in the 37 ˚C water bath for 2 minutes and then transferred to a T75 flask with the appropriate pre-warmed fresh growth medium and kept in the 37 °C incubator overnight for recovery.

2.2.2 Protein Manipulation

Cells growing in flasks or 6 well plates were washed with pre-warmed 1x PBS and trypsinised as described above. The cells were then collected and centrifuged at 1000rpm for 3 minutes. The supernatant was aspirated off and discarded. The cell pellet was washed in 1x PBS and again centrifuged at 1000rpm for 3 minutes. The supernatant was aspirated off and discarded and the cell pellet was resuspended in an appropriate amount of urea buffer. The solution was left for 30 minutes at RT, and then centrifuged at 13,200rpm for 20 minutes. The supernatant was then collected and used for protein quantification.

Mouse tissue was homogenized on dry ice in a pestle and mortar. The powered tissue was collected in a cold eppendorf and the appropriate amount of urea buffer was added. The resultant lysate was sonicated 3 times for 10 seconds and left for 30 minutes on ice. Samples were centrifuged at 13,200rpm for 20 minutes at 4˚C, and repeated centrifugations were performed until no pellet was obtained. The supernatant was collected and used for protein quantification.

Protein concentration determination

The protein concentrations of cell extracts were determined using the BioRad protein assay reagent system. 1µl of cell lysate was mixed with 200µl of 1x BioRad assay reagent and then measured at 595nm in the spectrophotometer (Anthos Labtech instrument). All samples were measured in duplicate and the absorbance was compared against a standard curve made at the same time from known concentrations of bovine serum albumin (BSA; Sigma-Aldrich) in the same solutions, using the same method.

SDS-polyacrylamide Gels

The Glass gel plates were cleaned with water and ethanol, dried and assembled in to the casting units (Biorad). The acrylamide content of the gels varied between 6% and 15% depending on the size of the protein of interest. The acrylamide gel was overlaid with 70% isopropanol solution and left to polymerise. After polymerisation, the isopropanol was washed away with water and replaced by a 4% stacking gel with a comb inset containing the appropriate number and size wells.

SDS-polyacrylamide gel electrophoresis (PAGE)

Known concentrations of protein were mixed with appropriate volumes of 5x SDS-PAGE Sample Loading Buffer and boiled for 5 min. Equal amounts of protein were loaded in each lane of the stacking gel. Gels were run at 120V for approximately 1.5hrs; depending on arcylamide percentage. Gels were run in 1x running buffer using the Biorad system. Pre-stained protein markers were purchased from New England Biolabs and used as size standards.

Immunoblotting

Once the SDS-PAGE running process was complete, the gel was transferred in a wet cassette unit and proteins blotted onto nitrocellulose membrane (Whatmann) for 2.5hrs at a constant voltage of 80V. The transfer tank was filled with 1x transfer buffer containing 20% ethanol. The nitrocellulose membrane was then stained with Ponceau S solution (purchased as a 0.1% (w/v) solution in 5% acetic acid) to determine the success of the protein transfer and equal loading of the lanes. The membrane was washed in water and blocked in 5% fat-free milk (Marvel, UK) in 1x TBS-Tween at RT for 1hr. Primary

antibody was added according to the recommended concentration, diluted in 5% fat-free milk/TBS-Tween for 3hrs RT or overnight at 4˚C. Following incubation with the primary antibody, the membrane was washed 3 times in TBS-T and incubated in 5% fat-free milk/TBS-Tween containing an appropriate secondary antibody for 1h at RT. The membrane was then washed 3 times for 5minutes in TBS-T followed by a final 3 rinses in distilled water. The results were visualised by enhanced chemiluminescent detection ECL (Amersham Biosciences) using X-ray films (Fujifilm). If it was necessary to reprobe the membrane with another primary antibody, the membrane was immediately reprobed or incubated in stripping buffer for 30 minutes at 55 ˚C. Following these steps, the membrane was reblocked in 5% fat-free milk/TBS-Tween, before being incubated with the new primary antibody.

Immunoprecipatation

Cells grown on 15cm dishes were washed with cold 1x PBS and lysed with NET 1% NP-40 IP buffer containing protease inhibitors. The cells were scraped using a sterile disposable cell scraper (Greiner). The lysate was transferred to an eppendorf and kept on ice for 20 minutes followed by centrifugation at 13,200rpm at 4˚C for 20 minutes. The resulting supernatant was transferred to a new eppendorf, and the protein concentration was determined using the Biorad assay system. 50µl of each lysate was removed to use as the input control. Protein G sepharose beads were washed in cold 1xPBS 3 times and made up to a ratio of 1:1 with PBS, beads were stored at 4˚C until required. Preclearing was performed by adding 30µl of slurry beads to each lysate and rotating for 60 minutes at 4˚C. Lysates were then centrifuged for 3 minutes at 4000rpm at 4˚C. Supernatant was then transfer to a new eppendorf and 10µl of antibody was added along with 30µl of fresh

protein G Sepharose beads. Lysates were then rotated overnight at 4˚C. Beads were washed 5 times in NET IP buffer with 0.2% NP-40 each time being centrifuged at 4000rpm for 3 minutes. After the last wash the supernatant was removed and 30µl of 5x sample buffer was added to the beads and mixed then boiled for 5 minutes. Samples were centrifuged at full speed for 30 seconds. Sample buffer was then loaded using a gel loading tip to prevent disturbance of the beads.

2.2.3 Cell based assays

Small Interfering RNA (siRNA) Transfection

A pool of 4 siRNA oligos specifically targeting iASPP and a control oligo (pGL2) targeting luciferase were obtained (Dharmacon, Inc.,Lafayette, CO). 40nM siRNA (4µl of 20µM stock) was added to 196µl of OptiMEM medium and 2.5µl of DharmaFECT 2 was added to 197.5µl of HBSS medium and left to stand for 10 minutes at RT. The 2 solutions were then mixed and left to stand for 20 minutes at RT. The final 400µl solution was then added to a 6 well tissue culture dish. Cells were plated in normal media on top of the siRNA solution at a density of 200,000 cells per well, the final volume was made up to 2ml of normal culture media per well. Samples for Western blot analysis were taken 72hrs later. The knockdown efficiency achieved for each cell line used in the functional study of iASPP is shown in figure 2.1.

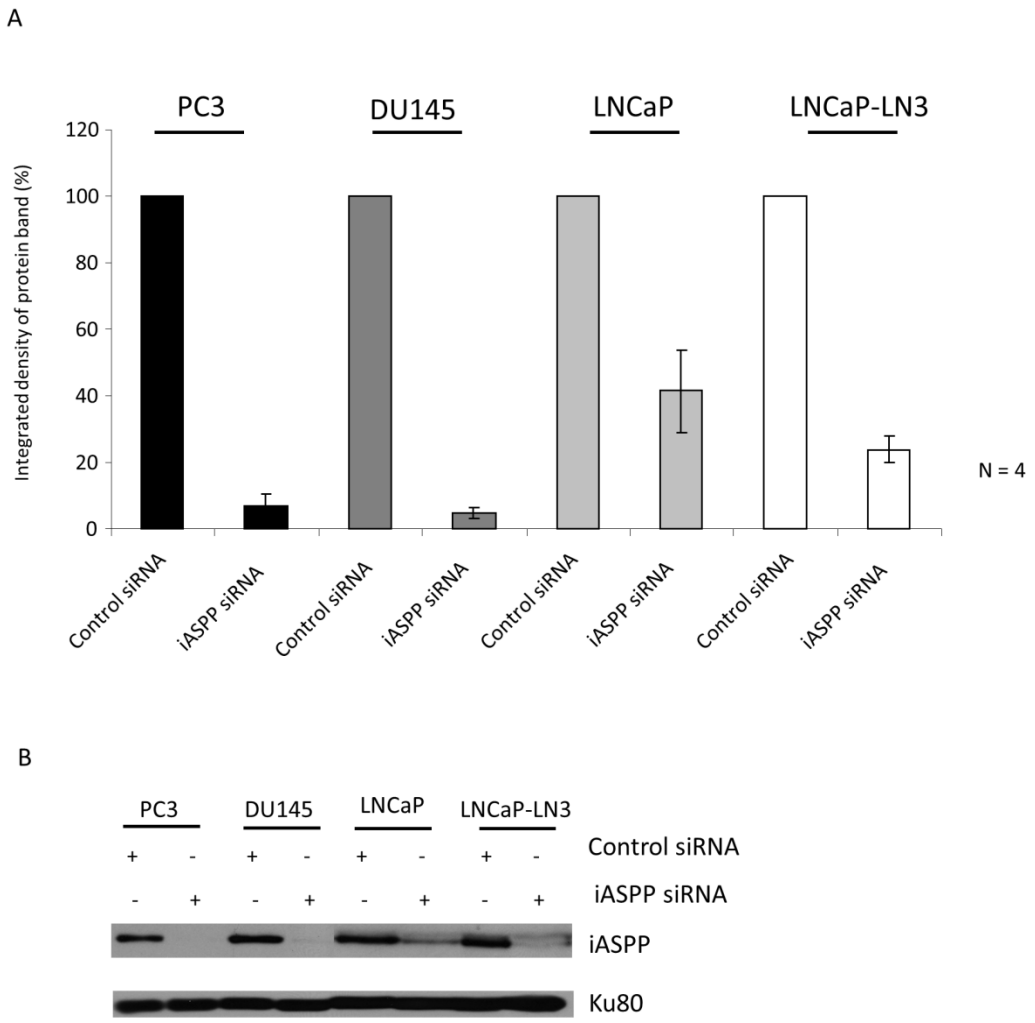

Figure 2.1 iASPP siRNA efficiency

(A) Mean integrated density quantification of iASPP protein bands 72hrs post transfection; siRNA mediated knockdown of PC3, DU145, LNCaP and LNCaP-LN3 was performed using 40nM of control or iASPP siRNA. Western blot bands were then normalised against control siRNA and quantified using image J analysis. Error bars indicate standard error. **(B)** siRNA mediated knockdown of iASPP 72hrs post transfection. 40µg of protein was loaded in the PC3 and DU145 lanes. 80µg was loaded in the LNCaP and LNCaP-LN3 lanes.

Immunocytochemistry

Cells were grown to 70%-80% confluency on coverslips in a 24-well plate. The cells were fixed in 4% PFA solution for 15 minutes after being washed twice with PBS. Cells were permeabilized with 0.1% Triton X solution for 5 minutes. The permeabilization solution was then removed by washing the wells with PBS, and cells were further blocked with 0.2% BSA for 20 minutes. The primary antibody was diluted in 0.2% BSA and incubated at room temperature for 1 hour. Subsequently, the wells were washed 3 times with PBS. Cells were then incubated with secondary antibody in 0.2% BSA along with DAPI which was used to stain nucleic acids, for 30 minutes at room temperature. This step was followed by another 3 washes with PBS. Finally, the coverslips were mounted onto microscope slides using mowiol-glycerol based solution. The slides were protected from light and kept at 4°C.

Cell Invasion Assay

Analysis of cell invasion was performed using a Matrigel invasion assay (BD Biocoat ™ Invasion Chamber, Becton Dickinson, Oxford, UK). For iASPP siRNA knockdown experiments, 100,000 cells were washed in PBS 72hrs after the siRNA treatment, resuspended in 500ml RPMI/1%FCS, and placed in the upper chamber of each 24-well plate Matrigel Chamber and 1ml RPMI/10%FCS was placed in the lower chamber. Cells were incubated for optimal times depending on cell line (PC3 – 24hrs, DU145 18hrs, LNCaP 72hrs and LNCaP-LN3 48hrs). Invaded cells on the lower surface of the Matrigel membrane were fixed in 4% paraformaldehyde and stained; with a cell stain purchased from Millipore, for cell counting. Cells were counted in nine central 40x magnification fields for each membrane.

Cell Motility Assay

siRNA treated DU145 cells were grown in 6 well plate for 72hrs until 90% confluent. Cells were washed and serum depleted media was added for 24hrs. Cells were then washed in PBS and a scratch was made in the middle of the well, normal culture media was added. The cells were incubated at 37°C on a timelapse microscope. An image was taken ever 15mins for 72hr of the scratched area. The time taken for the cells to cover 50% of the scratched area was calculated by using image J software.

Optimisation of Docetaxel treatment using MTT

Optimisation was performed on PC3, DU145, LNCaP and LNCaP-LN3 cells, 6,000 cells were seeded in each well of a 96 well plate. Cells were treated with 50ng, 100ng or 200ng of Docetaxel for; 24, 48 or 72hrs. Each treatment was performed in triplicate. Cell viability was then measured using MTT cell proliferation Assay (ATCC). 10ul of (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT) was added to each well and incubated for 4hrs at 37°C, until a purple precipitate was visible. 100µl of detergent reagent was then added and left in the dark at RT for a further 4hrs. The absorbance was measured for each well at a wavelength of 570nm, along with a reference wavelength of 750nm. The results used to determine the optimal concentration are illustrated in figure 2.2.

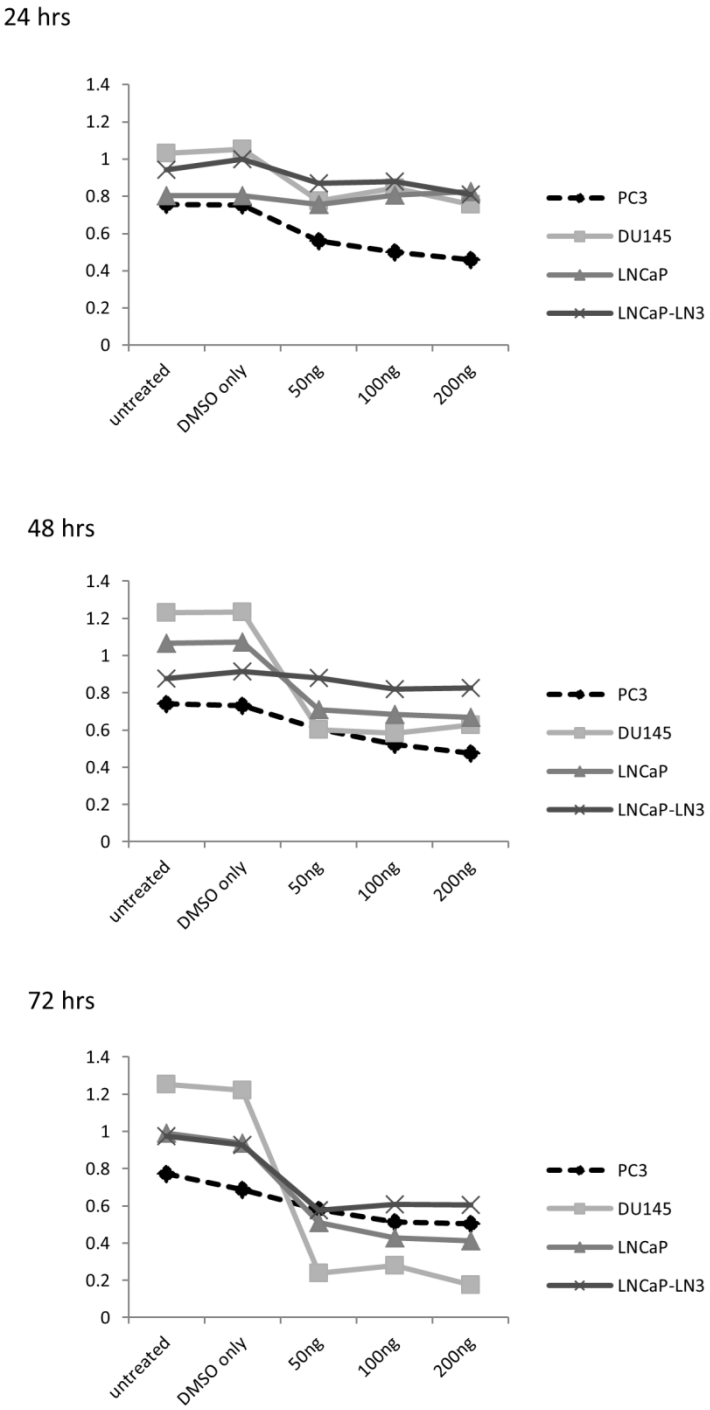

Figure 2.2 MTT cell viability assay after docetaxel treatment

Cell viability was measured in PC3, DU145, LNCaP and LNCaP-LN3 cells using MTT after either no treatment, treatment with DMSO alone or treatment with 50ng, 100ng or 200ng of Docetaxel for 24hrs, 48hrs or 72hrs.

Docetaxel Induced Apoptotic Cell Death Assay

siRNA targeted cells were treated with either DMSO or 50ng of docetaxel for 48h, cells were collected and washed with cold 1x PBS. Cells were fixed with 90% ethanol by slowing adding the ethanol drop by drop and frequently vortexing to mix. The cells were left overnight at 4°C. Cells were washed twice with PBS and stained with a PBS based solution containing 1mg/ml propidium iodide (PI) and 5mg/ml RNase A. Cells were incubated for 1hr on ice and cell cycle profiles were then determined by flow cytometry using a BD FACSCanto system.

Organotypic cultures

A cell suspension of 500,000 normal human fibroblasts was prepared in DMEM media for each organotypic culture. Gel plugs were then prepared to act as scaffolds for the fibroblasts. To make the gel plugs the following reagents were added into a 15ml falcon tube on ice. In the following order per gel: 1 volume of 500,000 fibroblasts, 1 volume of FBS, 1 volume of 10xDMEM (Gibco) 7 volumes of Collagen:Matrigel (1:1) (rat tail collagen type I (BD), Matrigel (BD)) to make a final volume of 1ml per gel. 1ml of mixture was added to a 24-well plate and incubated at 37°C for 1hr. This was followed by the addition of 1ml of DMEM culture media on top and left overnight at 37°C. Medium was then aspirated and 500,000 cells of interest (i.e. DU145 cells) were plated on top of the gel plug in appropriate culture media. Dish was returned to the incubator overnight. A previously autoclaved sterile nylon square, measuring 2.5cm by 2cm in size was placed in a petri dish and covered with a gel consisting of: 1 volume DMEM, 1 volume of FBS, 1 volume of 10xDMEM and 7 volumes of collagen to make up a final volume of 250µl per nylon sheet. If on mixing the gel solution was yellow 0.1M NaOH was added until the

solution turned pink. The gel covered nylon sheets were then incubated at 37°C for 15-20 minutes until the gel had polymerised. 10ml of 1% glutaraldehyde (sigma) in PBS was then added to the petri dish, and the nylon sheets were incubated at 4°C for 1 hr. They were then washed 3 times in PBS and once in DMEM culture media followed by the addition of 10ml of DMEM media and incubation overnight at 4°C. The nylon sheets were then mounted on autoclaved metal scaffolds measuring 2.5cm by 2cm in size and placed in a petri dish, and the previously prepared gel plugs containing the fibroblasts and the top layer of cells were placed on top of the nylon sheet. The petri dish was filled with appropriate culture media for the cell line until the liquid was just under the nylon sheet. Allowing the media to be drawn up into the nylon sheet but avoiding immersion into the media itself. The petri dish was incubated at 37°C for 10 days with the medium being changed every 48hrs. Gels were then fixed in formalin and processed in the same manner as tissue samples, followed by wax embedding. Sections were cut 10µm thick ready for further analysis by Haematoxylin and Eosin staining or immunofluorescence analysis.

Dihydrotestosterone (DHT) treatment

LNCaP cells were plated in 6 well plates at a cell density of 100,000 cells per well and left to grow in normal culture for 48hrs. Cells were washed in PBS and medium containing charcoal stripped FBS (one shot^{TM} purchased from Gibco) was added. Cells were cultured in androgen deprived conditions for 48hrs. The medium was removed and replaced with medium containing charcoal stripped FBS and either ethanol alone, 10µM of bicalutamide, 10µM of bicalutamide plus 10nM of DHT or 10nM of DHT. Cells were cultured for 8hrs, 16hrs, 24hrs or 36hrs and then harvested for western blot analysis.

2.2.4 Mouse study

Mice used in the study

Mouse age (weeks)	Genotype	Analysis
6	iASPP+/+	Prostate size, Morphology study, p63 expression, ki67 expression, TUNEL
6	iASPPΔ8/Δ8	Prostate size, Morphology study, p63 expression, ki67 expression, TUNEL
8	iASPP+/+	Protein extraction for cytokeratin and synaptophysin expression
8	iASPPΔ8/Δ8	Protein extraction for cytokeratin and synaptophysin expression
8	iASPP+/+	Protein extraction for cytokeratin and synaptophysin expression
8	iASPPΔ8/Δ8	Protein extraction for cytokeratin and synaptophysin expression
8	iASPP+/+	Protein extraction for cytokeratin and synaptophysin expression
8	iASPPΔ8/Δ8	Protein extraction for cytokeratin and synaptophysin expression
13	iASPP+/+	Prostate size, Morphology study, p63 expression, Brdu, TUNEL
13	iASPPΔ8/Δ8	Prostate size, Morphology study, p63 expression, Brdu, TUNEL
13	iASPP+/+	Prostate size, Morphology study, p63 expression, Brdu, TUNEL
13	iASPPΔ8/Δ8	Prostate size, Morphology study, p63 expression, Brdu, TUNEL
13	iASPP+/+	Prostate size, Morphology study, Brdu
13	iASPPΔ8/Δ8	Prostate size, Morphology study, Brdu
16	iASPP+/+	Prostate size, Morphology study, p63 expression, ki67 expression, TUNEL
16	iASPPΔ8/Δ8	Prostate size, Morphology study, p63 expression, ki67 expression, TUNEL
18	iASPP+/+	Prostate size, Morphology study, Brdu
18	iASPPΔ8/Δ8	Prostate size, Morphology study, Brdu
24	iASPP+/+	Prostate size, Morphology study, p63 expression, ki67 expression, TUNEL
24	iASPPΔ8/Δ8	Prostate size, Morphology study, p63 expression, ki67 expression, TUNEL
43	iASPP+/+	Protein extraction for p63 expression
43	iASPPΔ8/Δ8	Protein extraction for p63 expression
56	iASPP Cre +	Confirmation of iASPP deletion after tamoxifen treatment
60	iASPP Cre -	Confirmation of iASPP deletion after tamoxifen treatment

Table 2.5 Mice used in the characterisation of iASPP in an *in vivo* model of prostate development and epithelial homeostasis

All mice used in the study were paired with aged matched controls (littermates where possible).

Animals

Mice were bred and housed in the Henry Wellcome Building of Genomic Medicine. They were kept in IVC cages which had a sealed lid that acts as second part of the two-barrier system. Procedures were carried out following the Home Office Animals (Scientific Procedures) Act 1986.

Culturing of primary mouse prostate spheroids

1 part 10x Collagenase/Hyaluronidase was diluted with 9 parts DMEM-F12 supplemented with 5% FBS and placed in a 15ml falcon tube. Approximately 2 – 5ml of this solution was required for every 2 – 3 prostates dissociated. Prostates were dissected and placed in cold PBS, the lobes were further dissected and residual fat was removed using a dissection microscope. The prostate tissue was transferred to the 15ml falcon tube containing the Collagenase/Hyaluronidase/FBS/ DMEM-F12 solution and incubated for 3 hrs at 37°C. After dissociation, the cells were centrifuged at 350g for 5 minutes with the brake on and the supernatant was discarded. The pellet was then resuspended in 5 – 6ml of 0.25% Trypsin-EDTA and left on ice for 1hr. 10ml of cold Hanks' balanced Salt Solution Modified supplemented with 2% FBS was added. Cells were then centrifuged at 350g for 5 minutes with the brake on and the supernatant was discarded. 2ml of pre-warmed 5mg/ml Dispase and 200µl of 1mg/ml DNase I was added. The sample was pipetted up and down using a P1000 tip. 10ml of cold Hanks' balanced Salt Solution Modified supplemented with 2% FBS was added, and the suspension was filtered through a 40µm cell strainer into a new 50ml falcon tube. Cells were centrifuged at 350g for 5 minutes with the brake on and the supernatant was removed. Cells were resuspended in Matrigel and plated on a chamber slide. The slides were incubated for 10 minutes at 37°C

and then covered with Prostacult basal medium supplemented with 5% FBS plus supplements. Media was changed 24hrs later to serum-free complete Prostacult medium. The cells were left to form spheroids for 1 week. Medium was changed every 48hrs.

Mouse Tissue Processing.

Mouse organs were dissected immediately after sacrifice and fixed in 10% buffered formalin overnight. They were then dehydrated in an ethanol series and cleared in xylene using an automated machine. The tissue was then embedded in paraffin wax. Sections were cut at 4μm thickness for further experimental analysis.

Haematoxylin and Eosin staining

First, slides were de-paraffinized and hydrated through graded alcohols to water, by placing them in a solution of Histoclear for 5 minutes, twice, followed by 100% ethanol for 3 minutes, twice, 90% ethanol for 1 minutes followed by 1 minute in 70% ethanol. Sections were then washed in water 3 times and placed in Harris Haematoxylin (sigma) for 1 minute, followed by at least 5 washes in water or until the water was clear. They were then dipped 3 times in 1% acid ethanol and washed in water 3 times. Then placed in 2% $NaHCO_3$, 0.35% $MgSO_4$ in distilled water for 30 seconds followed by 3 washes in water. Slides were then submerged in Eosin (sigma) which consisted of 1g of Eosin per 100ml of 70% ethanol, acidified with 0.5ml of glacial acetic acid, for 1 minute and washed in water 5 times until the water was clear. Sections were then placed for 1 minute in 70% ethanol,twice, 1 minute in 100% ethanol twice, 5 minutes in Histoclear twice, and finally, mounted on a coverslip with mounting medium (Vector Labs) and left to dry overnight.

Alcian Blue Staining

Slides were de-paraffinized and hydrated in distilled water. The samples were then stained with alcian blue for 30 minutes and the excess removed by washing the slides under running tap water for 2 minutes. Samples were counterstained with nuclear fast red solution for 5 minutes, following which the excess was washed away with running tap water for 1 minute. The slides were dehydrated in increasing percentages of ethanol and further cleared in Histoclear. Slides were then mounted with resinuous mounting media.

Immunofluorescence using citric acid Buffer

Slides were de-paraffinized and hydrated through graded alcohols to water then subsequently immersed in boiling citric acid buffer, which had been heating in the microwave (800w) for 10 minutes, the container was wrapped in saran wrap to avoid evaporation and the sections were then returned to the microwave and placed on warm heat for 10 minutes. Slides were left in the buffer until they were cold enough to handle. They were then rinsed thoroughly with PBS. Sections were blocked with 5% normal goat serum in PBS for 30 minutes. They were then incubated in primary antibody, diluted in 5% fetal calf serum in PBS overnight at 4°C. The day after, slides were washed in PBS 3 times for 5 minutes. Secondary antibody was diluted in a solution of 5% normal goat serum in PBS. Sections were incubated with the secondary antibody for 40 minutes at RT, following which; slides were washed in PBS 3 times for 5 minutes. Finally, coverslips were mounted onto the slides using mowiol-glycerol based solution. The slides were kept at 4°C, protected from light, until they were observed using a confocal microscope.

Immunofluorescence using EDTA Buffer (p63 analysis)

Slides were de-paraffinized and hydrated through graded alcohols to water, and placed in 600ml of cold EDTA buffer (wrapped in saran wrap to avoid evaporation) and boiled in the microwave on high for 20 minutes. Slides were then left to cool for 20 minutes at RT. Slides were washed in water for 3 minutes followed by a wash in PBS. Slides were blocked for 30 minutes in 1% BSA in PBS at RT. Primary antibody was added and incubated overnight at 4°C. Sections were washed in PBS for 5 minutes 3 times and the secondary antibody diluted in 1% BSA in PBS was added for 1hr at RT. Secondary was then removed and a solution of DAPI in 1% BSA in PBS was applied for 5 minutes. The slides were washed in water for 5 minutes, 3 times and coverslips were mounted onto the slides using mowiol-glycerol based solution. The slides were kept at 4°C, protected from light, until they were observed using a confocal microscope.

Immunohistochemistry

Slides were de-paraffinized and hydrated through graded alcohols to water. Endogenous peroxidases were inactivated by placing slides in 3% H_2O_2 in methanol for 10 minutes. Slides were subsequently immersed in boiling citric acid buffer, which had been heating in the microwave (800w) for 10 minutes, the container was wrapped in saran wrap to avoid evaporation and the sections were then returned to the microwave and placed on warm heat for 10 minutes. Slides were then left in the buffer until they were cold enough to handle. They were then rinsed thoroughly with PBS. Sections were blocked with 5% normal goat serum in PBS for 30 minutes. They were then incubated in primary antibody, diluted in 5% fetal calf serum in PBS overnight at 4°C. The day after, slides were washed in PBS, 3 times for 5 minutes. Biotinylated secondary antibody was diluted in a solution

of 5% normal goat serum in PBS. Sections were incubated for 30 minutes at RT, following which; slides were washed in PBS 3 times for 5 minutes. Sections were incubated in an avidin/biotin-based peroxidase solution, for 15 minutes at RT, and washed in PBS, 3 times for 5 minutes. Subsequently, sections were incubated in a DAB (3, 3'-diaminobenzidine) peroxidase substrate solution for between 3 and 8 minutes. Slides were then rinsed in water and counterstained by immersion in Haematoxylin for 15 seconds. Finally, the slides were dehydrated through increasing graded alcohols, and immersed in Histoclear for 5 minutes before mounting on to a coverslip with mounting medium (Vector Labs) and left to dry overnight.

BrdU Proliferation Assay

Mice were injected with 30µg/g of BrdU 24hr before sacrifice (organs were then prepared as above) Sections were incubated with anti-rat BrdU and the protocol for immunofluorescence was followed.

Apoptosis Assay

Detection of apoptotic cells was performed using ApopTag Red *In Situ* Apoptosis Detection Kit purchased by Chemicon following the manufacturer's recommended protocol.

Immunofluorescence of primary mouse prostate spheroids

Prostate spheroids were grown on chamber slides for 7 days. The wells were washed 3 times with PBS before being incubated with 4% PFA for 30 minutes at RT. After this incubation, the cultures were washed for 10 minutes 3 times with PBS. The cultures were immersed in permeabilization solution for 30 minutes at RT. Subsequently, the spheroids were incubated with primary antibody diluted in permeabilization solution overnight at 4°C. Spheroids were washed for 10 minutes 5 times, with permeabilization solution. Secondary antibody was then added diluted in permeabilization solution for 3 hours at RT. Spheroids were then washed 5 more times with permeabilization solution, 10 minutes each wash. Cultures were kept in PBS at 4°C until analysis by confocal microscopy.

2.2.5 Human tissue study

Tissue microarrays

The tissue microarrays (TMAs) were generously provided by Prof. G. Thalmann (Department of Urology, Bern, Switzerland). The pathology scores were provided by Dr Florian Fritzsche and Dr Victoria Salter of the Ludwig Institute for Cancer Research (Oxford).

T3 tissue samples

The T3 tissue samples were provided by the Department of Pathology at the John Radcliffe Hospital (Oxford). The pathology scores were provided by Dr Clare Verrill from the Department of Cellular Pathology at the John Radcliffe Hospital (Oxford).

Immunohistochemistry

Human tissue was stained following the same immunohistochemistry protocol followed to analysis the mouse sections, using citric acid buffer for antigen retrieval. For iASPP analysis antigen retrieval was not performed on the human tissue sections.

2.2.6 Data analysis

Computer images

All autoradiographs were scanned using the Epson perfection 1660 photo scanner. Adobe photoshop 8.0 and Image J software were used to manipulate images (only as a whole for size, brightness and contrast). No signal was modified in relation to the raw image. FACS analysis was performed using BD FACSDiva software to analyse cell cycle profiles.

Cell counting

All cell counts were calculated using a minimum of 1000 cells. Four fields of view at a 20x magnification per slide were randomly selected for each prostate lobe. Three different slides were used per analysis, per animal. Analysis was performed using Image J software.

Statistical analysis

Patient survival analysis was performed by Kaplan Meier analysis, using SPSS statistics 20 software. Paired and unpaired T-test and Mann-Whitney U tests were performed using Graph pad software or excel. All statistical tests were performed as 2-tailed tests. Differences were considered significant at a value of $P \leq 0.05$.

Chapter 3: iASPP is required for prostate homeostasis

3.1 Introduction

The prostate is a male reproductive organ within the urogenital tract; urogenital organs consist of two systems; the reproductive system and the urinary system. They are often grouped together due to their anatomical proximity to one another and their common embryonic origin. Other urogenital organs in male mammals include the kidneys, ureters, testis, vas deferens, seminal vesicles, urethra and urinary bladder.

The prostate gland is composed of ducts which are comprised of two main cell types: luminal and basal cells, along with a third and smaller subpopulation of NE cells. The luminal cells form a continuous layer of polarised columnar secretory cells with basal nuclei. These cells are characterised by the expression of Ck8 and 18 and are AR positive. They are responsible for secreting components of prostatic fluid, such as PSA, in an androgen-dependent manner. The basal cells are located underneath the luminal layer, and sit on the basement membrane where they contribute to its structure by secreting components of the basement membrane. These cells are characterised by the expression of Ck5 and 14 they are TP63 positive, and have low or undetectable levels of AR. The basal cell compartment is the more proliferative layer of the prostate epithelium and the stem cell population is thought to reside in this layer.

The small population of NE cells have a regulatory role in the prostate as they are thought to regulate growth, differentiation, and the secretory activity of the prostate epithelium. they produce and secrete several different growth regulatory factors such as chromogranins, serotonin and synaptophysin, (Abrahamsson and di Sant'Agnese, 1993). The main way in which they exert their regulation is via paracrine signalling to adjacent epithelial cells. There are two types of NE cells termed ‘open’ and ‘closed’. The ‘open’

type has an apical cytoplasmic process which extends to the glandular lumen and has long specialised surface microvilli. The 'closed' type are surrounded by other epithelial cells and do not have direct contact with the lumen. Some of the growth factors known to be secreted by these cells have been detected in seminal fluid, raising the possibility that they may be actively secreted into seminal fluid in order to regulate sperm function (Sun et al., 2009). The development of the prostate predominantly takes place after birth, and so the long term effect of loss of any protein that plays a vital role in normal growth and maintenance of the prostate would be apparent after the developmental programme is complete, which in mice is at around 6 weeks of age.

iASPP has been implicated as being an essential protein in the regulation of normal differentiation in the stratified epithelium of the skin (Notari et al., 2011). However, the question as to whether or not iASPP plays a role in the homeostasis of the simple glandular epithelium of the prostate has not yet been answered. In order to characterise the role of iASPP in the prostate gland we used a recently generated transgenic mouse model. The generation of *iASPP* transgenic mice (iASPP$^{\Delta 8/\Delta 8}$ mice) has enabled the establishment of *in vivo* studies allowing the role of iASPP in development to be investigated. This analysis has aided the identification of a number of biological functions for iASPP beyond the regulation of p53-mediated apoptosis. It has also highlighted iASPP as being an important protein in mouse embryogenesis, as these mice are not born according to Mendelian segregation frequency, given that they have only an 11% birth rate of iASPP-deficient mice compared to the expected 25%. This finding has fuelled further research into iASPP's role in normal development. The organs that have so far been identified as being affected as a result of iASPP-deficiency are the skin (Notari et al., 2011), eyes and heart (unpublished data). These mice have been shown to have open

eye lids at birth which results in blindness caused by dehydration of the eyes. Moreover, they have been shown to have a shorter lifespan than normal which has been attributed to defects in the heart leading to sudden death due to cardiomyopathy. Finally, iASPP-deficient mice have peculiar 'wavy' or 'spiky' coats and this results from a defect in the orientation of the hair follicles as well as a thickening of the epidermis. These observations resulted in the discovery that iASPP is important for the homeostasis of stratified epithelium (Notari et al., 2011). Notari et al showed that iASPP is important in the prevention of premature senescence through its role as a regulator of the TP63. Loss of iASPP results in a marked reduction in markers of proliferative cells, such as Ck14, coupled by an increase in the markers of terminally differentiated cells, such as Ck1 and loricin. Considering the pivotal role of *p63* in the developing prostate, this prompted us to ask whether or not iASPP regulates TP63 expression in the prostate gland, and whether iASPP is essential for the maintenance of normal differentiation and homeostasis in this organ.

In order to carry out this study a number of murine prostate specimens were analysed. All of the mice used as part of the functional study were post-pubertal with ages ranging from 6 to 43 weeks. By analysing a cohort of these genetically modified mice new understanding of the role of iASPP in prostate development, both structurally and at the protein level, can be achieved. This chapter describes the morphology of the iASPP$^{\Delta 8/\Delta 8}$ prostate and the impact that functional loss of iASPP has on protein expression, cell lineage, proliferation and cell death.

3.2 Results

3.2.1 iASPP-deficiency causes a decrease in prostate size

To investigate the effect of iASPP loss on urogenital morphology a number of the mouse urogenital organs were macroscopically analysed these included the kidney, testis, bladder, seminal vesicles and prostate. The prostate gland of the iASPP$^{\Delta 8/\Delta 8}$ mice was found to be decreased in size compared to that of the control mice (figure 3.1B). The ventral and dorsolateral lobes were measured retrospectively with the assumption that the prostate lobes are elliptical in shape. The prostate samples were embedded in paraffin and cut into 4μm sections. Every tenth section was stained with haematoxylin and eosin (H&E) to visualise the cellular architecture. The largest area of each lobe was calculated based on width and height measurements obtained using image J software. Depth was then calculated by the amount of sections that had that particular lobe present. The final size was then calculated using the equation 'lobe volume = $(4/3)\pi r^1 r^2 r^{3'}$ [(r = radii) r^1=height, r^2=width r^3=depth]. Even though we observed a reduction in the volume of the prostate lobes of the iASPP$^{\Delta 8/\Delta 8}$ mice, it did not reach significance (iASPP$^{+/+}$ lobe areas were normalised to 100% versus. 76.1 ± 14.3 for the ventral lobe, P = 0.09 and 83 ± 14.5 for dorsolateral lobe, P = 0.20). The seminal vesicles varied in size in both the iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ mice, but there was no overall difference between the genotypes. However, there was a noticeable reduction in the size of the urinary bladder (figure 3.1A). This may be the result of a growth abnormality in the bladder, or it may be that the bladder has a reduced capacity for other reasons. For example it could be that these mice have a defect in the smooth muscle of the bladder thereby preventing it from expanding normally. However, to date the reason for this prominent reduction in bladder size is not known. There appeared to be no difference in the size or morphology of the kidneys or

testis (figure 3.1C). Next, the microscopic phenotype of the iASPP$^{\Delta 8/\Delta 8}$ mice was analysed. Due to the complex structure of the mouse prostate it is difficult to embed the lobes in a consistent orientation. The orientation of the organ is paramount when comparing protein expression between mice. For example it is well documented that expression levels of TP63 differ depending on the proximity to the urethra. The prostatic ducts branch and grow from the urethra, and so the number of progenitor cells are greater in areas proximal to the urethra compared with areas that are more distal. It is thought prostatic stem cells reside in areas proximal to the urethra, whereas intermediate cells are thought to be more distal.

In order to overcome problems with orientation of the tissue sections the entire prostate gland was embedded as a whole, with the ventral prostate at the front. The urethra was cut to ensure a flat surface, and the gland was then held in an anatomical position as seen in figure 3.2A. Once embedded the gland was sectioned from below. Sections were then selected using the urethra as a point of reference in order to ensure that a fair comparison of each lobe between mice was carried out. By using H&E staining of every tenth section cut from each block a map of the histology of the tissue could be obtained. By comparing 10 comparative sections from each pair of iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ mice the glandular and cellular morphology could be compared. All the iASPP$^{\Delta 8/\Delta 8}$ mice exhibited normal development of all four prostate lobes with microscopically normal glandular structures at the H&E level. All the lobes of the mouse prostate have distinct cellular differences. The anterior cells are cuboidal/columnar in shape and have abundant eosinophilic secretions. The ventral lobe consists of columnar epithelium and produces slightly basophilic secretions. The dorsal and lateral lobes can be grouped together for analysis as a dorsolateral lobe and this consists of simple cuboidal epithelium which secretes an

eosinophilic substance. The different cell morphologies could be clearly observed in both the iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ prostate tissue. (figure 3.2B&C). No obvious differences in the architecture of the glands, or of the cells of the iASPP$^{\Delta 8/\Delta 8}$ mouse, were observed at the H& E level compared with wild-type controls.

Taken together these findings suggest that the loss of iASPP expression may have a slight impact on the gross morphology of the prostate, resulting in a smaller organ. However, given that the iASPP$^{\Delta 8/\Delta 8}$ mouse does not have any obvious abnormalities in glandular or cellular architecture, it would appear that iASPP is not essential for the structural development of the prostate. Other changes in prostate function, such as its contractile ability, where not investigated and hence cannot be ruled out.

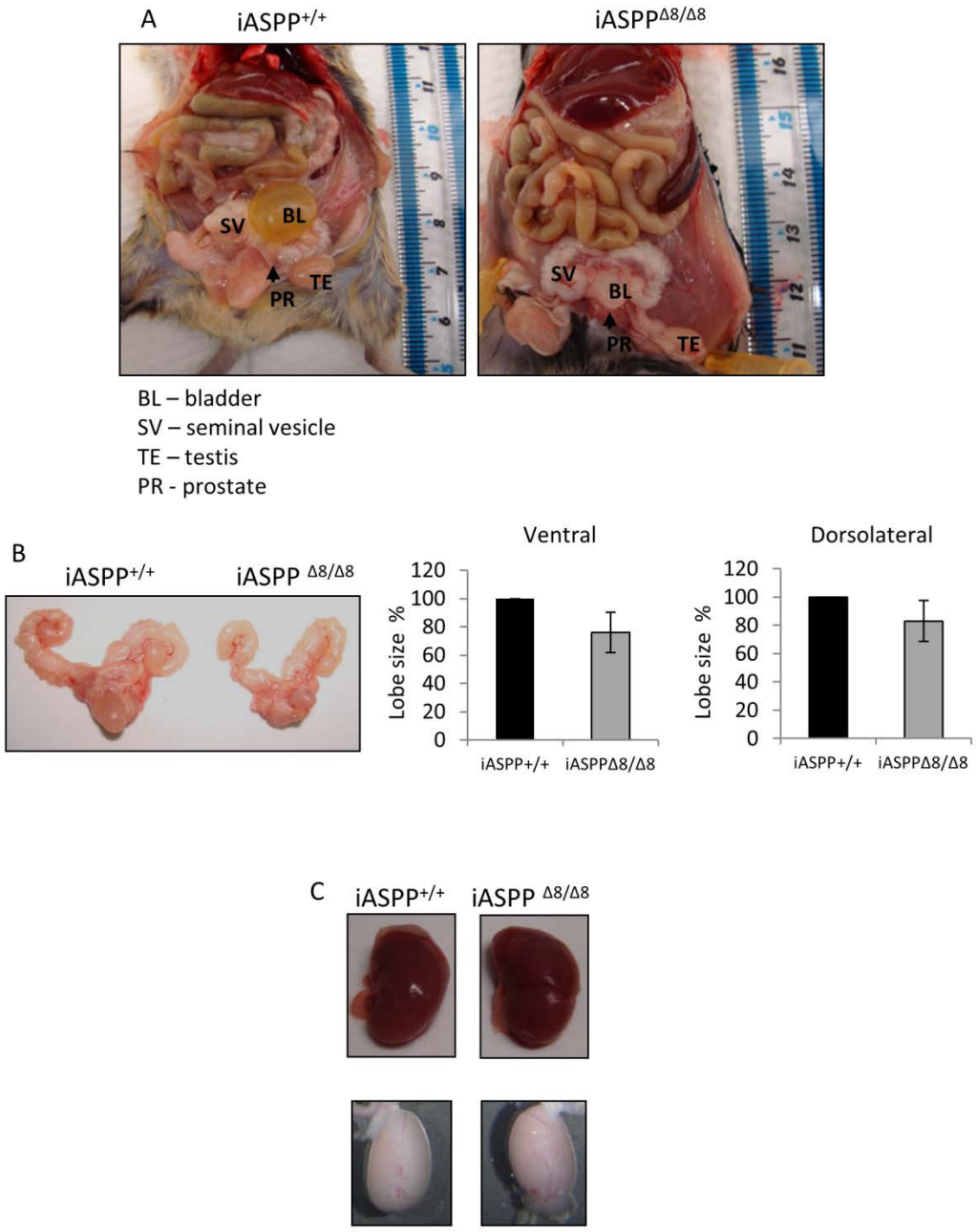

Figure 3.1 The morphological phenotype of the iASPP$^{\Delta 8/\Delta 8}$ mouse urogenital organs

(A) The macroscopic morphology of the prostate, bladder, seminal vesicles and testis. The organs were dissected and analysed from 7 age matched pairs of mice (**B)** The prostate of the iASPP$^{\Delta 8/\Delta 8}$ mice is smaller than the iASPP$^{+/+}$. The prostate size was calculated using the following equation 'lobe volume = $(4/3)\pi r^1 r^2 r^3$' [(r = radii) r^1=height, r^2=width r^3=depth]. Error bars indicate standard error. P values were calculated using a paired t-test**. (C)** The macroscopic morphology of the kidney and testis of iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ mice.

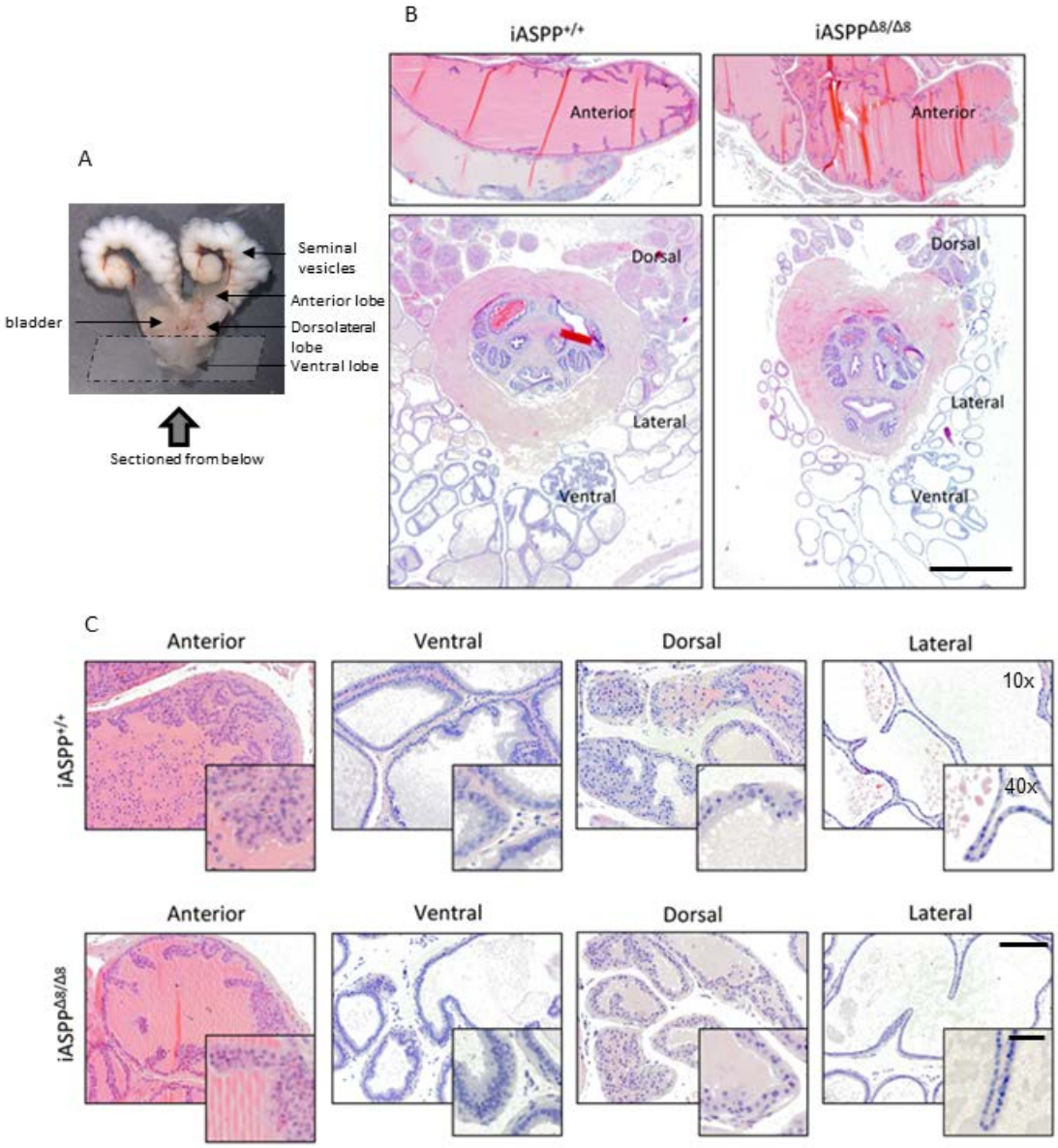

Figure 3.2 There is no obvious difference at the H&E level in glandular or cellular morphology between the iASPP$^{+/+}$ mice and the iASPP$^{\Delta 8/\Delta 8}$ mice.

(A) A depiction of the orientation of the prostate when paraffin-embedding. All sections were cut from below to ensure an accurate comparison between sections. **(B)** An overview of comparative sections from the iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ mice. Haematoxylin and eosin staining was used to show the histology of the anterior, dorsal, lateral and ventral lobes of the prostate. Scale bar - 1mm. **(C)** 10x magnification images illustrate the different histological features of the four lobes of the prostate. Squares shows 40x magnification to illustrate detail more clearly. Scale bars - 100µm, 50µm.

3.2.3 iASPP is expressed in both the basal and luminal cells of the mouse prostate

iASPP has been implicated as being an important regulator of the p53 family of transcription factors. TP63 is one of the most fundamental proteins involved in prostate development and homeostasis. Loss of *p63* results in the total absence of prostate development, and previous analyse revealed that TP63 is expressed in the basal layer of the prostate (Yang et al., 1998) and is associated with the progenitor cell population. In more recent studies iASPP has been found to be highly expressed in the nucleus of basal cells where it is known to interact with TP63, and its expression becomes cytoplasmic in differentiated cells (Notari et al., 2011, Chikh et al., 2011). This would indicate that iASPP is a cytoplasmic protein in its inactivated state and is recruited to the nucleus upon activation by an interacting transcription factor such as TP53 or TP63.

In order to investigate the location of iASPP in mouse prostate epithelial cells, and thus its potential role in maintaining homeostasis in this gland, sections of each lobe of the prostate were analysed by immunohistochemistry for the expression of iASPP and TP63 (figure 3.3). iASPP was found to be expressed in both the TP63-positive basal cell layer and the TP63-negative luminal layer. Its expression was observed in the nucleus and cytoplasm, which is in accordance with previous studies (Slee et al., 2004). The nuclear expression of iASPP in the basal cell layer supports the hypothesis that iASPP may interact with and regulate TP63 expression in prostate epithelial cells in a similar way as that described in skin. Interestingly, when iASPP expression and localisation were analysed by immunofluorescence using the same antibody as that used for light microscopy, iASPP localisation was found to be cytoplasmic and membranous, with no nuclear expression. (figure 3.4). These differences highlight the different sensitivity levels between the two techniques. Standard immunohistochemistry involves an

amplification step which may result in the detection of low levels of protein that may not be detected using immunofluorescence. However, the precise reasons for the observed differences in membrane staining are unclear. Due to these expression differences between techniques an element of caution is needed when analysing the iASPP staining in mouse tissue. The immunofluorescence stain did however confirm the absence of iASPP in the iASPP$^{\Delta 8/\Delta 8}$ prostate tissue. The epithelium was clean with no fluorescence signal, although the secondary antibody bound unspecifically to the secretions within the lumen (illustrated in the anterior lobe of figure 3.4). The prostatic secretions which remain within the lumen is inherently sticky, causing technical difficulties when staining. However, when the epithelium was evaluated the signal was reduced in iASPP$^{\Delta 8/\Delta 8}$ mice with only a minor background level of auto-fluorescence, which is a common finding when staining tissue sections. This findings confirm that the primary antibody is specific and that iASPP is absent from the prostate epithelial cells of iASPP$^{\Delta 8/\Delta 8}$ mice.

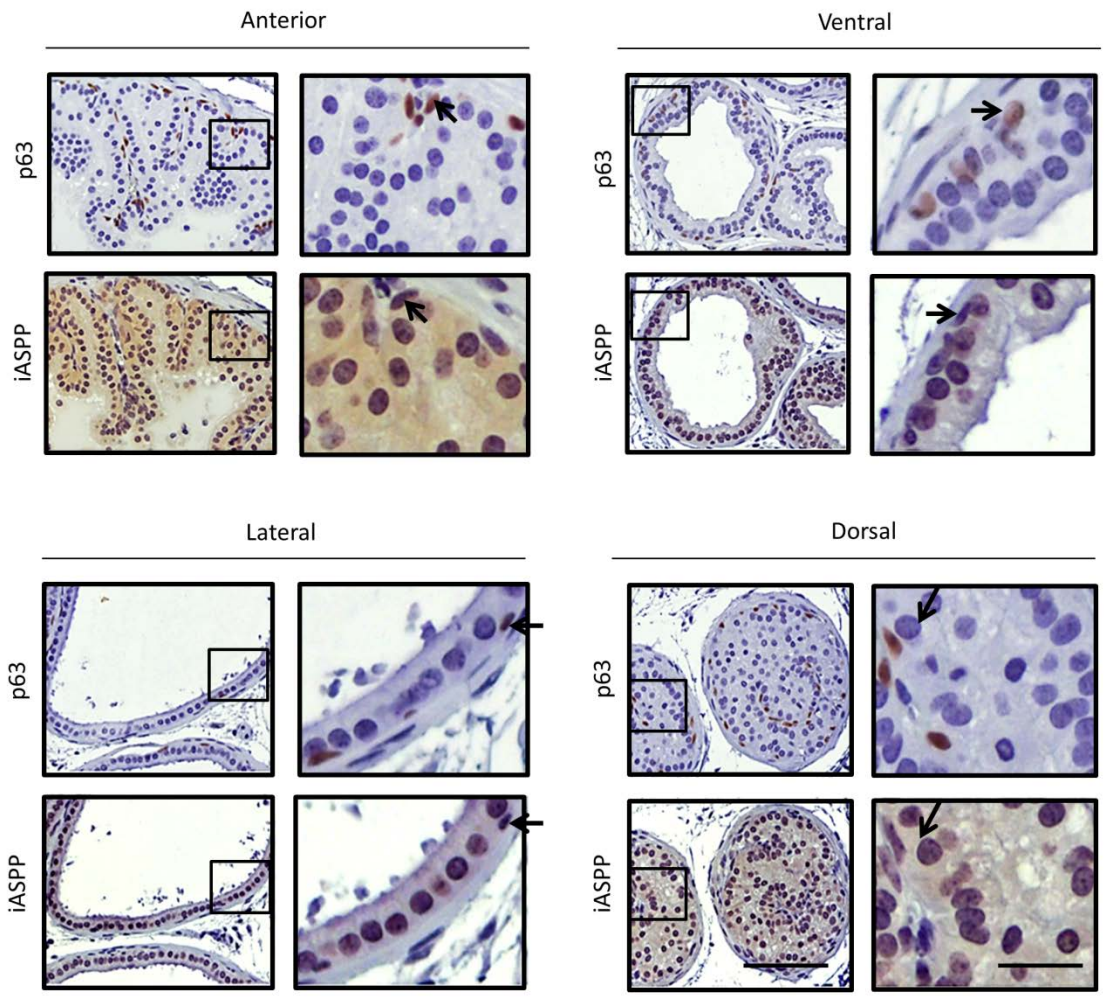

Figure 3.3 iASPP is expressed in both the basal and luminal cells of mouse prostate epithelium

Immunohistochemical staining of TP63 (upper panel) and iASPP (lower panel) counterstained with haematoxylin in wild-type mouse prostate tissue (20x magnification). Right hand squares show higher magnifications to illustrate detail more clearly. Scale bars - 100µm, 25µm.

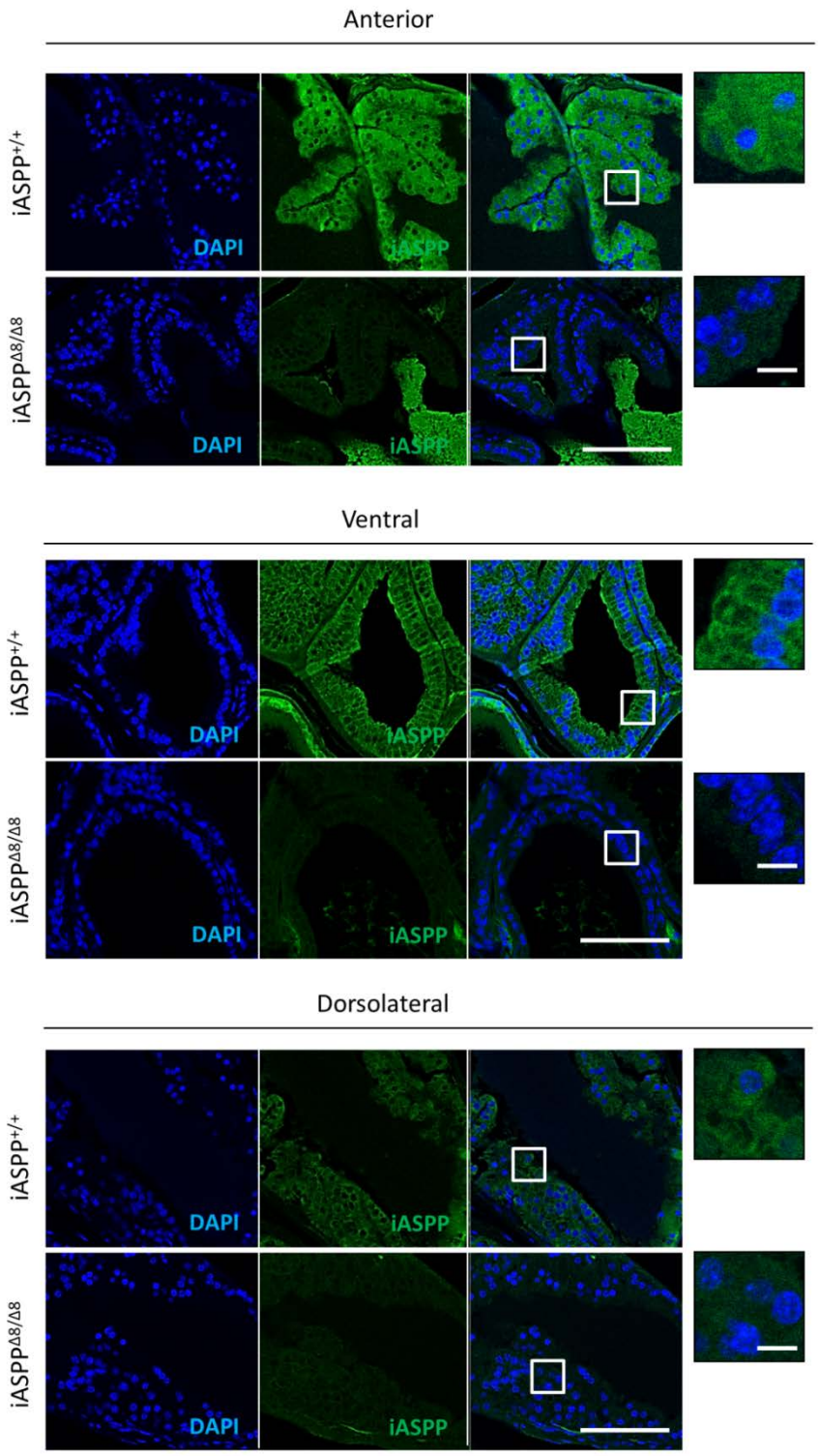

Figure 3.4 iASPP is expressed at the cell membrane

Immunofluorescence staining of iASPP (green), (upper panel) in the anterior lobe (middle panel), ventral (lower panel) and dorsolateral lobes of the $iASPP^{+/+}$ and $iASPP^{\Delta 8/\Delta 8}$ mouse prostate. Merge (right panel) All images were taken at 20x magnification. Squares show higher magnification to illustrate detail. Nuclei were counterstainied with DAPI. Scale bars - 75µm, 25µm

3.2.4 iASPP loss results in an increase in Ck19 positive intermediate cells

The prostate consists of two main cell types, termed basal cells and luminal cells, along with a small subpopulation of NE cells. Although the basal and luminal cells are two discrete populations there is also a population of intermediate cells that are in a transitional state. Intermediate cells are basal cells that have committed to a luminal cell phenotype and have entered the differentiation program. The cytokeratin expression for each particular cell type can be used to distinguish between these three populations. Cytokeratins are keratin-containing intermediate filaments found in the intra-cytoplasmic cytoskeleton of epithelial cells. There are two types of cytokeratin acidic type I and basic type II, and they are usually found in pairs comprising a type I and a type II cytokeratin. The acidic type I cytokeratins include Ck9 to Ck20, and the basic type II cytokeratins include Ck1 to Ck8, and their expression is both tissue and cell specific. In prostate epithelium basal cells express Ck5 and Ck14, and luminal cells express Ck8 and Ck18, and intermediate cells express both basal and luminal cytokeratins together with Ck19. Ck19 is the exception to the general rule regarding cytokeratin pairs as it exists in an unpaired state.

Cytokeratin expression was assessed in order to investigate whether iASPP loss impacts on cell lineage or on the number of cells committed to each lineage, cytokeratin expression was assessed. Both of the main cell types were found to be present in the iASPP-deficient mice as demonstrated by immunofluorescence staining for Ck5 and Ck8 (figure 3.5A). Both basal and luminal cells were clearly distinguishable by their known cytokeratin markers. In order to determine the levels of cytokeratin expression the prostate was dissected from 3 iASPP$^{\Delta 8/\Delta 8}$ mice and 3 age-matched control iASPP$^{+/+}$ mice. The lobes were removed from the seminal vesicles, urethra and bladder to ensure that

pure prostate tissue was obtained and the anterior, dorsolateral and ventral lobes were pooled together for each mouse. Protein was then extracted from the tissue in order to compare cytokeratin expression using a western blot (figure 3.5B). To quantify the difference in expression levels the band intensity from the western blot was measured using Image J software. All bands were normalised against the actin loading control. A small increase in Ck5 expression was observed although this was not statistically significant (mean band intensity for $iASPP^{+/+}$ 13.2 ± 7.9 versus $iASPP^{\Delta 8/\Delta 8}$ 17.7 ± 6.1, P = 0.228). There was no difference in Ck8 expression (mean band intensity for $iASPP^{+/+}$ 16.5 ± 3.5 versus $iASPP^{\Delta 8/\Delta 8}$ 14.7 ± 3.7, P = 0.667). Interestingly, there was a statistically significant increase in the expression of the intermediate cell marker Ck19 in the $iASPP^{\Delta 8/\Delta 8}$ tissue compared with the wild-type (mean band intensity for $iASPP^{+/+}$ 10.7 ± 5.9 versus $iASPP^{\Delta 8/\Delta 8}$ 19.6 ± 4.2, P = 0.043). Ck5 is expressed in both basal cells and intermediate cells therefore an increase in Ck5 expression coupled with an increase in Ck19 suggests that the intermediate cell population is enriched in the $iASPP^{\Delta 8/\Delta 8}$ mice. This was a fascinating observation which led us to investigate whether this increase in intermediate cells was a consequence of an alteration in basal cell numbers.

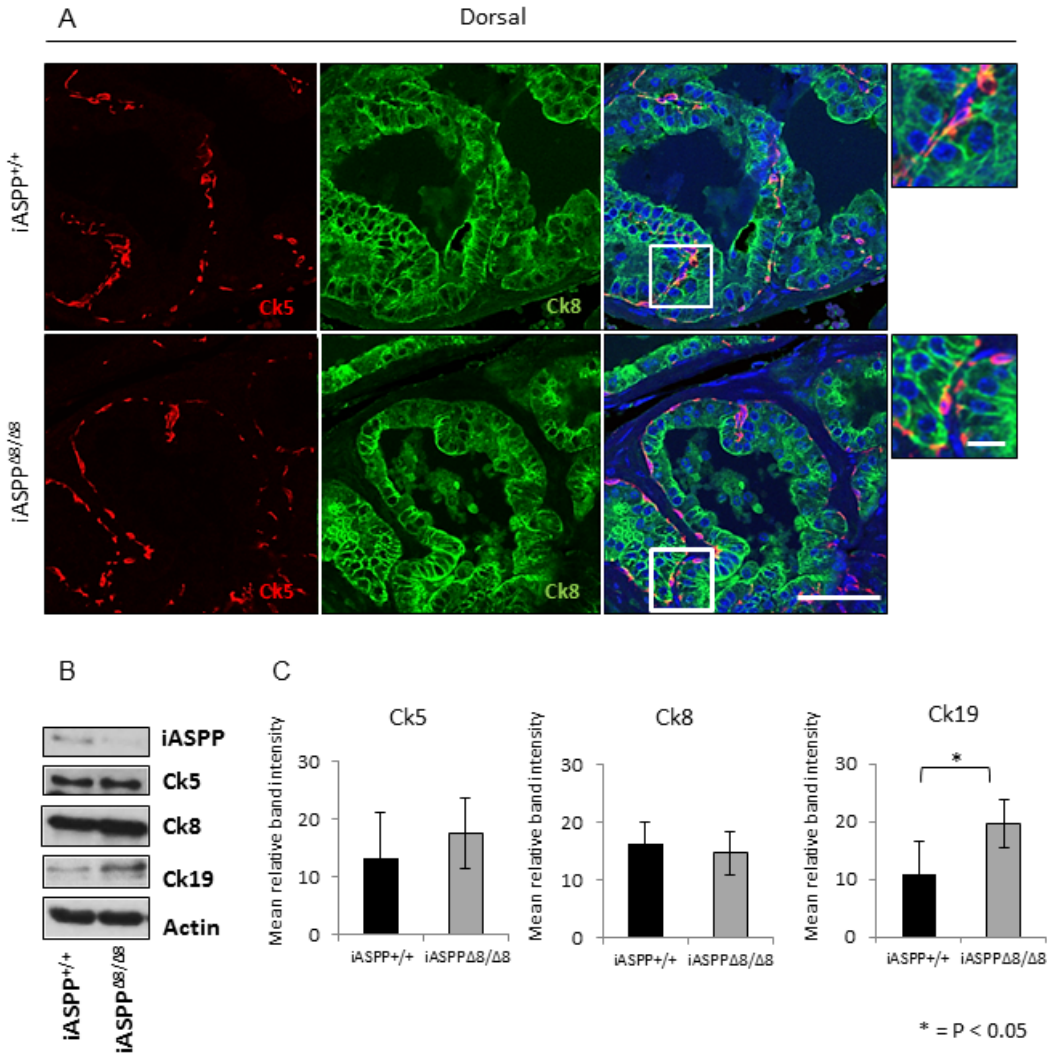

Figure 3.5 Cytokeratins 5 and 19 are increased in the prostate of iASPP$^{\Delta 8/\Delta 8}$ mice

(A) Immunofluorescence staining of cytokeratin markers (left panel) Ck5 (red) in basal cells, (middle panel) Ck8 (green) in luminal cells, and (right panel) a merged image of both cytokeratins in iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ dorsal lobes of the prostate. All the lobes were stained and this is a representative image of the staining pattern seen throughout. All images were taken at 20x magnification. High magnification images are shown to illustrate detail. Nuclei are counterstained with DAPI. Scale bars - 100µm, 25µm. **(B)** A western blot showing cytokeratin expression from both iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ prostate tissue. **(C)** Quantification of mean relative band intensity of Ck5, Ck8 and Ck19 from 3 iASPP$^{+/+}$ mice and 3 iASPP$^{\Delta 8/\Delta 8}$ mouse prostates. Standard errors are indicated and P values were calculated using a paired t-test.

3.2.5 iASPP loss results in a decrease in p63 positive cells

To date the ASPP proteins have not been demonstrated to possess any intrinsic enzymatic activity, and their functional effects are mediated through protein/protein interactions and the modulation of transcription factors (Sullivan and Lu, 2007). *In vitro* studies have shown that iASPP can bind to TP63 with a relatively weak affinity compared to its binding to TP53 (Robinson et al., 2008). However, *in vivo*, it is known that TP63 is the key transcription factor that regulates the commitment of precursor cells to the prostate epithelial cell lineage. iASPP has recently been identified as being important for regulating the proliferative potential of TP63-positive basal cells in the epidermis of the skin (Notari et al., 2011). Therefore it is intriguing to investigate whether iASPP is necessary to regulate TP63 expression in prostate epithelial cells and thus maintain the TP63-positve basal cell population. In order to answer this question TP63 expression was evaluated and quantified in both $iASPP^{+/+}$ and $iASPP^{\Delta 8/\Delta 8}$ prostate tissue.

TP63 is exclusively expressed in the basal cells of prostate epithelium and these cells have high proliferative potential and have been shown to have the capacity to regenerate all the cell lineages after prostate regression following androgen deprivation. As basal cells enter a program of differentiation TP63 expression is lost and this is coupled by the loss of Ck14, due to the fact that Ck14 expression is regulated by TP63's transcriptional activity. In contrast Ck5 continues to be expressed as the cell moves along the differentiation pathway from a basal cell to an intermediate cell phenotype.

Immunofluorescence staining was used to evaluate the expression of TP63 (figure 3.6A). Comparative slides were selected for each prostate lobe from both $iASPP^{\Delta 8/\Delta 8}$ and age-matched $iASPP^{+/+}$ mice. Each section was stained for both TP63 and Ck5 in order to identify the basal cell layer. A significant decrease in TP63-positivity was observed in

iASPP$^{\Delta 8/\Delta 8}$ mouse prostate epithelium. In order to quantify this observation, tissue was dissected from both iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ prostate tissue for protein extraction and western blot analysis (figure 3.6B). Using this approach a clear difference in TP63 expression was observed. To conclude this analysis TP63 expression was quantified in a number of sections selected from 5 iASPP$^{+/+}$ and 5 iASPP$^{\Delta 8/\Delta 8}$ mice. Each lobe was quantified separately in order to observe any potential differences that may exist between lobes in terms of levels of TP63 expression. There was a statistically significant reduction in TP63 expression in the iASPP$^{\Delta 8/\Delta 8}$ mice compared with iASPP$^{+/+}$ mice in all three lobes (dorsal and lateral lobes were grouped together as dorsolateral lobe for this analysis), (anterior lobe, mean number of TP63-positive cells per 100 luminal cells iASPP$^{+/+}$ 22.3 ± 1.7 versus iASPP$^{\Delta 8/\Delta 8}$ 14.4 ± 1.1, P = 0.008), ventral lobe iASPP$^{+/+}$ 13.5 ± 0.4 versus iASPP$^{\Delta 8/\Delta 8}$ 9.5 ± 1.0, P = 0.016, dorsolateral lobe iASPP$^{+/+}$ 24.7 ± 2.2 versus iASPP$^{\Delta 8/\Delta 8}$ 18.7 ± 1.0, P = 0.032) (figure 3.6C). This finding led us to question whether iASPP is essential for TP63 expression, or whether iASPP is essential to maintain an appropriate level of TP63 expression in the mouse prostate. In order to answer this question iASPP expression was depleted using siRNA in the benign cell line RWPE-1 (figure 3.6D). This cell line is one of the few prostate cell lines that has a high level of TP63 expression. siRNA-mediated knockdown of iASPP in these cells showed no change in the level of TP63, thereby confirming that iASPP is not essential for TP63 expression, but rather is necessary for the maintenance of the TP63-positive cell lineage. Taken together these results suggest that loss of iASPP function pushes the differentiation program forward, resulting in a reduced number of highly proliferative TP63-positive basal cells and an increased number of Ck19-positive intermediate cells with a lower proliferative potential (Tran et al., 2002).

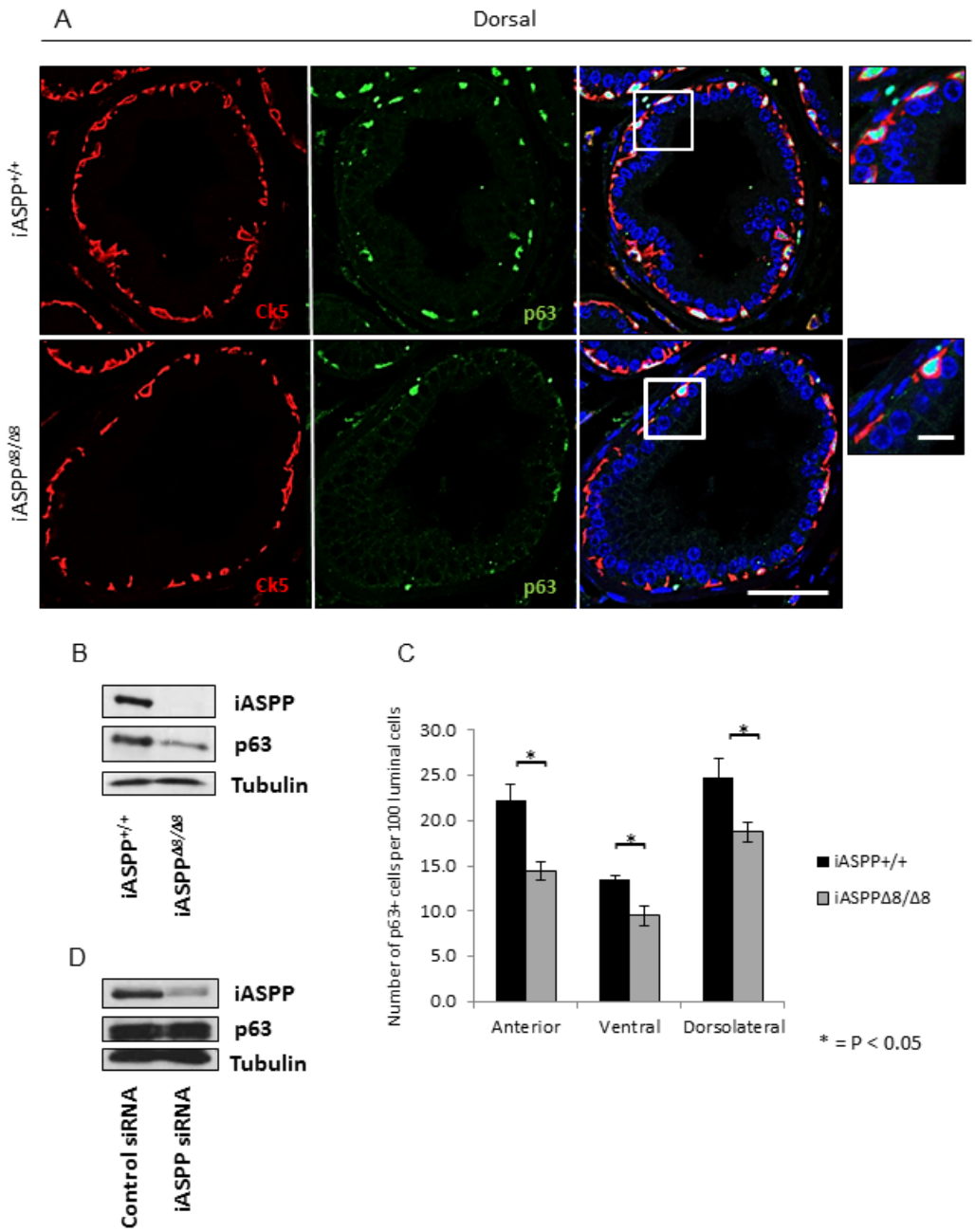

Figure 3.6 Loss of iASPP significantly reduces the number of TP63-positive cells in the mouse prostate

(A) Immunofluorscence staining of Ck5 (red) and TP63 (green) in the dorsal lobe of the mouse prostate. All individual lobes were stained and this is a reprentative image. All images were taken at 40x magnification. Squares show higher magnification to illustrate detail. Nuclei were counterstainied with DAPI. Scale bars - 50µm, 25µm. **(B)** The protein expression levels of TP63 were evaluated by western blotting in iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ prostate tissue **(C)** Quantification of TP63 expression. Comparative sections were selected for each lobe from 5 iASPP$^{+/+}$ mice and 5 iASPP$^{\Delta 8/\Delta 8}$ mice and the number of TP63-positive cells per 100 luminal cells was counted. Standard errors are indicated and P values were calculated using a Mann Whitney U test. **(D)** The protein level of TP63 in RWPE-1 cells after iASPP siRNA-mediated knockdown.

3.2.6 iASPP-deficiency does not affect prostate lineage commitment

In endodermally derived UGS TP63 appears to inhibit differentiation of progenitor cells toward other endodermally-derived epithelia outside of the urogenital tract, such as the intestinal epithelium. Intestinal epithelium is completely devoid of TP63 expression. In experiments where *p63*$^{-/-}$ UGS tissue was grafted under the renal capsule it was consistently observed that there was an absence of cells with a basal phenotype whereas mucinous cells with a close resemblance to colonic epithelium were observed (Signoretti et al., 2005, Kurita et al., 2004). These findings were validated by staining the tissue with Alcian blue which stains mucin-producing secretory cells such as goblet cells. The colonic-like cells seen in these experiments did indeed stain positively with Alcian blue suggesting that the absence of *p63* leads to an inability of the UGS precursor cells to fully commit to a prostate cell lineage.

Our earlier observation that TP63 expression is reduced in iASPP$^{\Delta 8/\Delta 8}$ mice raised the possibility that iASPP-deficiency might affect the ability of prostate cells to completely commit to the prostate cell lineage. To investigate this tissue sections taken from each lobe of the iASPP$^{\Delta 8/\Delta 8}$ mouse were stained with Alcian blue (figure 3.7). No Alcian blue staining was observed in any of the prostate glands analysed. Therefore the reduction of TP63 expression resulting from iASPP-deficiency was not severe enough to result in cells progressing to form mucinous epithelium-like cells, suggesting that iASPP loss does not impact on the full commitment of UGS precursor cells to the prostate cell lineage.

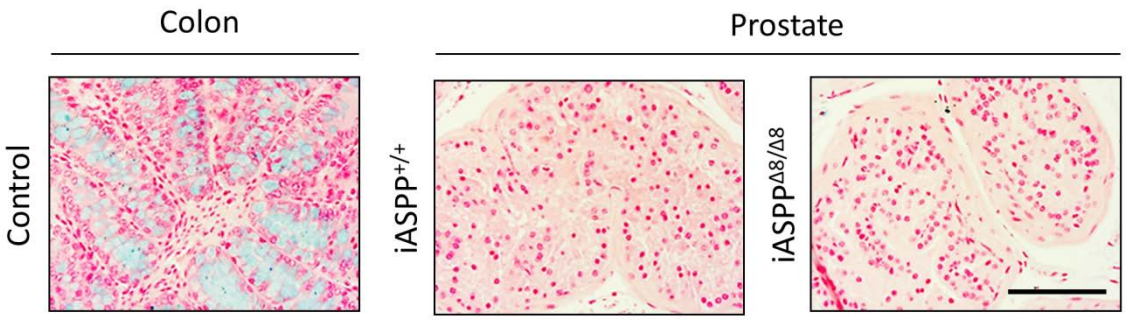

Figure 3.7 iASPP-deficiency does not affect the commitment of UGS precursor cells to a prostate cell lineage

Sections were stained with Alcian blue (pH 2.8) and counterstained with nuclear fast stain. A section of wild type colon tissue was collected as a control. All lobes were analysed; the above is a representative image. All images were taken at 20x magnification. Scale bar - 100μm

3.2.7 Synaptophysin expression is reduced in the prostate of the iASPP$^{\Delta 8/\Delta 8}$ mouse

NE cells are a far rarer cell type than basal or luminal cells in the prostate. NE cells are distinguishable by their secretion of a number of neurotransmitters and secretory products such as Chromogranin A, serotonin and synaptophysin. NE cells are thought to regulate the growth, differentiation and secretory activity of the prostatic epithelium through paracrine, endocrine and neurocrine mechanisms. Synaptophysin is a synaptic vesicle glycoprotein that participates in synaptic transmission, and is a marker that is frequently used to identify this infrequent cell type.

In order to investigate whether the loss of iASPP affects the NE cell lineage immunofluorescence staining was carried out on iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ mouse prostate tissue (figure 3.8A). However, the very small number of NE cells present in the prostate meant that no comparative analysis could be performed by simply analysing cell numbers in comparative sections. We therefore took an alternative approach whereby synaptophysin expression levels were measured by a western blot analysis of protein

extracts taken from pure prostate tissue from 3 pairs of iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ mice (figure 3.8B). Given that synaptophysin is expressed in nerve bundles and the stromal compartment of mouse prostate as well as in occasional NE cells within the epithelium, it is possible that any differences observed between the iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ mice in the expression of this NE marker might actually be due to other differences in the prostate rather than changes in the NE cell population. However, given that all prostate lobes were removed and pooled for each mouse the other sources of synaptophysin should contribute equally to in all the cell lysates. We observed a clear reduction in synaptophysin levels in the tissue taken from the iASPP$^{\Delta 8/\Delta 8}$ mice compared to tissue taken from the wild-type mice (all four lobes were pooled for this analysis). The immunofluorescence analysis (figure 3.8A) also confirmed that the reduction in synaptophysin expression observed in the iASPP$^{\Delta 8/\Delta 8}$ mice was not due to a reduction in background expression as both the iASPP$^{+/+}$ and the iASPP$^{\Delta 8/\Delta 8}$ tissue exhibited a high fluorescence signal around the basement membrane of the glands. This reduction in synaptophysin expression was quantified using image J software to measure the band intensity from the western blot (figure 3.8C). All bands were normalised against the actin loading control (mean iASPP$^{+/+}$ 22.8 ± 4.8 versus iASPP$^{\Delta 8/\Delta 8}$ 9.0 ± 3.0, P = 0.032).

The reduced expression of synaptophysin in the absence of functional iASPP is an intriguing observation as it is well-documented that *p53* loss during prostate tumorigenesis increases the number of NE cells (Zhou et al., 2006, Chen et al., 2012). There is evidence to suggest that TP53 exerts a growth inhibitory mechanism upon the NE cell population via an interleukin 8 (IL8) - $CXCR_2$ – p53 pathway which keeps these cells in a quiescent state. Inactivation of this pathway, perhaps through the acquisition of a *p53* mutation, may be responsible for the rapid proliferation of NE cells seen in

conditions such as prostatic small cell carcinoma (Chen et al., 2012). iASPP may therefore play a role in regulating the number of NE cells through its inhibitory interaction with *p53*. Loss of functional iASPP may increase the p53-mediated inhibition of the number of NE cells within prostate epithelium. However, any interpretation of the changes observed in synaptophysin expression in this model should consider the fact that this marker is also expressed in neurons; therefore a reduction in the number of nerve bundles within the prostate of the iASPP$^{\Delta 8/\Delta 8}$ mice cannot be excluded.

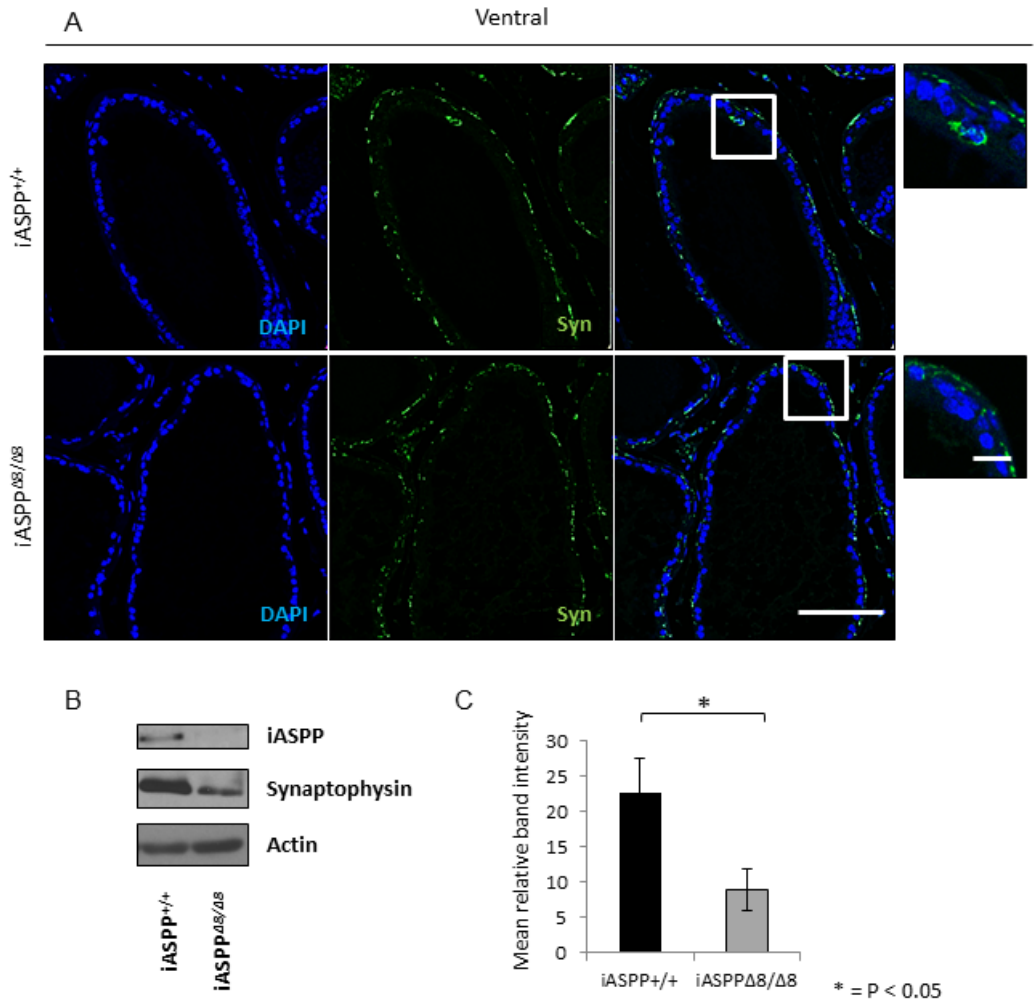

Figure 3.8 Synaptophysin expression is reduced in the prostate of iASPP$^{\Delta 8/\Delta 8}$ mice

(A) Immunofluorescence staining of neuroendocrine cells with synaptophysin (green) (middle panel). Merged image (right panel) in the ventral lobe of the iASPP+/+ mouse prostate. All lobes were stained and this is a reprentative image. All images were taken at 20x magnification. Square shows higher magnification to illustrate detail. Nuclei were counterstainied with DAPI. Scale bars - 75µm, 25µm. (B) Western blot analysis of synaptophysin levels in both iASPP+/+ and iASPPΔ8/Δ8 prostate tissue. (C) Quantification of relative protein expression of synaptophysin. Standard errors are indicated and the P value was calculated using a paired t-test.

3.2.8 Epithelial cell-to-cell contacts appear unaffected by iASPP-deficiency

iASPP shows a slightly different pattern of expression in the mouse prostate epithelium depending on whether immunofluorescence or immunohistochemistry is used. Using immunofluorescence iASPP is seen to be localised to the cell junctions and the cytoplasm, whereas it is in the cytoplasm and nucleus using immunohistochemistry. We therefore used junctional markers to investigate the possibility that loss of iASPP affects the integrity of cell-cell contacts, especially as previous studies suggest that members of the ASPP family are implicated in the integrity of cell-to-cell contacts. ASPP2 has been identified as being a component of tight junctions and co-localises with Par-3 at the apical region of epithelial cells suggesting that ASPP2 regulates cell polarity (Sottocornola et al., 2010). iASPP was recently found to have an important association with the intercalated discs of the heart and was shown to be located at the polar-end of cardiomyocytes, suggesting that iASPP has an integral role in maintaining the structural integrity of the heart (personal communication from Mario Notari). Taken together these findings suggest that iASPP may play a role in the maintenance of cell-to-cell contacts within prostate epithelium. In order to investigate this possibility a panel of junctional markers was used to analyse the presence of tight junctions, gap junctions and adherens junctions. Each of these types of junction has a different role to play in maintaining the homeostasis of normal healthy epithelium. Tight junctions act as a barrier by sealing the epithelial cells together as a continuous sheet thereby preventing the passage of molecules and ions into the paracellular space. Claudin-1 expression was analysed in our prostate samples as this is a marker of tight junctions (figure 3.9). Claudin-1 is normally located at the apical and basolateral aspect of prostate epithelial cells and has been identified as being an important protein in prostate gland integrity (Seo et al., 2010). We observed no

difference in the localisation or expression of claudin-1 in the iASPP$^{\Delta 8/\Delta 8}$ mice compared to control.

In order to investigate the effect of loss of iASPP function on gap junction assembly connexin-43 expression was analysed. Gap junctions are formed by the connexin proteins and permit the free passage of small molecules and ions between cells. Connexin proteins normally assemble as hexamers in the cell membrane to form connexons that align with connexons in adjacent cells in order to form intercellular pathways. Connexin-43 and connexin-32 have been identified as prostate-specific connexin proteins (figure 3.9). The expression of connexin-43 has been shown to be disrupted in human prostate cancer (Tsai et al., 1996). No difference in connexin-43 expression or localisation was observed in the iASPP$^{\Delta 8/\Delta 8}$ prostate epithelium when compared with iASPP$^{+/+}$ controls.

Next, in order to evaluate the effect of iASPP-deficiency on adherens junction formation the expression of both E-cadherin and β-catenin was analysed (figure 3.10). Adherens junctions provide strong mechanical attachments between adjacent cells. β-catenin links E-cadherin to the plasma membrane via the adapter protein α-catenin. It also plays a role in mediating the interplay of adherens junction molecules with the actin cytoskeleton. Both E-cadherin and β-catenin play an integral role in the stability and strength of cell adhesions between neighbouring cells. Down regulation of E-cadherin and membrane-bound β-catenin have both been associated with prostate cancer progression (Jaggi et al., 2005). We observed no difference in either the expression or localisation of E-cadherin and membrane-bound β-catenin in the iASPP$^{\Delta 8/\Delta 8}$ mouse prostate tissue compared with iASPP$^{+/+}$ controls.

Taken together these results indicate that the cell-to-cell junctions of prostate epithelial cells are not affected by the loss of function of iASPP. However it should be remembered that only a limited number of proteins were evaluated in this aspect of the project. Cellular junctions are complex structures and are comprised of numerous components, therefore carrying out an analysis of just a small number of these components may not fully exclude the possibility that iASPP may be an active junctional protein. Loss of iASPP function could weaken a junctional complex without necessarily leading to its disruption. In order to unequivocally address the question as to whether or not iASPP plays a functional role at the cell-to-cell junction, an extensive evaluation of a large number of junctional markers would have to be carried out. Even this approach might not fully elucidate whether iASPP plays a role in junctional stability and/or strength as it might only pin-point protein expression or localisation changes. To evaluate strength of cell: cell junctions a mechanical stress would have to be applied to the tissue. In order to evaluate the barrier function of tight junctions a measure of permeability would have to be undertaken.

Taken together these results suggest that iASPP does not appear to play a role in regulating the expression or localisation of common markers of cell; cell junctions.

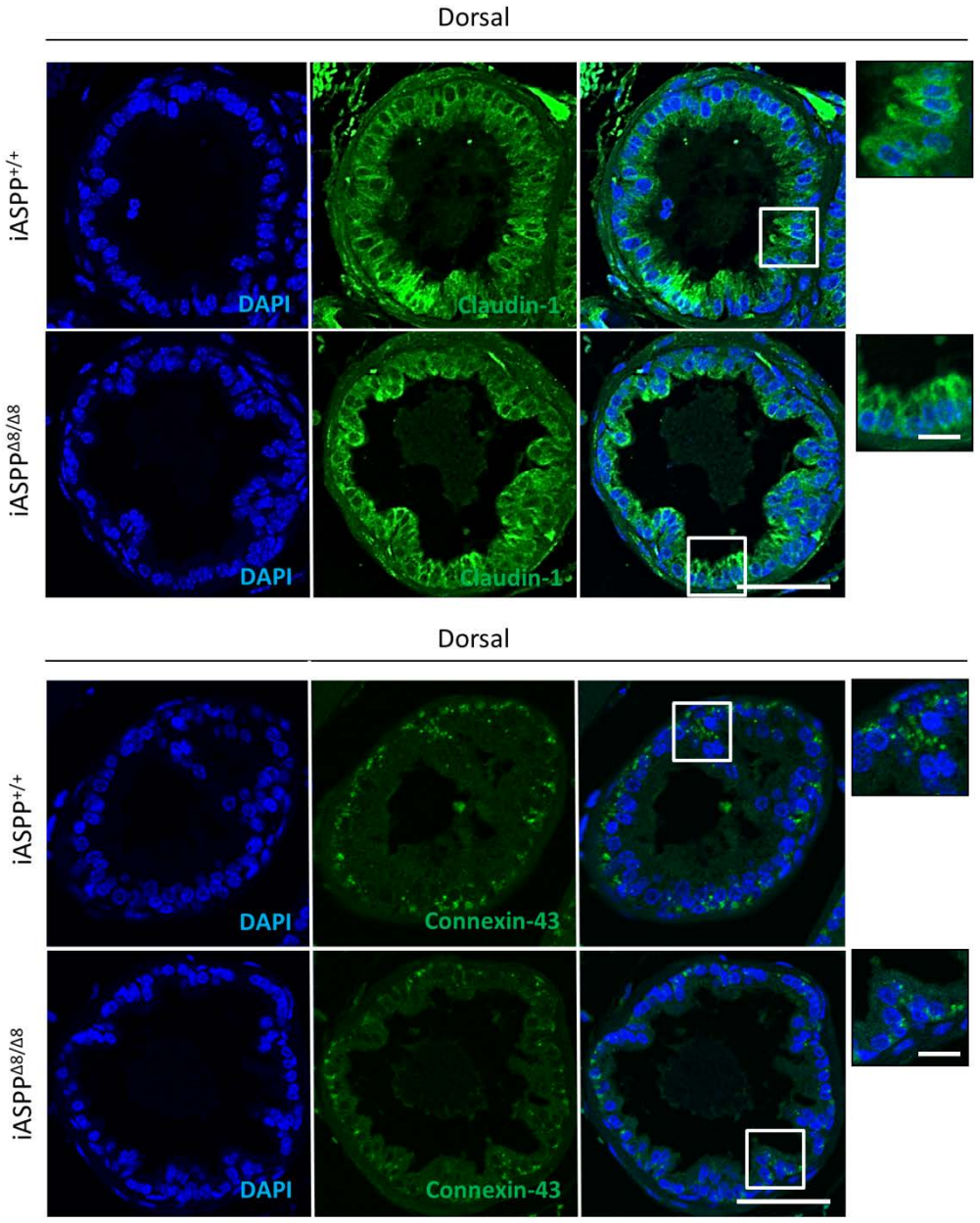

Figure 3.9 Tight junction and gap junction proteins are present at the cell-cell membrane in both iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ mouse prostate epithelial cells

Immunofluorescence staining of the tight junction proteins claudin-1 (upper panel, green, dorsal prostate) and connexin-43 (lower panel, green, dorsal prostate) in iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ mouse prostate tissue. All lobes were stained and these are representative images. All images were taken at 40x magnification. Squares show higher magnification to illustrate detail. Nuclei were counterstainied with DAPI. Scale bars - 50μm, 25μm.

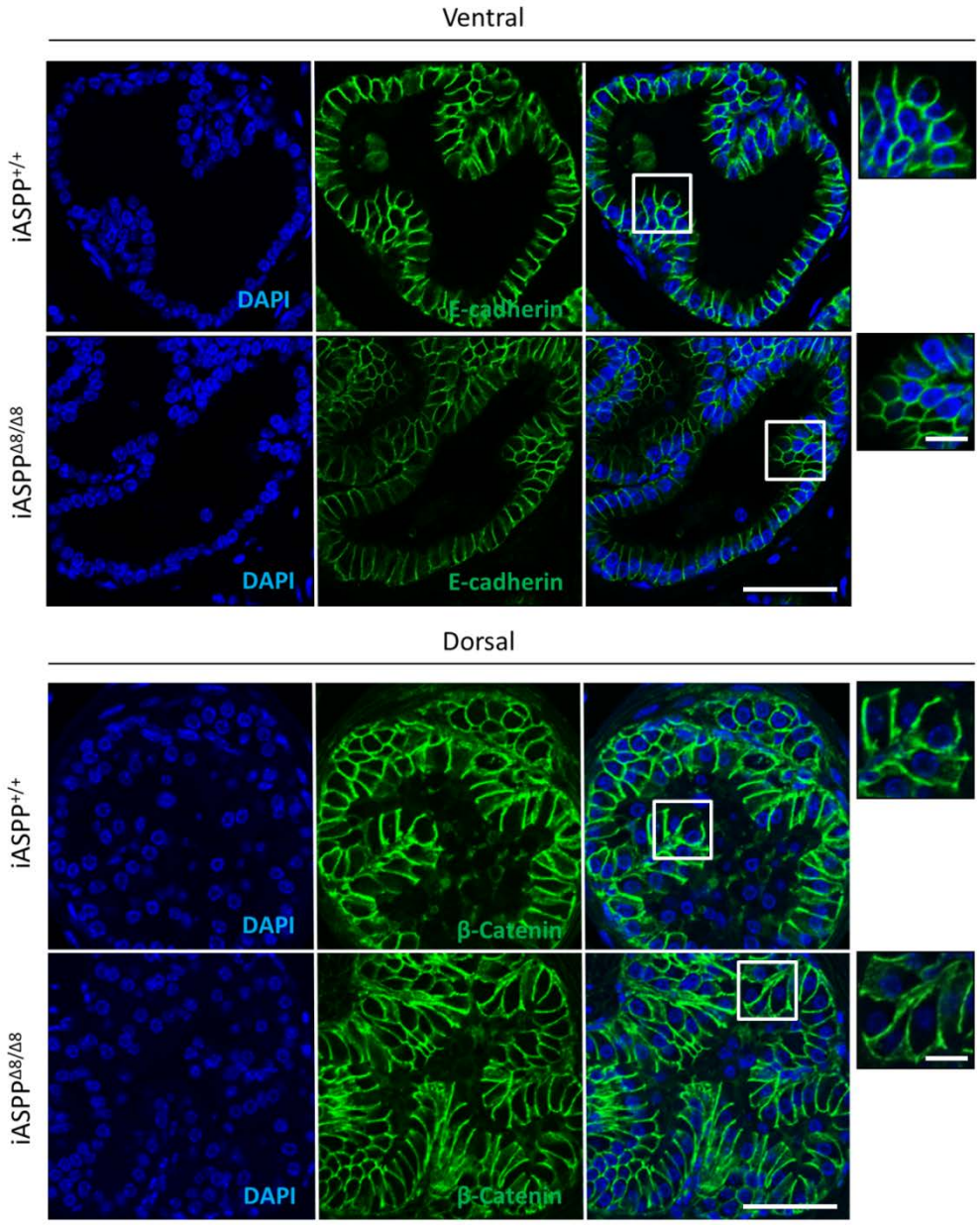

Figure 3.10 Adherens junction proteins are present at the cell-cell membrane in both iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ mouse prostate epithelial cells.

Immunofluorescence staining of adherens junctional proteins E-cadherin (upper panel, green, ventral prostate) and β-catenin (lower panel, green, dorsal prostate) in iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ mouse prostate tissue. All lobes were stained and these are representative images. All images were taken at 40x magnification. Squares show higher magnification to illustrate detail. Nuclei were counterstainied with DAPI. Scale bars - 50μm, 25μm.

3.2.9 iASPP-deficiency does not affect the muscle component of the mouse prostate

Stromal-epithelial interactions have been shown to play an important role in normal prostate development. Maintenance of cell and tissue homeostasis is dependent upon the dynamic balance of cell proliferation, differentiation, and apoptosis through interactions between cells and their microenvironment. The unique prostatic epithelial cell phenotypes are in part induced and maintained by the interaction between these epithelial cells and adjacent stromal cells through intimate intercellular signalling pathways. During normal prostate gland development appropriate and reciprocal signalling between these two cellular compartments is essential. Androgen stimulation of the developing stroma causes the differentiation of prostate epithelium. Without this cross-talk prostate development would not take place (Cunha, 1994). The prostate stroma is composed primarily of smooth muscle, fibroblasts and myofibroblasts. These stromal cells secrete growth factors, produce extra cellular matrix, and express a number of key receptors including the androgen receptor (AR) and estrogen receptor. Smooth muscle cells are characterised by the expression of desmin and α-actin, whereas fibroblasts express vimentin and myofibroblasts express procollagen-1. Other less prevalent cells of the stroma include endothelial cells, neuroendocrine cells and inflammatory cells (Niu and Xia, 2009). The stroma has a greater level of complexity than the epithelium owing to its involvement in a high number of biological processes such as cell adhesion, muscle development and contraction (Berquin et al., 2005).

iASPP has recently been implicated in maintaining the integrity of the intercalated discs of the heart. There is evidence to suggest a possible link between iASPP, desmoplakin and desmin in strengthening the link between the cytoskeleton and the sarcolemma membrane in order to maintain the structural and mechanical integrity of the cell during

contraction (personal communication from Mario Notari). This relationship was observed in cardiac muscle, however, given our earlier observation that the urinary bladder of the iASPP$^{\Delta 8/\Delta 8}$ mouse was smaller than that of its comparative wild-type, we wondered whether there could be a defect in the smooth muscle of the bladder, possibly preventing normal muscle contraction. Any potential abnormality of bladder muscle could lead to an inability to hold a normal capacity of urine. If the loss of iASPP causes a general defect in muscle cells in the body this might be apparent in the smooth muscle cells of the prostate. To investigate this hypothesis tissue sections from both iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ mouse prostates were stained with desmin and α-smooth muscle actin (α-SMA) (figure 3.11). No difference was observed between the iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ prostate tissue, suggesting that iASPP does not play a role in maintaining the integrity of the smooth muscle component of the mouse prostate stromal compartment.

It is worthwhile noting that we only evaluated the integrity and organisation of the smooth muscle cells which make up the majority of the stroma. Changes in number or organisation of the remaining cells present in the stromal compartment of the iASPP$^{\Delta 8/\Delta 8}$ mouse cannot be ruled out at this stage. However no striking stromal differences were observed during the histological analysis of this tissue.

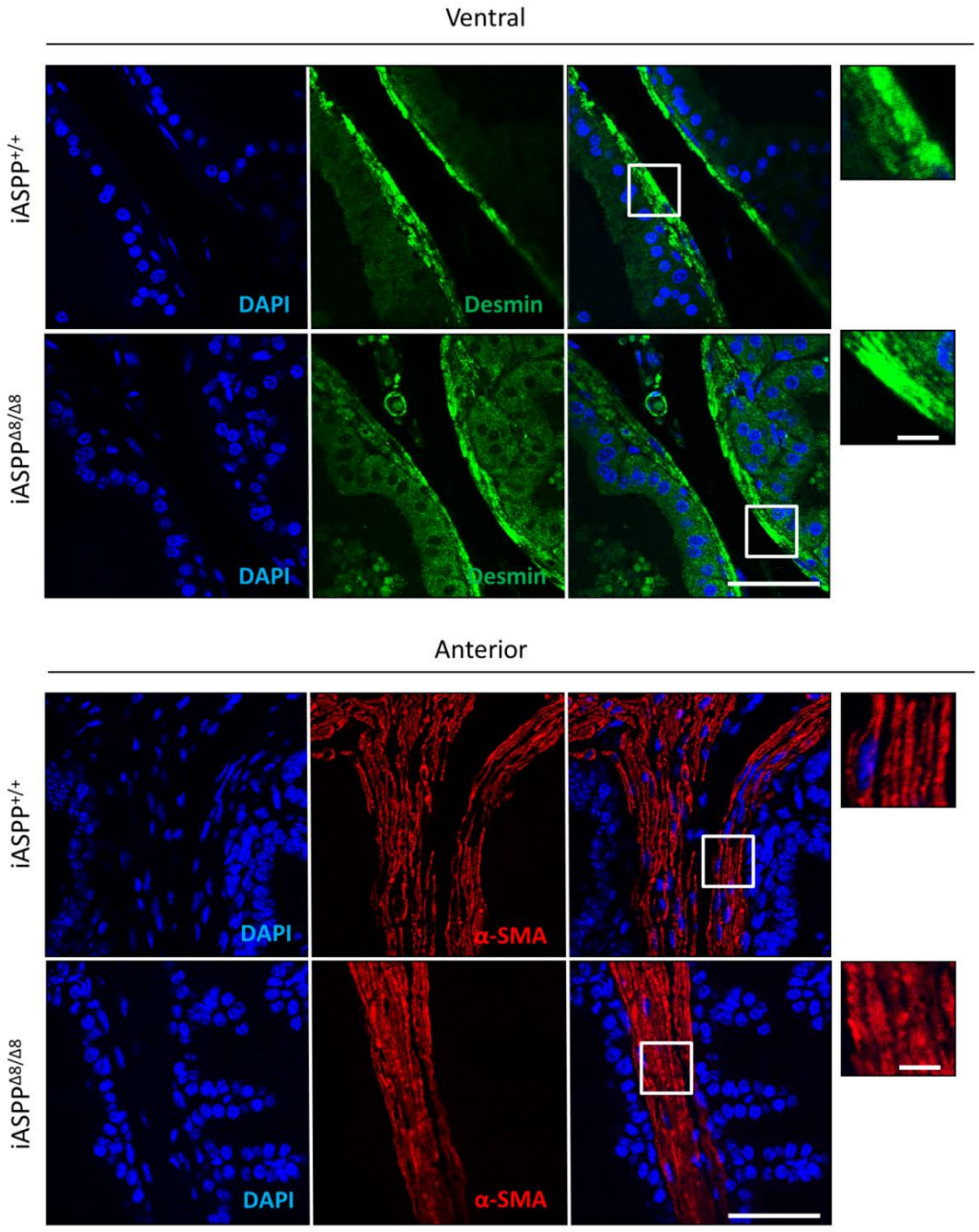

Figure 3.11 iASPP loss does not affect the smooth muscle component of the prostate

Immunofluorescence staining of smooth muscle cell proteins desmin (upper panel, green, ventral prostate, α-SMA (lower panel, red, anterior prostate) in iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ mouse prostate tissue. All lobes were stained and these are representative images. All images were taken at 40x magnification. Squares show higher magnification to illustrate detail. Nuclei were counterstainied with DAPI. Scale bars - 50μm, 25μm.

3.2.10 iASPP loss reduces the rate of cell proliferation in the prostate of the iASPP$^{\Delta 8/\Delta 8}$ mouse

The baseline rate of epithelial cell proliferation in the adult mouse prostate is low, and once the gland is fully developed the rate of cell turnover is slow in order to maintain tissue homeostasis. The loss of iASPP in a cancer setting has been shown to result in a decrease in the rate of cancer cell proliferation. This has been clearly demonstrated in a number of different studies in a range of different malignancies such as lung, colon, liver, bladder and prostate cancer (Chen et al., 2010, Liu et al., 2013b, Lin et al., 2011, Liu et al., 2011, Zhang et al., 2011). The functional role of iASPP in normal tissue has not been well studied. Even though the proliferation rate in the normal prostate is low active epithelial cell proliferation is essential in order to ensure the maintenance of healthy tissue.

To evaluate the rate of proliferation in iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ prostate tissue, sections were stained with the proliferation marker Ki67 (figure 3.12A). Ki67 is present during all active phases of the cell cycle, but is absent from resting cells. During interphase Ki67 accumulates in the nucleus, whereas in mitosis it is relocated onto the surface of the chromosomes. Comparative slides were selected for each lobe from both iASPP$^{\Delta 8/\Delta 8}$ mice and age-matched iASPP$^{+/+}$ controls. All lobes were quantified separately in order to allow for any potential differences that may exist in the rate of epithelial cell proliferation between particular anatomical lobes. We observed a reduction in ki67 expression in the iASPP$^{\Delta 8/\Delta 8}$ mice compared to iASPP$^{+/+}$ mice in all three lobes (dorsal and lateral lobes were grouped for this analysis) (anterior lobe, mean number of ki67-positive cells per 100 epithelial cells counted iASPP$^{+/+}$ 3.7 ± 2.1 versus iASPP$^{\Delta 8/\Delta 8}$ 2.2 ± 1.9 P = 0.273, ventral lobe, mean iASPP$^{+/+}$ 1.0 ± 0.5 versus iASPP$^{\Delta 8/\Delta 8}$ 0.8 ± 0.5, P = 0.626, dorsolateral

lobe mean iASPP$^{+/+}$ 2.4 ± 1.9 versus iASPP$^{\Delta 8/\Delta 8}$ 0.7 ± 0.7, P = 0.295) (figure 3.12B). Although a consistent reduction in the number of proliferating epithelial cells was observed the difference did not reach statistical significance (p>0.05). This may be explained by the small sample size together with the fact that the mice were aged between 6 and 24 weeks. It is known that during normal aging of mice the number of proliferating cells naturally decreases. In both the iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ prostate tissue of the 24 week old mouse no ki67 staining was observed. Therefore any analysis of tissue taken from mice older than a few months of age might make the data difficult to accurately interpret. In order to overcome this issue, the rate of cellular proliferation was assessed in a cohort of 7 mice; (1 iASPP$^{+/+}$ and 1 iASPP$^{\Delta 8/\Delta 8}$ mouse aged 18 weeks, and 2 iASPP$^{+/+}$ and 3 iASPP$^{\Delta 8/\Delta 8}$ mice aged 13 weeks). These mice were more comparable in age than in our earlier analysis and had only 5 weeks of age between them, compared to 18 weeks in our first experiment. Mice were injected with a single pulse of 5-bromo-2-deoxyuridine (BrdU) 24hrs before they were sacrificed. BrdU is a uridine derivative and a structural analog of thymidine and it can be incorporated into DNA during the synthesis-phase of the cell cycle as a substitute for thymidine, thereby serving as a marker for proliferation. Comparative slides were selected from each lobe for both iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ mice. Immunofluorescence was carried out in order to visualise the incorporated BrdU (figure 3.12C). All lobes were quantified separately. A reduction in cellular proliferation was observed in the iASPP$^{\Delta 8/\Delta 8}$ in comparison to control mice (dorsal and lateral lobes were grouped for this analysis) (anterior lobe, mean of BrdU-positive cells per 100 total cells counted iASPP$^{+/+}$ 1.4 ± 0.6 versus iASPP$^{\Delta 8/\Delta 8}$ 0.6 ± 0.2, P = 0.329, ventral lobe, mean iASPP$^{+/+}$ 0.9 ± 0.4 versus iASPP$^{\Delta 8/\Delta 8}$ 0.5 ± 0.1, P = 0.132, dorsolateral lobe, mean iASPP$^{+/+}$ 0.8 ± 0.2 versus iASPP$^{\Delta 8/\Delta 8}$ 0.5 ± 0.1, P = 0.137) (figure 3.11D). The difference

in proliferation again did not reach statistical significance and this might be due to the restraints of a small sample size. Even though this data did not show statistical significance it did show a clear trend towards a reduction in the number of proliferating cells in the epithelium of the iASPP$^{\Delta 8/\Delta 8}$ mice. This indicates that iASPP may play a role in regulating the rate of epithelial cell proliferation in the mouse prostate.

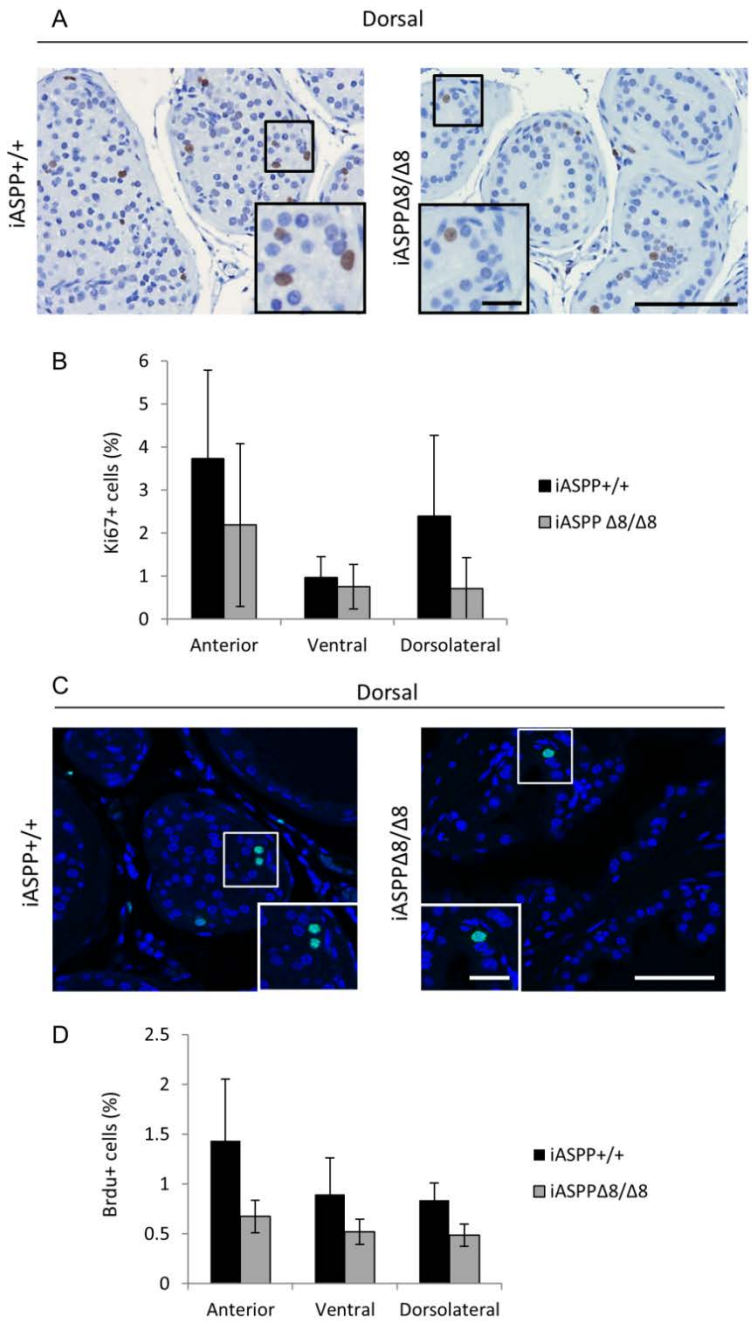

Figure 3.12 iASPP-deficiency reduces the rate of cellular proliferation

(A) Immunohistochemical staining of ki67 in the dorsal lobe of iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ prostate tissue. All images were taken at 20x magnification. Squares show higher magnification to illustrate detail. Scale bar - 100µm. **(B)** Comparative sections were selected for each lobe from 3 iASPP$^{+/+}$ mice and 3 iASPP$^{\Delta 8/\Delta 8}$ mice and the percentage of ki67 positive cells per total cells was calculated. Standard errors are indicated and P values were calculated using a paired t-test **(C)** Immunofluorescence staining of BrdU (green) in the dorsal lobe of both iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ prostate tissue. All images were taken at 20x magnification. Squares show higher magnification to illustrate detail. Nuclei were counterstainied with DAPI. Scale bar - 100µm, 50µm. **(D)** Comparative sections were selected from each lobe for 3 iASPP$^{+/+}$ mice and 4 iASPP$^{\Delta 8/\Delta 8}$ mice the percentage of BrdU positive cells per total cells was calculated. Standard errors are indicated and P values calculated using a paired t-test

3.2.11 iASPP loss results in a reduced number of apoptotic cells

The cellular function iASPP is most well-known for is its inhibitory influence on the apoptotic function of *p53*. iASPP binds to the DNA binding domain of TP53 preventing it from interacting with ASPP1 or ASPP2, thereby resulting in the attenuation of *p53's* apoptotic response. Previous studies have demonstrated that the loss of iASPP in *p53* wild-type cancer cells leads to an increase in apoptosis in response to a number of drug treatments such as etoposide and daunorubicin (Liu et al., 2009). However, these studies investigated the impact of iASPP-deficiency after cells have been challenged with an apoptosis inducer. Moreover, these cells are already molecularly altered with changes occurring in many proteins regulating cellular proliferation and apoptosis. Cancer cells are more vulnerable than normal cells to apoptosis. In a scenario when a normal cell may choose to senesce a cancer cell no longer has this ability, and so almost invariably undergoes a program of cell death. Most of the work carried out to elucidate the function of iASPP has been done with regards to its fundamental role in cancer. Little is known about its role in normal tissue homeostasis.

Given that the trend towards a decrease in the number of proliferating cells was observed in the prostate tissue of the $iASPP^{\Delta 8/\Delta 8}$ mouse, we were intrigued to understand whether this decrease in proliferation might be compensated by a decrease in apoptosis. Intuitively, an increase in apoptosis may be expected as a result of loss of iASPP function; however these mice were not challenged in order to elicit a p53-response. In order to investigate the possibility that loss of iASPP in normal tissue leads to a change in the rate of apoptosis a Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay was performed using tissue taken from both $iASPP^{+/+}$ and $iASPP^{\Delta 8/\Delta 8}$

mouse prostates. TUNEL is a means of measuring nuclear DNA fragmentation which is a hallmark of apoptotic cells (figure 3.13A).

Comparative slides were selected from each prostate lobe for both iASPP$^{\Delta 8/\Delta 8}$ mice and age-matched iASPP$^{+/+}$ mice and all lobes were quantified separately. We observed a reduction in the number of apoptotic cells in the iASPP$^{\Delta 8/\Delta 8}$ mice compared to the iASPP$^{+/+}$ mice in all three prostate lobes (dorsal and lateral lobes were grouped for this analysis) (anterior lobe, mean number of TUNEL-positive cell per 100 total cells counted iASPP$^{+/+}$ 1.5 ± 0.5 versus iASPP$^{\Delta 8/\Delta 8}$ 1.3 ± 0.8, P = 0.793, ventral lobe, mean iASPP$^{+/+}$ 0.4 ± 0.2 versus iASPP$^{\Delta 8/\Delta 8}$ 0.2 ± 0.1, P = 0.383, dorsolateral lobe, mean iASPP$^{+/+}$ 0.8 ± 0.5 versus iASPP$^{\Delta 8/\Delta 8}$ 0.3 ± 0.1, P = 0.313) (figure 3.13B). Even though a reduction in the number of apoptotic cells was observed this result did not reach statistical significance.

Given that we observed a reduction in the rates of proliferation and apoptosis in iASPP$^{\Delta 8/\Delta 8}$ prostate compared with wild-type controls one might postulate that the loss of iASPP results in premature cellular senescence, with cells neither cycling nor dying. The slow rate of proliferation is balanced by a slow rate of apoptosis, as fewer cells enter into the cell cycle. Taken together these observations complement the work of Notari et al. whereby a similar phenotype has been observed following loss of iASPP function in the epidermis of the skin (Notari et al., 2011).

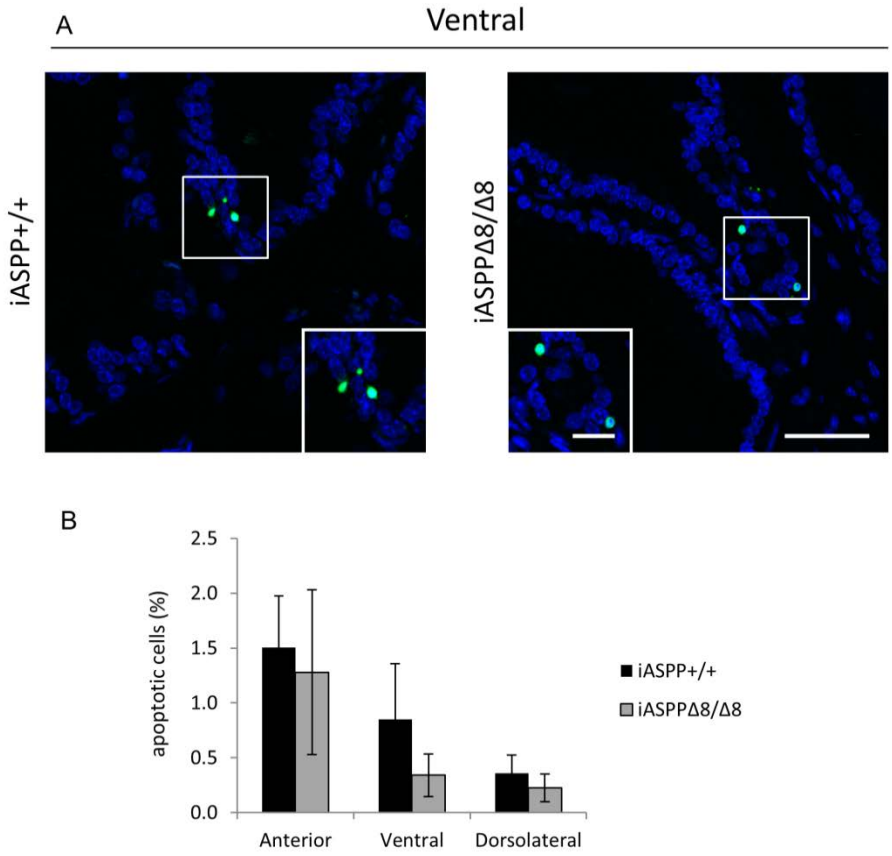

Figure 3 13 iASPP loss results in a reduced number of apoptotic cells

(A) Immunofluorescence staining of TUNEL (green) in the ventral lobe of both iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ prostate tissue. All images were taken at 20x magnification. Squares show higher magnification to illustrate detail. Nuclei were counterstained with DAPI. Scale bar - 100µm, 50µm. **(B)** Comparative sections were selected for each lobe for 5 iASPP$^{+/+}$ mice and 5 iASPP$^{\Delta 8/\Delta 8}$ mice and the percentage of apoptotic cells (TUNEL positive cells) was calculated. Standard errors are indicated and P values were calculated using a paired t-test.

3.3 Discussion

Recently a transgenic mouse strain was generated that has a deletion of exon 8 in the murine *ppp131rl* gene which results in the complete absence of its iASPP protein product. iASPP$^{\Delta 8/\Delta 8}$ mice allow the function of iASPP to be studied with regards to the development of organ structure and function. To date the prostate of the iASPP$^{\Delta 8/\Delta 8}$ mice has not been characterised. Prostate epithelium relies heavily on *p63* expression for normal development and homeostasis and iASPP is a known regulator of the *p53* family of genes of which *p63* is a member. We were therefore keen to understand whether iASPP plays an essential role in normal prostate biology.

By collecting a number of prostate specimens from both iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ mice the histology, cell lineage and cell identity of the prostate epithelium could be analysed. Moreover, we were able to analyse key biological processes such as differentiation, proliferation and apoptosis all of which are normal biological processes associated with healthy tissue and the maintenance of homeostasis. The main aim of this characterisation was to investigate whether any of these processes were compromised, changed or lost in the iASPP$^{\Delta 8/\Delta 8}$ mice. Even though iASPP has a clear role in development as is clearly highlighted by the observation that these mice are not born according to Mendelian segregation theory, the surviving offspring appear to develop normally into adulthood. iASPP mice do not present any clinical evidence of tumourigenesis or any benign abnormal lesions in the prostate.

Our macroscopic analysis of the prostate gland of the iASPP$^{\Delta 8/\Delta 8}$ mice revealed that it was slightly smaller in size compared with the wild-type organ. Despite this observation no gross difference in the histology of the prostate tissue was seen, suggesting that the loss of iASPP is not sufficient to cause any changes in glandular or cellular morphology.

Analysis of our complete iASPP staining data indicates that the iASPP protein appears to be localised to the nucleus, cytoplasm and membrane of prostate epithelial cells. Differences in iASPP localisation were seen depending on the staining techniques used highlighting the fact that the exact staining technique and procedure used for antigen retrieval may alter antibody sensitivity resulting in very different the end results in terms of tissue staining. Nuclear proteins are normally more difficult to detect and so may need an extra amplification step. The specificity of the iASPP antibody has been previously shown to be reliable on western blot detection as the iASPP band is absent from tissue lysates taken form the iASPP$^{\Delta 8/\Delta 8}$ mouse tissue. The observed differences in staining therefore probably indicate that the antigenicity of the mouse tissue after fixation and heat exposure may be compromised. Our subsequent analysis of iASPP expression in human tissue (see chapter 4) confirmed that iASPP is indeed localised to the nucleus, cytoplasm and membrane of prostate epithelial cells and this gives a level of validation to the staining observed in the mouse tissue using both immunofluorescence and immunohistochemistry.

Even though no difference was initially observed in glandular or cellular morphology between the iASPP$^{+/+}$ and iASPP$^{\Delta 8/\Delta 8}$ mouse prostates, the loss of iASPP did appear to have an impact on the differentiation program. In normal tissue it is important to have a balance between the number of progenitor cells and the number of committed and terminally differentiated cells. This balance allows the tissue to function at a healthy level preventing any over-growth or involution of the tissue. It is also important to have the correct number of progenitor cells for tissue regeneration in the event of tissue damage or, in the case of the prostate, to react to any fluctuations in androgen levels. The iASPP$^{\Delta 8/\Delta 8}$ mice were found to have a significant reduction in the number of TP63-

positive cells and a significant increase in the number of intermediate cells. This finding suggests that iASPP is necessary to maintain the proliferative potential of TP63-positive cells, and that its loss results in a shift in the differentiation program towards a less proliferative cell phenotype. However, the observed reduction in TP63 expression did not appear to have any effect on prostate epithelial cell identity or commitment to the prostate cell lineage, as the iASPP$^{\Delta 8/\Delta 8}$ prostate tissue stained negative for the intestinal cell marker Alcian blue.

Interestingly, the iASPP$^{\Delta 8/\Delta 8}$ mice were found to have a significant decrease in synaptophysin expression. Synaptophysin is a marker of NE cells therefore this observation suggested that changes in cellular differentiation seen in the iASPP$^{\Delta 8/\Delta 8}$ tissue might be the result of alterations in cell fate decisions. Both intermediate cells and NE cells are thought to originate from TP63-positive progenitor cells. It would appear that iASPP-deficiency may cause an alteration in the decision-making process as to whether a differentiating TP63-positive basal cell becomes an intermediate cell or a NE cell. This is an intriguing possibility as NE cells are thought to play an important role in regulating the differentiation and growth of the prostate. Therefore, the changes in differentiation could also be linked to the reduction in NE cell number.

No difference was observed in the cell-to-cell contacts or in the cellular organisation of the smooth muscle cells of the stromal compartment of the prostate. iASPP loss did however appear to affect the proliferation rate of prostate cells given that there was a trend towards a reduction in both ki67 and BrdU positive cells in the iASPP$^{\Delta 8/\Delta 8}$ mice compared to the wild-type. This reduction in cellular proliferation appeared to be compensated by a decrease in the rate of apoptosis as demonstrated by TUNEL staining. These findings suggest that a loss of iASPP compromises the rate of cellular turnover in

the prostate epithelium. A decrease in proliferation rate coupled with a decrease in the rate of apoptosis might also suggest that there is a higher proportion of senescent cells in the iASPP$^{\Delta 8/\Delta 8}$ prostate compared with the iASPP$^{+/+}$ prostate.

Taken together these findings show, for the first time to our knowledge that iASPP is involved in maintaining the TP63-positive cell lineage in the mouse prostate, and that iASPP plays a key role in maintaining a correct balance of cellular differentiation in this organ. The alterations observed in cellular differentiation, cell fate decisions, proliferation and apoptosis are novel insights into the role of iASPP in murine prostate biology.

Chapter 4: iASPP is associated with prostate cancer progression

4.1 Introduction

Prostate cancer is the second most common male-related cancer worldwide and the commonest male malignancy in the UK. In 2009 40,841 men were diagnosed with prostate cancer and over 10,000 men died from this disease in the UK (www.cancerresearchuk.org). One of the main challenges in prostate cancer treatment is the extreme variability of its clinical course. Prostate cancers can be relatively indolent or latent tumours that will result in no clinical symptoms throughout the life-time of the patient. These patients almost invariably die with their cancer, and not from it. However, some cases of prostate cancer can take an aggressive course and spread quickly throughout the body, affecting adjacent organs such as the urinary bladder, rectum and seminal vesicles, and metastasising to distant sites, most frequently to lymph nodes, bone, liver and lung (Schulz et al., 2003). These patients unfortunately have a very poor clinical outcome. Once a patient has been diagnosed with metastatic disease their likelihood of dying from prostate cancer surpasses their risk of dying from other causes. It is therefore of paramount importance to find markers that can aid in risk stratification, enabling clinicians to confidently categorise patients into low-, intermediate- and high-risk of their disease progressing to an aggressive fatal malignancy.

One area of prostate cancer biology that remains controversial is the cell type that gives rise to and maintains prostate carcinoma (Goldstein et al., 2010) Luminal cells were previously thought to be the cells of origin of prostate cancer, as the malignancy is characterised by a loss of basal cell markers such as TP63, along with an expansion of cells with a luminal phenotype expressing the androgen receptor (AR) and prostate-specific antigen (PSA). However, there is considerable evidence that the histological

hallmarks of the disease do not necessarily correlate with its cellular origin, and that it is the basal cells that contain the prostate cancer-initiating cells (Schalken and van Leenders, 2003, Maitland et al., 2011). It is postulated that although many of the identifying markers of the basal cell population such as p63 and Ck14 are no longer expressed, these basal cells still maintain their ability to proliferate at a high rate and express pro-survival proteins such as bcl-2 at a high level (McDonnell et al., 1992), which are normally only detected in the basal layer of normal prostate epithelium.

The loss of TP63 expression during the progression to invasive prostate cancer, is accompanied by an inability of prostate epithelial cells to undergo apoptosis, which is a process normally regulated by both *p63* and *p53*. *p53* mutations in prostate cancer are uncommon given that only 10-30% of organ confined malignancies harbour mutant *p53*. However, this figure rises substantially to around 70% of cases of locally advanced and hormone-resistant metastatic prostate cancer (Navone et al., 1993). Due to the relatively low *p53* mutation rate in organ-confined prostate cancer it is likely that the inability of wild-type *p53* to initiate and execute apoptosis may be explained by molecular alterations elsewhere in the apoptotic pathway.

iASPP is an inhibitory member of the ASPP family of proteins and is known to inhibit p53-mediated apoptosis and regulate TP63 function. iASPP has been found to be highly expressed in the basal layer of stratified epithelium (Notari et al., 2011) and has been shown to be up-regulated in a number of human cancers such as breast carcinoma, acute leukaemia, non-small cell lung cancer and prostate cancer (Bergamaschi et al., 2003, Zhang et al., 2005, Chen et al., 2010, Zhang et al., 2011). iASPP is known to prevent premature cellular senescence by maintaining the proliferative potential of TP63-positive cells via its inhibitory effects on TP63's transcriptional activity. However, in malignant

prostate tissue, we hypothesised that iASPP's primary role is to directly inhibit p53-mediated apoptosis and thereby promote prostate tumour cell survival. In a recent publication iASPP has been identified as being a target of cyclin B1 phosphorylation. This phosphorylation results in the inhibition of iASPP dimerization, thereby promoting both the nuclear entry of monomeric iASPP and exposure of its p53 binding site, which in turn results in the increased inhibition of *p53* (Lu et al., 2013). Cyclin B1 has been shown to be overexpressed in prostate cancer and this correlates with an increased in Gleason grade (Kallakury et al., 1999). This overexpression of cyclin B1 could therefore play an instrumental role in inhibiting *p53*-mediated apoptosis.

Even though *p53* mutations are rare in prostate cancer those patients that harbour a mutation have been shown to have a worse clinical outcome and are significantly more prone to the formation of metastases. In recent years the mutant *p53* "gain-of-function" hypothesis has highlighted the fact that *p53* mutations can actively contribute to disease progression. In the light of these findings it would be fascinating to understand whether iASPP can not only regulate wild-type *p53* in order to drive and facilitate prostate cancer progression, but also whether it can also interact with mutant *p53*, and positively regulate its detrimental effects during the progression of this malignancy.

In order to determine the expression pattern and localisation of iASPP in benign and malignant prostate tissue, and investigate any potential relationship between changes in iASPP protein expression and an aggressive prostate cancer phenotype, benign and malignant prostate tissue was analysed from patients with clinical follow-up. iASPP expression was analysed in conjunction with clinical follow-up data in order to determine whether iASPP has any prognostic value as a biomarker. This chapter describes the significant changes in iASPP expression and localisation and provides new

insight into ways in which iASPP may promote prostate cancer progression and tumour cell survival.

4.2 Results

4.2.1 iASPP co-localises with TP63 in the basal layer of benign human prostate tissue, and is significantly increased in Prostate cancer samples compared with BPH samples.

In recent studies of squamous epithelium iASPP has been shown to co-localise with TP63 expression in the nuclei of basal cells, whereas it's expression becomes cytoplasmic in differentiated epithelial cells (Notari et al., 2011, Chikh et al., 2011). In order to determine whether iASPP and TP63 co-localise in the basal cells of the prostate, and to investigate the expression patterns of iASPP in benign and malignant human prostate tissue, we performed a tissue microarray (TMA) immunohistochemical analysis of samples from 12 patients with benign prostatic hyperplasia (BPH) and 203 patients with prostate cancer. These prostate cancer samples were from men who had undergone a radical prostatectomy for the treatment of their prostate cancer. All patients were hormone naïve and had up to ten years of associated clinical follow-up data. Although BPH is not strictly normal prostate tissue, it is still considered to be a valid control as BPH is extremely common and almost ubiquitous in later life, and it is not thought to be a precancerous condition.

iASPP was observed to co-localise with TP63 in the nuclei of basal cells within BPH samples (figure 4.1A). It was also detectable at low levels in the basolateral aspect of luminal cells and at the cell membrane. Immunohistochemical analysis of iASPP

expression in BPH and prostate cancer samples showed an increase in staining intensity in the prostate cancer samples compared to BPH samples (figure 4.1B).

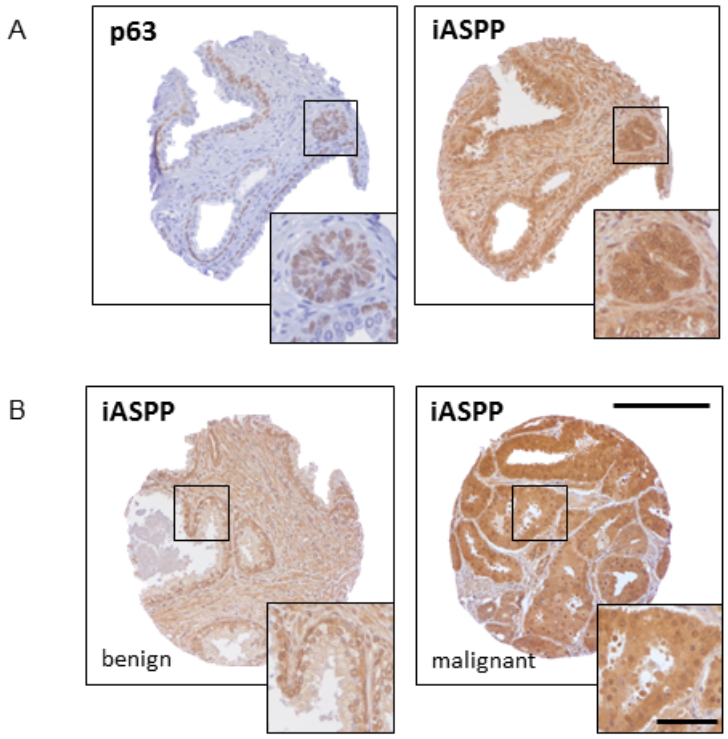

Figure 4.1 iASPP co-localises with TP63, and iASPP expression is increased in prostate cancer samples compared to benign samples

(A) Immunohistochemical analysis of TP63 (left) and iASPP (right) illustrating co-localisation of the two proteins in the basal cells of benign prostate epithelium (B) Immunohistochemical analysis of iASPP in BPH samples (left) and prostate cancer samples (right). All images were taken at 20x magnification. Squares show higher magnification to illustrate detail. Scale bars – 200µm, 50µm.

4.2.2 Changes in iASPP expression in prostate cancer tissue compared with BPH

In order to characterise further the observed increase in nuclear and cytoplasmic iASPP expression in prostate cancer tissue cores compared with benign controls, iASPP intensity was scored by a histopathologist; all cores were scored blind. Nuclear and cytoplasmic staining intensity were graded as follows: absent (0), weak (1), intermediate (2) or strong (3). The maximum staining within each core was recorded. Quantification of this expression revealed a significant increase in both nuclear ($P = 0.002$) and cytoplasmic iASPP ($P = 0.01$) in prostate cancer compared to BPH samples (figure 4.2B), suggesting that elevated levels of iASPP bestows an advantage on tumour cells and may promote prostate cancer progression.

Interestingly, a number of distinct different expression patterns were detected during the core analysis. For example some samples showed high nuclear iASPP and low cytoplasmic expression, a number of samples displayed high cytoplasmic expression and low nuclear expression, and some samples exhibited both high nuclear and high cytoplasmic expression (figure 4.2A). This raises a question regarding the potential underlying differences in the genetic profiles of each group. We wondered whether differences in molecular pathways, such as *p53* status could shed some understanding as to the reason why these different expression patterns were observed.

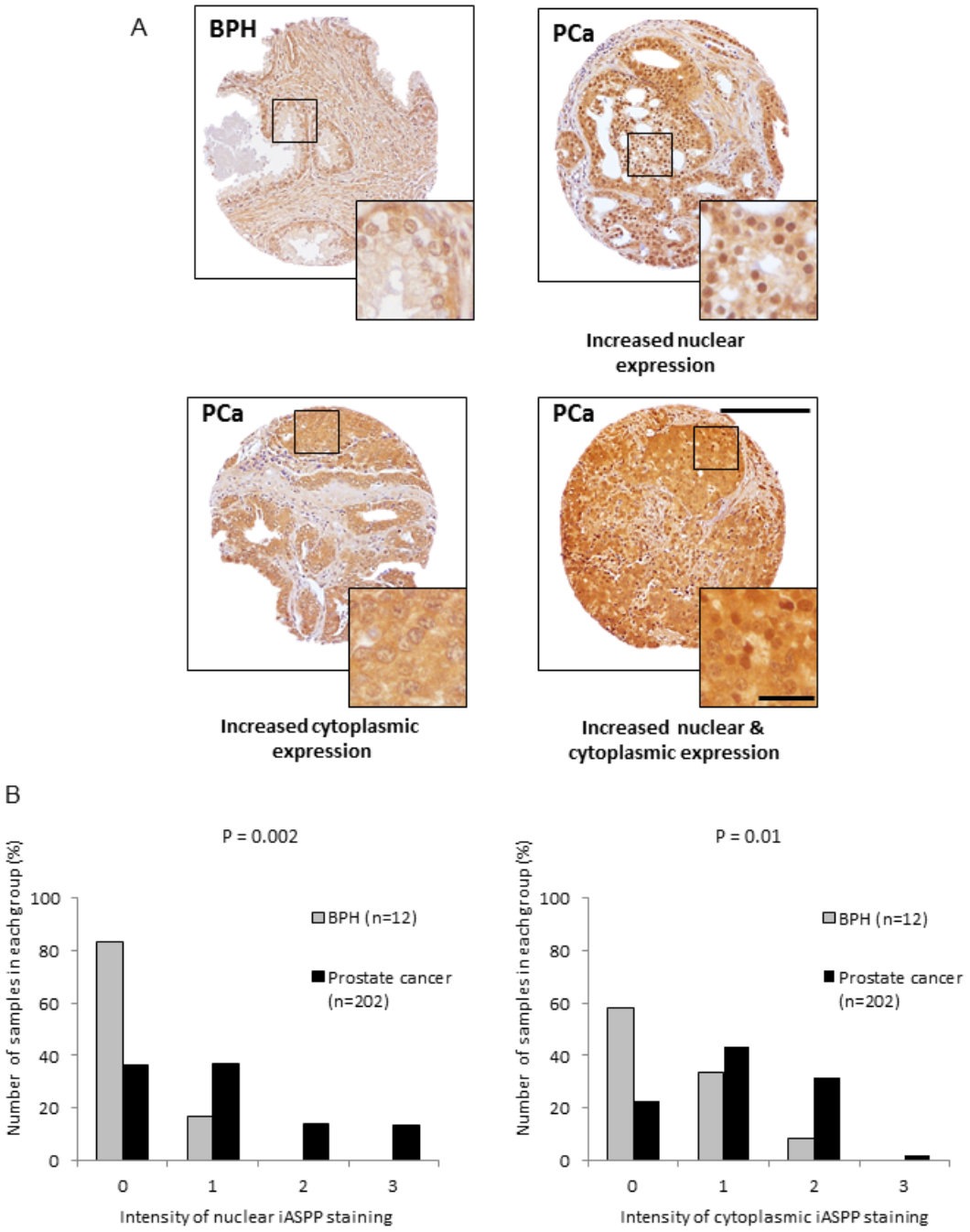

Figure 4.2 iASPP expression is increased in prostate cancer samples compared to benign

(A) Immunohistochemistry of the different iASPP expression patterns in BPH (top left) and prostate cancer (top right, bottom left and bottom right). All images were taken at 20x magnification. Squares show higher magnification to illustrate detail. Scale bars - 200µm, 50µm. **(B)** Nuclear and cytoplasmic iASPP intensity were scored in 12 BPH tissue samples and 202 prostate cancer samples. The scoring was recorded as follows: absent (0), weak (1), intermediate (2) or strong (3). The maximum staining within each core was recorded. P values were calculated using a Man whitney U test.

4.2.3 iASPP expression is increased in normal adjacent prostate in cancer tissue compared to BPH

One intriguing observation that was made during our analysis of the prostate cancer cores was that iASPP appeared to be up-regulated in tissue that was deemed to be architecturally normal adjacent prostate (NAP) tissue in relationship to the areas of prostate cancer. Many studies use NAP, which is defined by the presence of an intact TP63-positive basal cell layer, as an experimental control. However, a paper published in Nature in 2001 showed that the expression of many genes are altered in NAP, compared to that of truly normal prostate tissue (Dhanasekaran et al., 2001). The majority of researchers in the prostate cancer field believe that the cancer-initiating cells are within the basal cell layer, and so molecular alterations may already have occurred in NAP tissue before any architecture changes have arisen. Taken together these previous observations suggested to us that iASPP expression might be altered in NAP compared to truly benign prostate samples. Moreover, given that iASPP is a known regulator of *p63*, it is conceivable that an increase in iASPP expression could contribute to malignant transformation by maintaining the proliferative potential of the TP63-positive tumour-initiating cells. In order to test the hypothesis that iASPP expression is elevated in NAP within prostate cancer cores, separate scores were given for the malignant areas of each core and the adjacent TP63-positive basal cell areas of epithelium, wherever possible. The staining intensity (figure 4.3A) and scores for both the TP63-positive basal cells and the TP63-negative luminal cells were then compared to the scores given to BPH samples (figure 4.3B). iASPP expression was significantly higher in both the nuclei and cytoplasm of basal ($P = 0.005$ nuclear, $P = 0.024$ cytoplasmic) cells and luminal ($P = 0.038$ nuclear, $P = 0.026$ cytoplasmic) cells in NAP compared to BPH samples. These findings indicate

that the up-regulation of iASPP protein expression is an early event in the malignant transformation of prostate epithelial cells.

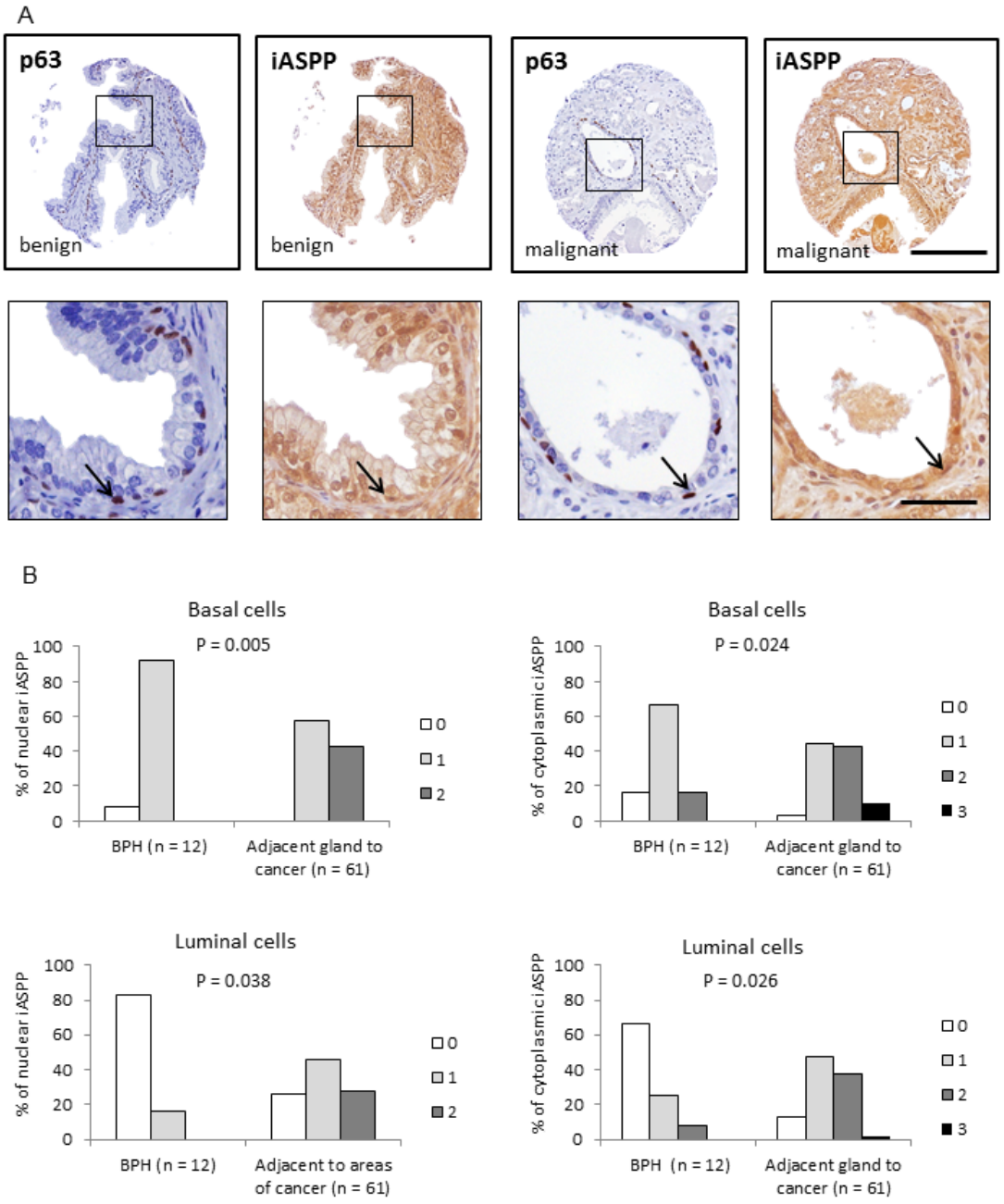

Figure 4.3 iASPP is increased in normal adjacent prostate epithelium in cancer samples compared to BPH

(A) Immunohistochemistry analysis of iASPP and TP63 expression in BPH and normal adjacent prostate (NAP) areas in prostate cancer tissue. All images were taken at 20x magnification. Squares below show higher magnification to illustrate detail. Scale bars - 200µm, 50µm. **(B)** Nuclear and cytoplasmic iASPP intensity were scored in both the basal cells and the luminal cells in 12 BPH tissue samples and 61 prostate cancer samples with NAP. The scorings were given as follows: nuclear intensity; absent (0), weak (1), intermediate (2), for cytoplasmic intensity absent (0), weak (1), intermediate (2) or strong (3). The maximum staining within each core was recorded. P values were calculated using a Man whitney U test.

4.2.4 iASPP expression is increased in both wild-type and mutant *p53* prostate cancer samples

In 2003 Bergamaschi et al. demonstrated that the expression of iASPP stimulated Ras-mediated transformation of cells by a factor of 15, and that the expression of iASPP also enhanced the transforming activities of Ras plus E7 or E1A. However, iASPP was not found to affect the transforming ability of Ras and mutant *p53* (Bergamaschi et al., 2003). These findings led to the assumption that iASPP activity was confined to regulating wild-type *p53* but not mutant *p53*.

Unlike most tumour suppressor genes, which are predominantly inactivated as the result of a deletion or truncation, the vast majority of cancer-associated mutations in the *p53* gene are missense mutations and single base-pair substitutions. This observation has led to the proposal of a "gain-of-function" hypothesis which states that a mutation of *p53* is not equivalent to simply losing wild-type *p53* function. Rather, the strong selection for maintained expression of a select group of mutant *p53* proteins suggests a positive role for certain *p53* mutants in tumorigenesis (Freed-Pastor and Prives, 2012). These gain-of-function activities could be the result of mutant *p53*-mediated regulation of novel target genes, or an interaction between mutant *p53* and cellular proteins. One of the most common *p53* mutations, $p53^{R175H}$, has been shown to be responsible for an up-regulation of cell cycle-associated M-phase genes in prostate cell lines resulting in the acceleration of cell cycle progression. As well as the up-regulation of a unique group of developmental genes such as *twist* and *snail*, the expression of this subset of genes is thought to play an instrumental role in the induction of an EMT-like state, thereby promoting invasion and metastasis (Kogan-Sakin et al., 2011). Another common *p53* mutation, $p53^{R273H}$ has been implicated in increased proliferation (Kogan-Sakin et al.,

2011). To date mutant *p53* has been implicated in numerous aspects of tumour progression such as DNA synthesis and cell proliferation, survival, angiogenesis, invasion and migration. Changes of residues 143, 175, 248, 273 and 281 of the TP53 protein have all been associated with a gain-of-function phenotype in transfected p53-null cell lines (van Oijen and Slootweg, 2000). These findings support the theory that mutations in the *p53* gene are more advantageous to cancer cells than a complete loss of p53 function however whether these mutations function by co-operating with co-regulators of wild-type *p53* is still poorly understood. However with regards to prostate cancer biology, it is already widely recognised that *p53* mutations lead to a more advanced and aggressive metastatic phenotype (Navone et al., 1999).

iASPP is a known binding partner of wild-type TP53 and it executes a fundamental task in the mediation of the apoptotic pathway. However, its ability to interact with mutant TP53 has not been fully elucidated. If iASPP is able to interact and regulate mutant TP53 it is conceivable that an increase in iASPP expression might be a consequence of mutant TP53 accumulation and could result in the promotion of *p53's* gain-of-function activity. In order to investigate this possibility an immunohistochemical analysis of a tissue microarray was carried out. We used TP53 expression in order to determine whether there was a change in iASPP expression in tissue samples with high p53 accumulation compared with those with low or undetectable levels of TP53. Sections with over 30% of cells expressing TP53 antigen were classed as having a *p53* mutation (Navone et al., 1993). Although this does not unequivocally define the presence of a *p53* mutation it was our preferred method as sequencing data was not available for the clinical samples at our disposal. Therefore in our experiments *p53* status could only be determined by TP53 expression.

Prostate cancer cores were classified according to their *p53* status (figure 4.4A). Both nuclear and cytoplasmic iASPP expression was increased in prostate cancer samples with wild-type *p53* when compared to BPH samples (P = 0.004, P =0.012 respectively). Moreover, iASPP expression was also significantly increased in prostate cancer samples harboring a *p53* mutation when compared to BPH (P = 0.003, P = 0.044 respectively) (Figure 4.4B). Furthermore, it appeared that iASPP expression was higher in mutant *p53* samples compared to prostate cancer samples with wild-type *p53*. This finding suggests that iASPP is further increased in response to the accumulation of mutant TP53 protein. However, due to the small sample size of n = 5 in our cohort this last result did not reach statistical significance for either nuclear or cytoplasmic expression of iASPP (P >0.05). A selected cohort with known *p53* mutation status, or a larger independent cohort, is needed to achieve the numbers necessary to conclude whether or not iASPP is significantly increased in response to mutant TP53 protein accumulation.

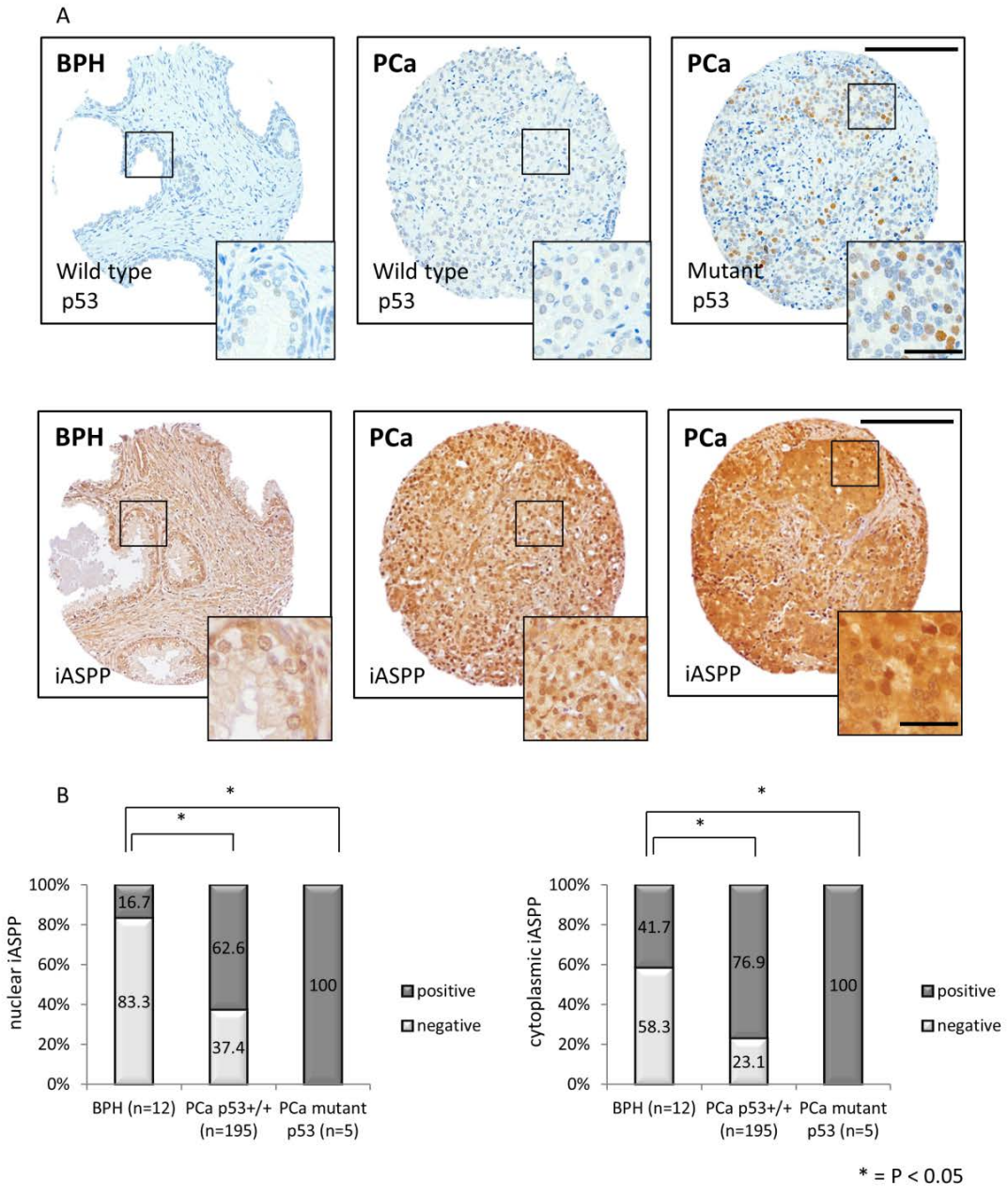

Figure 4.4 iASPP is increased in both wild-type and mutant *p53* prostate cancer samples

(A) Immunohistochemistry analysis of TP53 (upper panel) and iASPP (lower panel) in BPH and prostate cancer samples. All images were taken at 20x magnification. Squares below show higher magnification to illustrate detail. Scale bars - 200μm, 50μm. **(B)** Nuclear and cytoplasmic iASPP intensity was scored either positive or negative in 12 BPH tissue samples and 195 prostate cancer samples with wild-type *p53* (defined as < 30% TP53 staining) and 5 prostate cancer samples with mutant *p53* (defined as > 30% TP53 staining). P values were calculated using the Fisher's exact test.

4.2.5 Increased iASPP expression is associated with poor clinical outcome

All of the patients included in the iASPP protein expression study had undergone a radical retropubic prostatectomy between 1989 – 2001, and they were aged between 44 – 75 years (median age of 63 years). All patients were hormone naïve and had up to ten years of clinical follow up data. Prostate cancer is often a slow growing disease, and so in order to conclude whether the increase in iASPP expression has any clinical relevance, and/or prognostic value, it is important to have an appropriate time frame to analyse the clinical course and prostate cancer-specific survival (PCSS) of the cohort of patients.

Patients were grouped according to whether they had absent/weak (scores 0/1) or moderate/strong (scores 2/3) for both nuclear and cytoplasmic iASPP expression at the time of their surgery in order to assess PCSS regardless of their Gleason score or stage (figure 4.5). Even though there was a small separation in PCSS between patients who had high nuclear (figure 4.5.A) or high cytoplasmic (figure 4.5B) iASPP expression at the time of their radical prostatectomy, this difference did not reach statistical significance. However, when patients were grouped according to their Gleason score, patients with Gleason sum 7 prostate cancer who exhibited moderate/strong nuclear iASPP had a statistically significantly poorer PCSS, than patients with absent/low nuclear iASPP (figure 4.5C). Furthermore, the same trend was observed for patients who presented with a Gleason score greater than 7, and moderate/strong cytoplasmic iASPP, compared with those who exhibited absent/low cytoplasmic iASPP (figure 4.5D). Taken together, these findings would suggest that iASPP plays a significant role in disease progression.

To investigate this hypothesis further, we assessed the probability of metastasis formation according to iASPP expression. Prostate cancer cells can metastasise to the bone and it is believed that there are reciprocal cellular interactions between prostate cancer cells and

bone stroma, resulting in proliferation of both prostate cancer and bone stromal cells. During osteolytic metastasis formation, bone resorption releases a variety of growth factors such as Wnt proteins that enhance prostate cancer cell proliferation. In turn, metastatic prostate cancer cells express oestolytic factors that cause the release of growth factors to the bone, promoting an oestoblastic growth phase and leading to the formation of osteoblastic lesions in the bones (Hall et al., 2006).

Patients were grouped according to their Gleason score. Patients with a Gleason sum greater than 7, who exhibited moderate/strong cytoplasmic iASPP and who had previously demonstrated a reduction in PCSS, were also statistically significantly more likely to form bone metastases compared with patients with absent/low cytoplasmic iASPP (figure 4.5F). This was not the case for the nuclear iASPP as this was not found to associate with bone metastasis formation.

This data provides evidence that iASPP expression may be involved in metastasis and disease progression, and that increased iASPP expression in the primary tumour is a prognostic marker of poor clinical outcome.

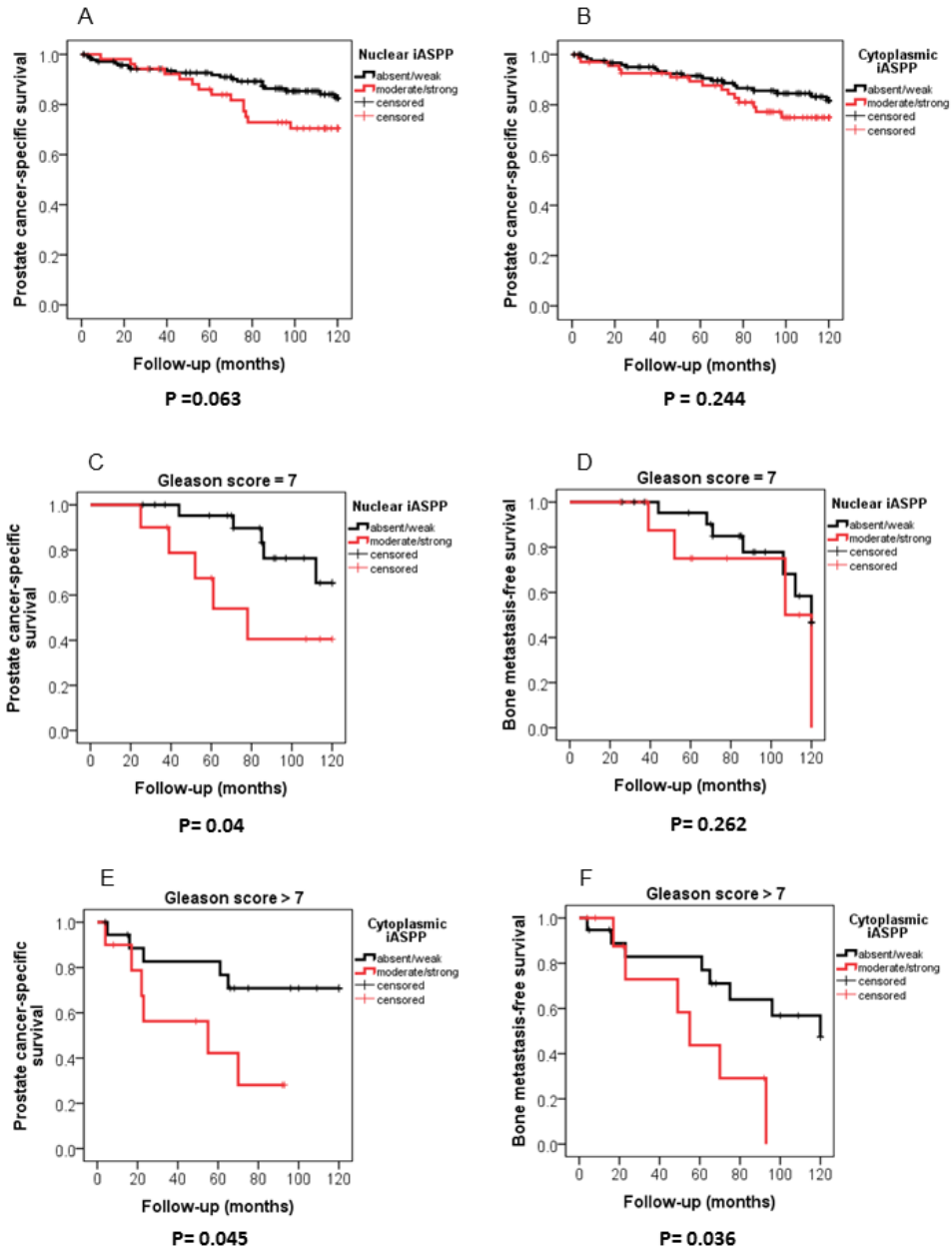

Figure 4.5 Increased iASPP expression is associated with poor clinical outcome

(A) Prostate cancer-specific survival (PCSS) of patients with absent/weak nuclear iASPP (black) compared with patients with moderate/strong nuclear iASPP (red) at the time of their radical prostatectomy **(B)** PCSS of patients with absent/weak cytoplasmic iASPP (black) compared to patients with moderate/strong cytoplasmic iASPP (red) at the time of their radical prostatectomy **(C)** PCSS of patients with Gleason grade 7 prostate cancer and absent/weak nuclear iASPP (black) compared with patients with moderate/strong nuclear iASPP (red). **(D)** PCSS of patients with Gleason grade greater than 7 and absent/weak cytoplasmic iASPP (black) compared to patients with moderate/strong cytoplasmic iASPP (red) **(E)** Bone metastasis-free survival of patients with Gleason grade 7 prostate cancer and absent/weak nuclear iASPP (black) compared with patients with moderate/strong nuclear iASPP (red). **(F)** Bone metastasis-free survival of patients with Gleason grade greater than 7 and absent/weak cytoplasmic iASPP (black) compared with patients with moderate/strong cytoplasmic iASPP (red). P values are calculated using Kaplan Meier survival analysis using a log rank test.

4.2.6 Increased nuclear iASPP expression is associated with poor clinical outcome in patients with non-organ confined prostate cancer

Often patients who undergo a radical prostatectomy are thought to have organ-confined pT2 disease or occasionally locally advanced spread such as pT3a or pT3b disease. The prostate is covered by a thin lining called the capsule. Prostate cancer that is organ confined and denoted as pT2 is located entirely within the limits of the prostate and thus within the capsule. However, in a number of patients, pathological examination of the prostate following radical prostatectomy reveals that the cancer cells have spread out of the capsule of the prostate. This is known as extracapsular extension and is denoted as pT3 disease. pT3 disease results in a poorer clinical outcome than pT2 disease. The molecular alterations in the prostate cancer foci allow these cells to invade through the stroma and prostate capsule, and survive in a new microenvironment, are the subject of much research.

Nuclear iASPP is known to inhibit p53-mediated apoptosis, thereby promoting cell survival. Recently, iASPP has been shown to be up-regulated in metastatic melanoma samples compared to primary melanoma samples. This up-regulation was found to result in protection from cell death during mitosis when the cell is at its most vulnerable (Lu et al., 2013). These observations prompted us to analyse whether there might be a difference in iASPP expression between men with organ-confined prostate cancer and those with non organ-confined disease.

Men with pT1 or pT2 prostate cancer were grouped as having organ-confined disease, whereas men with pT3 prostate cancer were defined as having non-organ confined disease. There was no difference in PCSS for patients with organ-confined disease, with absent/weak nuclear iASPP compared with moderate/strong nuclear iASPP ($P = 0.746$).

Similarly there was no difference in PCSS for men with absent/weak cytoplasmic iASPP compared with patients who exhibited moderate/strong cytoplasmic iASPP (P = 0.724) (figure 4.6A & C). Interestingly for the cohort of patients who were diagnosed as having non organ-confined pT3 prostate cancer, men who had moderate/strong nuclear iASPP at the time of their surgery had a statistically significantly poorer PCSS rate than those who exhibited absent/weak nuclear iASPP (P = 0.009) (figure 4.6B). The same trend was observed with regards to cytoplasmic iASPP although this did not reach statistical significance (figure 4.6D).

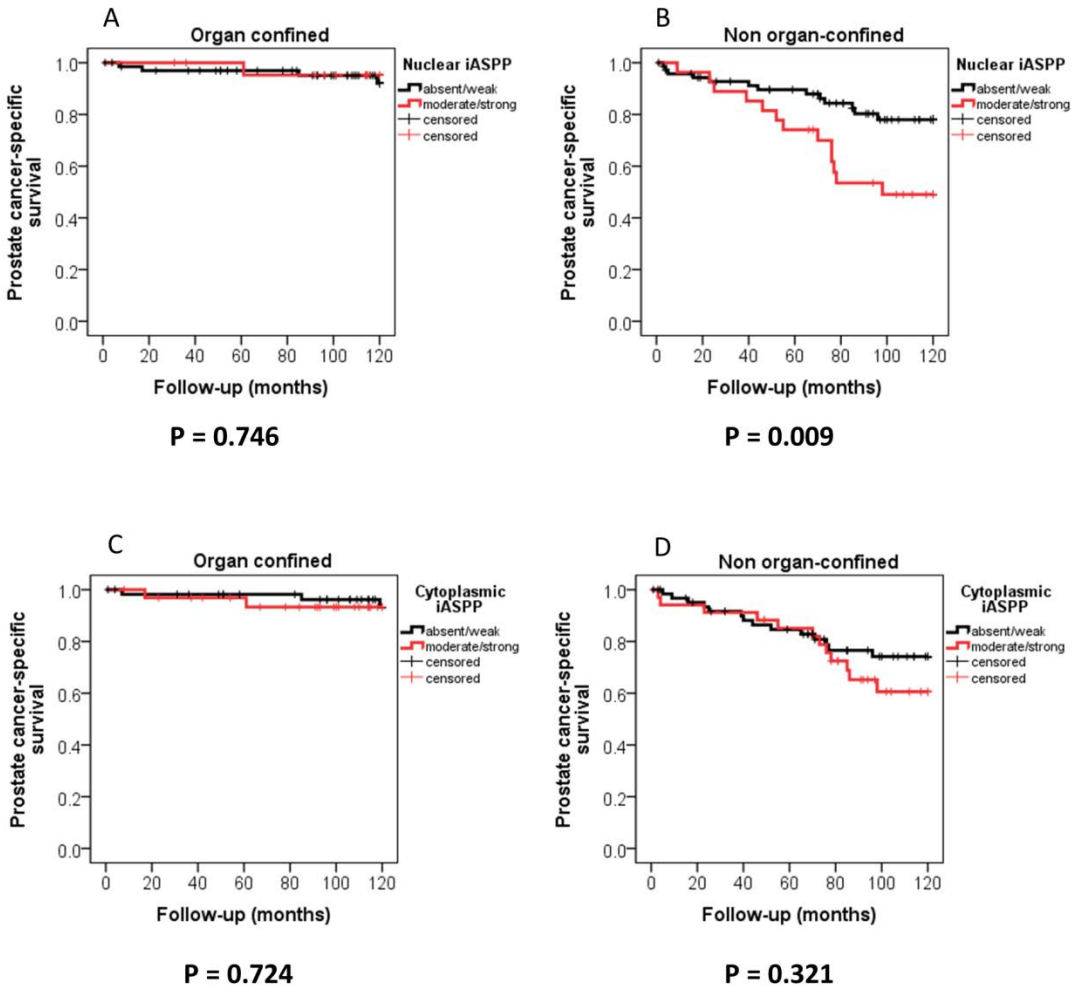

Figure 4.6 Increased nuclear iASPP is associated with poor clinical outcome in patients with pT3 prostate cancer

(A) A comparison of nuclear iASPP expression [absent/weak (black) versus moderate/strong (red)] in patients with organ-confined prostate cancer. **(B)** A comparison of nuclear iASPP expression [absent/weak (black) versus moderate/strong (red)] in patients with non organ-confined prostate cancer. **(C)** A comparison of cytoplasmic iASPP expression [absent/weak (black) versus moderate/strong (red)] in patients with organ-confined prostate cancer. **(D)** A comparison of cytoplasmic iASPP expression [absent/weak (black) versus moderate/strong (red)] in patients with non organ-confined prostate cancer. P values were calculated using Kaplan Meier survival analysis using a log rank test.

4.2.7 Nuclear and cytoplasmic iASPP is significantly increased in cells invading through the prostatic capsule

To further investigate iASPP expression in terms of disease progression and metastasis, 27 locally advanced non organ-confined pT3 prostate cancer cases were analysed in order to evaluate the potential relationship between elevated iASPP expression and the cells invading into or through the prostate capsule. The prostate capsule is not a distinct structure but outer fibromuscular tissue which is an inseparable component of the prostatic stroma. Although the capsule is not a well-defined structure it may be a physical barrier which prostate cancer cells need to cross in order to locally invade. The molecular mechanisms by which these cells are able to facilitate this invasive phenotype are poorly understood.

The increased expression of both nuclear and cytoplasmic iASPP observed in the TMA study was found to be associated with poor clinical outcome. Moreover, nuclear iASPP was significantly increased in the pT3 cancers suggesting that the expression of nuclear iASPP is important for the invasive potential and survival of prostate cancer cells. These findings led us to investigate whether iASPP expression patterns were different in the cells located at the leading edge of the tumour compared with cells located deep within the tumour. Such a change may suggest a role for iASPP in the development of locally advanced disease. In order to investigate this hypothesis a urology histopathologist scored nuclear, cytoplasmic and membranous iASPP expression in the cells at the leading edge of pT3a prostate cancer, and in the cells that were contained within the prostate (defined by > 1mm from the prostate capsule) along with cells in benign glands adjacent to the areas of cancer. Scoring was graded in the same manner as previously described i.e. 0 – 3 (0 = absent, 1 = weak, 2 = moderate, 3 = strong) depending on staining intensity. iASPP

was found to be statistically significantly increased in the nuclei of the invading cells at the leading edge of the tumour compared with the intraprostatic tumour cells (P = 0.001). It was also found to be increased in intraprostatic tumour cells compared with benign cells (P = 0.05). The difference in expression between invading cells and benign cells was also highly statistically significant (P < 0.0001) (figure 4.7). These observed changes validate the earlier findings that nuclear iASPP promotes invasion in prostate cancer. Its localisation in the nucleus suggests that iASPP is preventing apoptosis via its interaction with TP53, thereby promoting cell survival.

Cytoplasmic iASPP expression was also assessed in cells at the leading edge of pT3a prostate cancers. iASPP is known to be a cytoplasmic protein when it is in its inactivated state. Lu et al demonstrated that the N-terminus forms a dimer with the C-terminus of iASPP holding the protein in an inactivated state. Only after activation is this complex broken, unmasking iASPP's nuclear localisation signal and allowing it to translocate into the nucleus and act as a transcriptional co-regulator. However, whether iASPP has an active role within the cytoplasm is still not known. iASPP was found to be statistically significantly up-regulated in the cytoplasm of the leading edge tumour cells compared with the intraprostatic cells (P = 0.039). It was also found to be increased in the intraprostatic cells compared with the benign cells (P = 0.001). The difference in expression between invading cells and benign cells was again highly statistically significant (P < 0.0001) (figure 4.8). The substantial increase in cytoplasmic iASPP in cells invading through the prostate capsule suggests that either iASPP expression is increased to compensate for an increase in TP53 expression, or that cytoplasmic iASPP plays a p53-independent role in cancer progression by promoting invasion and/or cell survival.

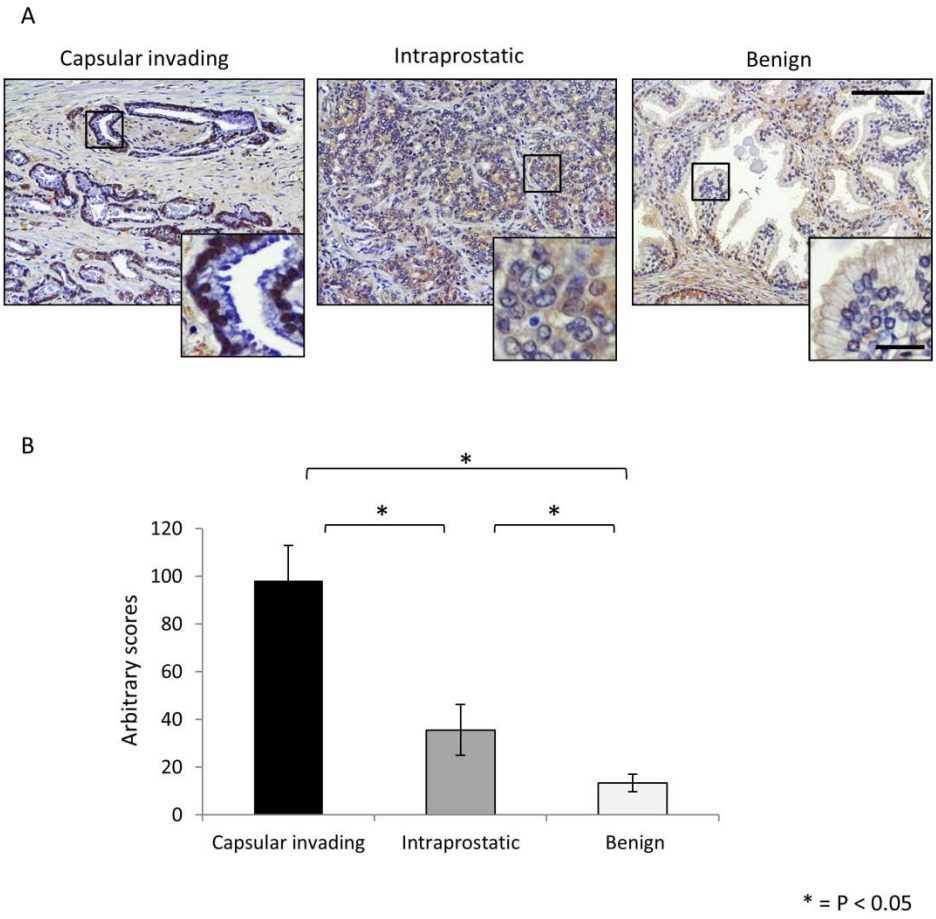

Figure 4.7 Nuclear iASPP is increased in cells invading through the prostatic capsule

(A) Immunohistochemistry analysis of nuclear iASPP expression in cells invading through the prostate capsule in pT3a prostate cancer (left), intraprostatic cells contained within the prostate (middle), and benign cells adjacent to areas of prostate cancer (right) All images were taken at 20x magnification. Squares below show higher magnification to illustrate detail. Scale bars - 200µm, 50µm. **(B)** Quantification of nuclear iASPP expression in three different areas of the tumour sample. Error bars indicate standard error. P values were calculated using an unpaired t-test.

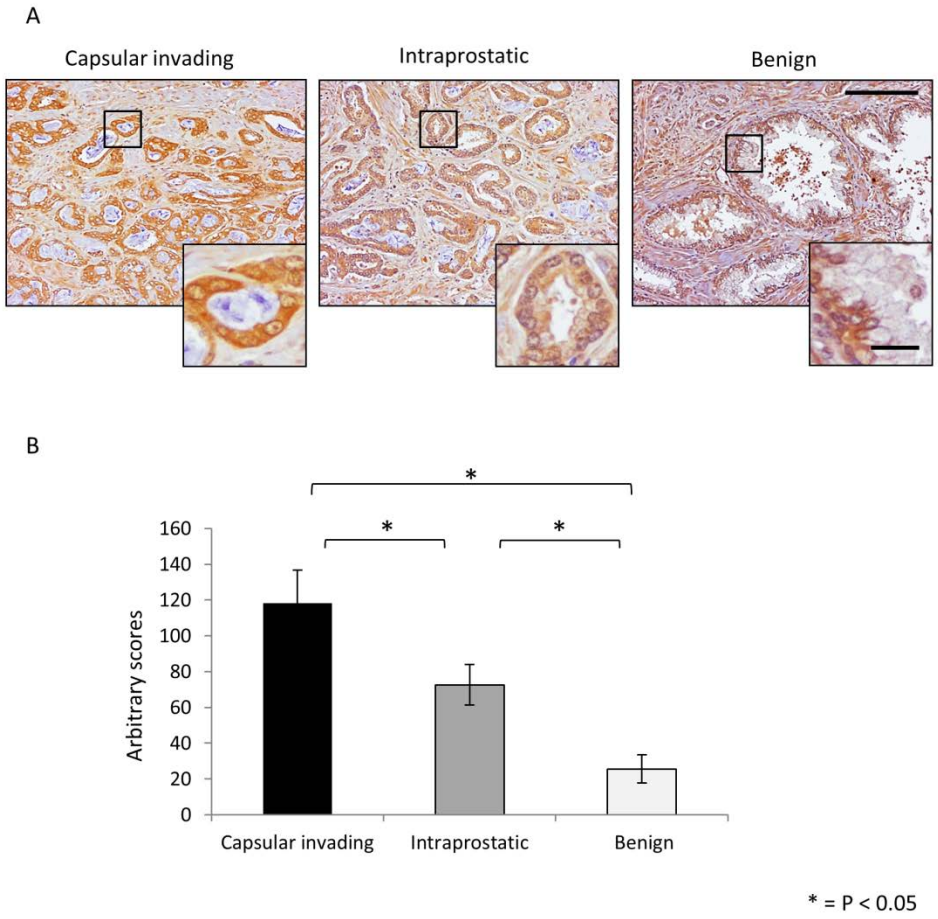

Figure 4.8 Cytoplasmic iASPP is increased in cells invading through the prostatic capsule

(A) Immunohistochemistry analysis of cytoplasmic iASPP expression in cells invading through the prostate capsule in pT3a prostate cancer (left), intraprostatic cells contained within the prostate (middle), and benign cells adjacent to areas of prostate cancer (right) All images were taken at 20x magnification. Squares below show higher magnification to illustrate detail. Scale bars - 200µm, 50µm. **(B)** Quantification of cytoplasmic iASPP expression in three different areas of the tumour sample. Error bars indicate standard error. P values were calculated using an unpaired t-test.

4.2.8 Membranous iASPP expression is reduced in cells invading through the prostatic capsule

Even though iASPP was first identified as a protein that actively contributes to the regulation of apoptosis via its functional role as a transcriptional co-regulator, it now appears that iASPP may be a protein with a number of different distinct cellular functions. Recent functional studies have revealed iASPP as a potential junctional protein (unpublished data). Immunohistochemical and immunofluorescence analysis of benign tissue in the mouse and in the human samples along with immunocytochemistry in a number of cell lines, has clearly demonstrated that iASPP is also located at the membrane. Functional studies in heart tissue and cardiomyocytes have shown that iASPP may be important in maintaining mechanical strength as a desmosomal component (unpublished data). If iASPP plays a structural role at the membrane of epithelial cells its expression pattern may be altered in cancer cells that have gained metastatic capabilities.

In order for cells to metastasise they must shed many components of their epithelial phenotype resulting in an epithelial-to-mesenchymal transition (EMT) like state. Whether cancer cells undergo true EMT is controversial, however there is substantial evidence that EMT-like states exist and are important for various aspects of prostate cancer progression and metastasis (Nauseef and Henry, 2011). EMT is a normal process in embryogenesis and wound healing. By activating this morphogenetic program cancer cells are able to exploit it to profoundly change their own morphology, motility and ability to invade through the surrounding stroma. EMT can be induced by growth factors such as transforming growth factor β (TGF-β), epidermal growth factor (EGF) and transcription factors such as snail and twist. Some of the main alterations in protein expression associated with EMT are a down-regulation of E-cadherin and desmoplakin, coupled by

an up-regulation of twist, snail, vimentin and the secretion of fibronectin (Nauseef and Henry, 2011, Kogan-Sakin et al., 2011, Smith and Odero-Marah, 2012). These changes allow the cancer cells to breach the basement membrane and invade the stroma and eventually to metastasise outside of the prostate disseminating to distant anatomical sites.

The immunohistochemical staining of iASPP in both the TMA study and the study of pT3 prostate cancer samples clearly demonstrated membranous iASPP localisation in the epithelial cells of the BPH samples, and in areas of benign tissue adjacent to areas of cancer. We thereby hypothesised that membranous iASPP might be an important structural component of cell-to-cell contacts, and that changes in cell behaviour and gene expression related to an EMT-like state might cause a down-regulation of cell-to-cell contacts, resulting in a reduced membranous iASPP expression. To investigate this hypothesis membranous iASPP expression in the different compartments of the pT3 tumour samples was scored in the same manner as previously described i.e. 0 – 3 (0 = absent, 1 = weak, 2 = moderate, 3 = strong staining intensity). Scores were given to the membrane staining of cells at the leading edge of the tumour invading through the prostate capsule, intraprostatic cancer cells, and cells situated in benign glands. Membranous iASPP expression was found to be significantly reduced in the cells invading through the prostate capsule compared to benign cells ($P = 0.023$) (figure 4.9). However, the decrease between the intraprostatic and the capsular invading cells did not reach significance ($P = 0.076$). These preliminary findings do however raise the question as to whether iASPP plays an integral role in normal epithelial organisation.

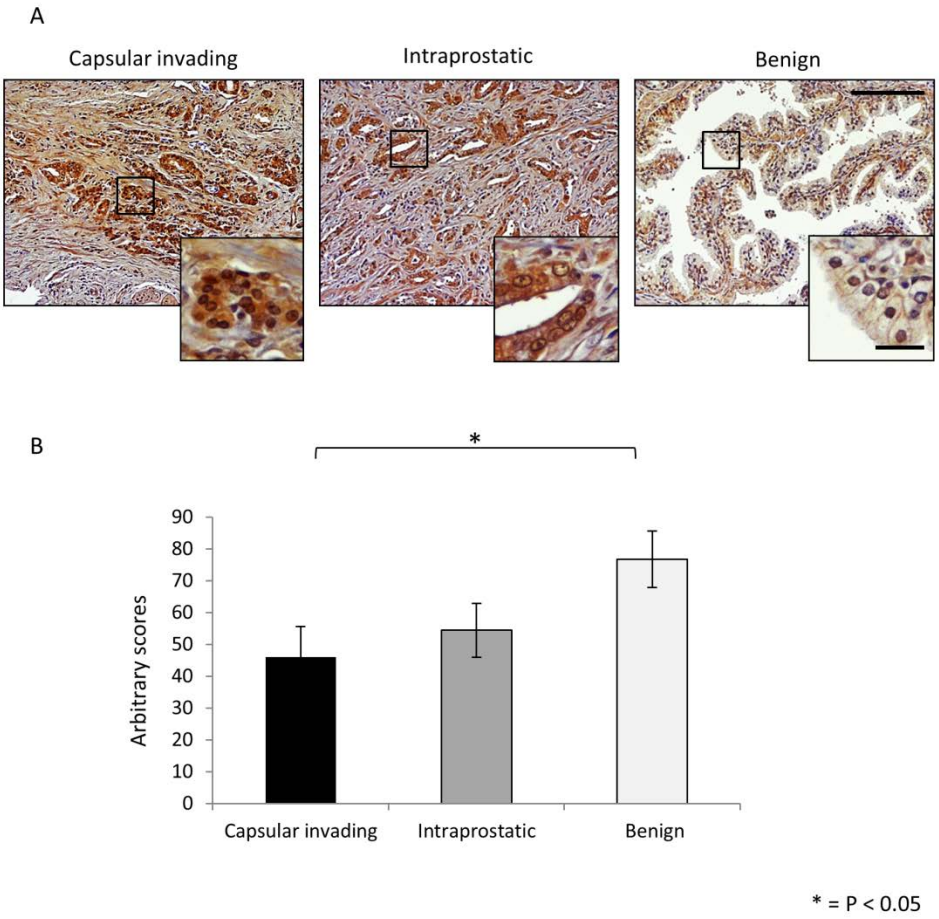

Figure 4.9 Membranous iASPP is reduced in cells invading through the prostatic capsule

(A) Immunohistochemistry analysis of membranous iASPP expression in cells invading through the prostate capsule in pT3a prostate cancer (left), intraprostatic cells contained within the prostate (middle), and benign cells adjacent to areas of prostate cancer (right) All images were taken at 20x magnification Squares below show higher magnification to illustrate detail. Scale bars - 200µm, 50µm. **(B)** Quantification of membranous iASPP expression in three different areas of the tumour sample. Error bars indicate standard error. P values were calculated using an unpaired t-test.

4.2.9 Increased nuclear iASPP is associated with mutant *p53* expression

In order to test on the hypothesis that nuclear iASPP expression is increased in response to TP53 accumulation, and may functionally interact with mutant *p53* to facilitate disease progression, we analysed TP53 expression in the pT3 prostate cancer samples for the three areas previously described (i.e. capsular invading, intraprostatic and benign cell populations). Scores for iASPP expression were given in the same manner as previously described. TP53 expression was clearly up-regulated at the leading edge of the tumour with high expression in the capsular invading cells (figure 4.10A). In contrast, this expression was statistically significantly lower in the intraprostatic cancer cells ($P = 0.007$). A statistically significant decrease was also observed in the cells situated in benign glands compared with the intraprostatic cancer cells ($P = 0.011$) and the cells at the leading edge ($P < 0.0001$) (figure 4.10B).

These findings, along with those in the TMA analysis (figure 4.4), suggest that there is a positive correlation between the expression of nuclear iASPP and TP53. To further investigate this relationship, the TP53 scores were categorised as either low or high TP53 expression, depending on the original scores (< 1.5 = low, > 1.5 = high). All the samples with low TP53 expression were grouped together and all the samples with high TP53 expression were grouped together. Their corresponding nuclear iASPP scores were then evaluated. Samples with low TP53 expression exhibited lower nuclear iASPP expression compared with those with high TP53 expression. This trend was evident in both the capsular-invading tumour cells and the intraprostatic tumour cells (figure 4.10C).

Taken together, these results indicate that TP53 and nuclear iASPP interact to promote prostate cancer tumourigenesis.

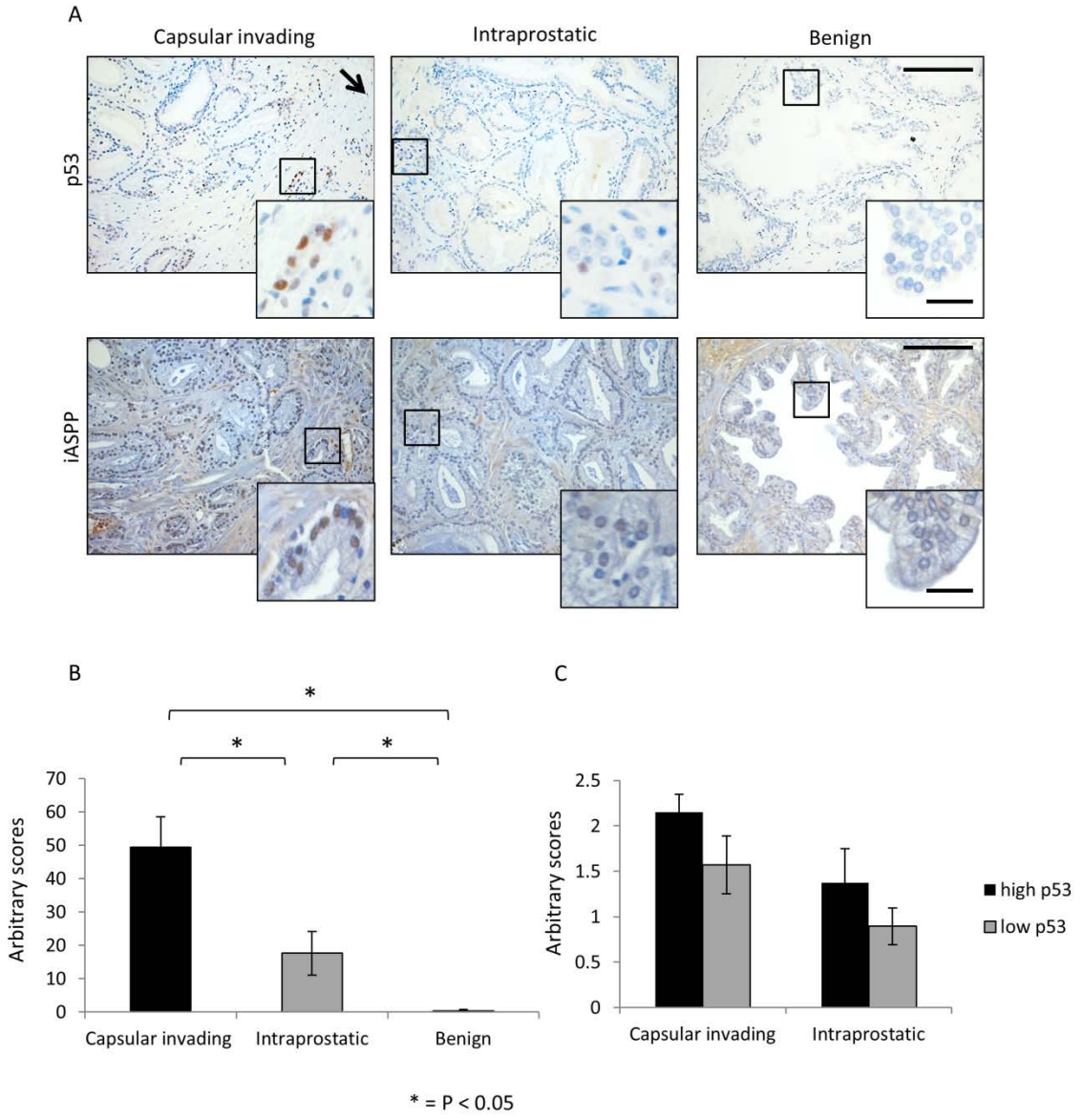

Figure 4.10 Nuclear iASPP expression is up regulated in cells with high levels of TP53 expression

(A) Immunohistochemistry analysis of TP53 expression (top) and nuclear iASPP expression (bottom) in cells invading through the prostate capsule in pT3a prostate cancer (left), intraprostatic cells contained within the prostate (middle), and benign cells adjacent to areas of prostate cancer (right) arrow denotes the direction of the invading leading edge. All images were taken at 20x magnification. Squares below show higher magnification to illustrate detail. Scale bars - 200µm, 50µm. **(B)** Quantification of TP53 expression in three different areas of the tumour sample. Error bars indicate standard error and P values were calculated using an unpaired t-test. **(C)** Mean iASPP expression level in samples with high or low TP53 detection in capsular invading prostate cells and intraprostatic prostate cells. Error bars indicate standard error.

4.3 Discussion

The use of a large cohort of patients with varying grades and stages of both organ-confined and non organ-confined prostate cancer has enabled us to obtain new insights into the biology that drives disease progression and metastasis. iASPP has been reported to be up-regulated in a number of different cancers, however despite this, no detailed evaluation of its cellular localisation and expression levels has been reported in human prostate cancer samples. Using a cohort of 203 cancer patients and 12 BPH patients we were able to demonstrate that an increase in nuclear and cytoplasmic iASPP was associated with poor clinical outcome and metastasis.

Cytoplasmic iASPP was up-regulated in the prostate cancer cases compared to the BPH and this regulation was shown to associate with poor PCSS in men with a Gleason sum score greater than 7. Moreover, this up-regulation was also associated with the formation of bone metastases. Cytoplasmic iASPP was also increased in prostate cancer samples with a high accumulation of TP53. iASPP is inherently cytoplasmic, and is only recruited to the nucleus upon activation by an appropriate stimulus. The up-regulation of cytoplasmic iASPP with regards to mutant *p53* may be a compensatory effect owing to the increased levels of TP53, or it may have a regulatory role of its own. Whilst these possibilities are yet to be addressed, our findings suggest that high cytoplasmic iASPP expression could have clinical prognostic value.

Nuclear iASPP was found to be up-regulated in prostate cancer cases compared with BPH, and this was associated with poor PCSS in patients with a Gleason sum score equal to 7. It was also found to be significantly increased in non-organ confined disease. These initial findings support the work of Liu Z et al. who investigated iASPP expression in head and neck squamous cell carcinoma and found that both nuclear and cytoplasmic

iASPP were significantly elevated in cancer samples with cytoplasmic iASPP having prognostic value (Liu et al., 2012). Given the link we established between poor PCSS and increased nuclear iASPP expression in non organ-confined disease, we further investigated iASPP expression in pT3a, non organ-confined prostate cancer. pT3a patients have extracapsular extension upon pathological examination of the prostate after radical prostatectomy, with tumour cells invading the prostate capsule. In order for cells to invade through the basement membrane, into the surrounding stroma, and eventually through the capsule of the prostate, alterations in gene expression occur which allow cells to take on an EMT-like state. Both nuclear and cytoplasmic iASPP expression were found to be increased in the cancer cells invading through the prostate capsule. Interestingly, this was coupled with a down-regulation of membranous iASPP. EMT is known to have a drastic effect on the expression and localisation of junctional proteins. Our observations raise the possibility that iASPP may also play a fundamental role in the stability and strength of cell-to-cell contacts. This could also explain the observed increase in cytoplasmic iASPP expression, as iASPP may be lost from the membrane and become mislocalised into the cytoplasm, resulting in elevated levels of cytoplasmic iASPP expression.

Our results show that elevated iASPP is associated with both poor PCSS and metastasis formation. Nuclear iASPP most likely promotes cell survival through its interaction with TP53. It was fascinating that our TMA cores with mutant *p53* exhibited high expression of nuclear iASPP and this observation prompted us to hypothesise that nuclear iASPP might co-operate with mutant *p53*. If this was to be true, it could be hypothesised that this interplay may result in iASPP co-regulating the transcription of novel mutant *p53* genes, thereby promoting *p53* gain-of-function. Owing to a small sample size of only 5 patients

in the TMA study exhibiting high levels of TP53 it was impossible to make any firm conclusions, although a positive trend was observed. However, when we analysed the pT3 tumours, nuclear iASPP expression increased in areas of the leading edge of the tumour with high levels of TP53 expression. To date iASPP has only been implicated as a regulator of wild-type *p53*, however our findings are the first indicators that iASPP may also interact with gain-of-function mutant *p53* and drive a more aggressive, metastatic cancer cell phenotype.

One intriguing possibility that these findings raise is that nuclear, cytoplasmic and membrane-bound iASPP are from the same pool of cellular iASPP, but that they behave and localise differently based purely on post-translational modifications in response to different environmental stresses. Alternatively these three protein pools may be defined to locate in different places and carry out different functional roles. A third possibility is that whether iASPP's functional role is more complex and may incorporate both of these theories, iASPP is located in different cellular compartments in normal homeostatic balanced cells, but has the ability to translocate to another cellular compartment in response to particular stresses imposed on the cell.

The iASPP expression pattern is significantly changed in prostate cancer samples with elevated levels leading to poor prognosis. These results provide some insight into how prostate cancer cells may be evading apoptosis, progressing to a more aggressive phenotype. These findings identify iASPP as a potential prognostic marker and have shed light on novel ways in which iASPP may be promoting the survival and metastasis of prostate cancer cells outside of the normal confines of the prostate.

Chapter 5: iASPP reduction alters prostate cancer cellular behaviour *in vitro*

5.1 Introduction

Research into the molecular mechanisms underlying the various aspects of prostate cancer requires the use of *in vivo* and *in vitro* model systems. In order to validate observations seen in animal models and human tissue samples, and to achieve a thorough understanding of the functional role of specific proteins in a cancer cell model *in vitro* studies have proved to be invaluable. Using protein manipulation technology the mechanisms employed by cancer cells can be deciphered and our knowledge of disease initiation and progression can be furthered.

We have shown, using *in vivo* studies of both mouse and human tissue, that iASPP has an integral role in tissue homeostasis. When the localisation and expression levels of iASPP are altered these changes have effects on normal tissue homeostasis, and, promote disease progression potentially bestowing a survival advantage upon cells that have undergone malignant transformation. We have found that elevated nuclear and cytoplasmic iASPP expression are associated with poor clinical prognosis. Interestingly, decreased membranous iASPP was also observed in cancer cells that had gained metastatic potential when they were compared with benign cells. This chapter describes the effect of loss of iASPP function in a number of cancer cell lines, and attempts to address some of the mechanisms behind iASPP's role in the detrimental phenotypes observed in human prostate cancer.

5.2 Results

5.2.1 iASPP expression and localisation differs in prostate cancer cell lines with varying *p53* status

In order to investigate the role of iASPP in prostate cancer cells, the PC3, DU145, LNCaP and LNCaP-LN3 cell lines were used. The first three cell lines are the best characterised and the most heavily studied in prostate cancer research. PC3 cells were derived from a lumbar vertebral metastasis from a 62 year old man and have been used as a valuable research tool since 1979. DU145 were the first established prostate cancer cell line and these cells were derived from a tumour mass excised from the brain of a 69 year old man. LNCaP cells were isolated from a needle aspiration biopsy of a lymph node metastatic lesion from a 50 year man. Finally, LNCaP-LN3 is a derivative of the parental LNCaP cell line which has been selected for metastatic potential. These cells were recovered from spontaneous lymph node metastasis arising from an original injection site within the mouse prostate. The number 3 denotes the number of times these cells were recovered and subsequently re-injected back into the mouse prostate (Sobel and Sadar, 2005).

Firstly we analysed the expression level of iASPP in these four cell lines (figure 5.1). These cell lines have reported differences in their *p53* status, PC3 are *p53* null due to a frameshift mutation in *p53* that results in a premature stop codon, which in turn, results in no detectable protein product. DU145 cells have a mutation in both alleles of the *p53* gene, one allele has a mutation in exon 6, *P223L*, and the other carries a mutation in exon 8, *V274F*. These mutations have been shown to complement each other, resulting in *p53* gain-of-function (Gurova et al., 2003). LNCaP and LNCaP-LN3 cells express wild-type *p53*.

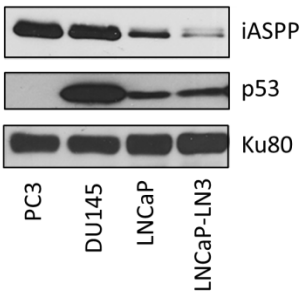

Figure 5.1 iASPP expression varies in cells with different *p53* status

A western blot of cell extracts from PC3, DU145, LNCaP, and LNCaP-LN3 cells.

Under normal unstressed conditions iASPP expression was found to be higher in cells with altered *p53* function (i.e. PC-3 and DU145 cells), compared with cells expressing wild-type *p53* (LNCaP and LNCaP-LN3 cells). This agrees with data from Laska and colleagues proposing that iASPP and *p53* form a negative feedback loop regulating the genotoxic stress responses of a cell (Laska et al., 2010). Next, in order to further our understanding of the potential role of iASPP in prostate cancer progression, we analysed its' localisation within the cell. Immunocytochemistry was performed in all four cell lines to establish whether there were any differences in iASPP expression and/or in localisation (figure 5.2). iASPP was found to only be expressed in the cytoplasm of PC3 cells; this may be a consequence of the absence of TP53. It was found to be both cytoplasmic and nuclear in LNCaP and LNCaP-LN3 cells, whilst iASPP was found to be in the cytoplasm, nucleus and membrane of DU145 cells. This finding in DU145 cells complements the work carried out in Chapter 4 in the analysis of human tissue samples, as it concurs the theory that iASPP may not only play a role in mediating p53 induced apoptosis but may also promote junctional integrity.

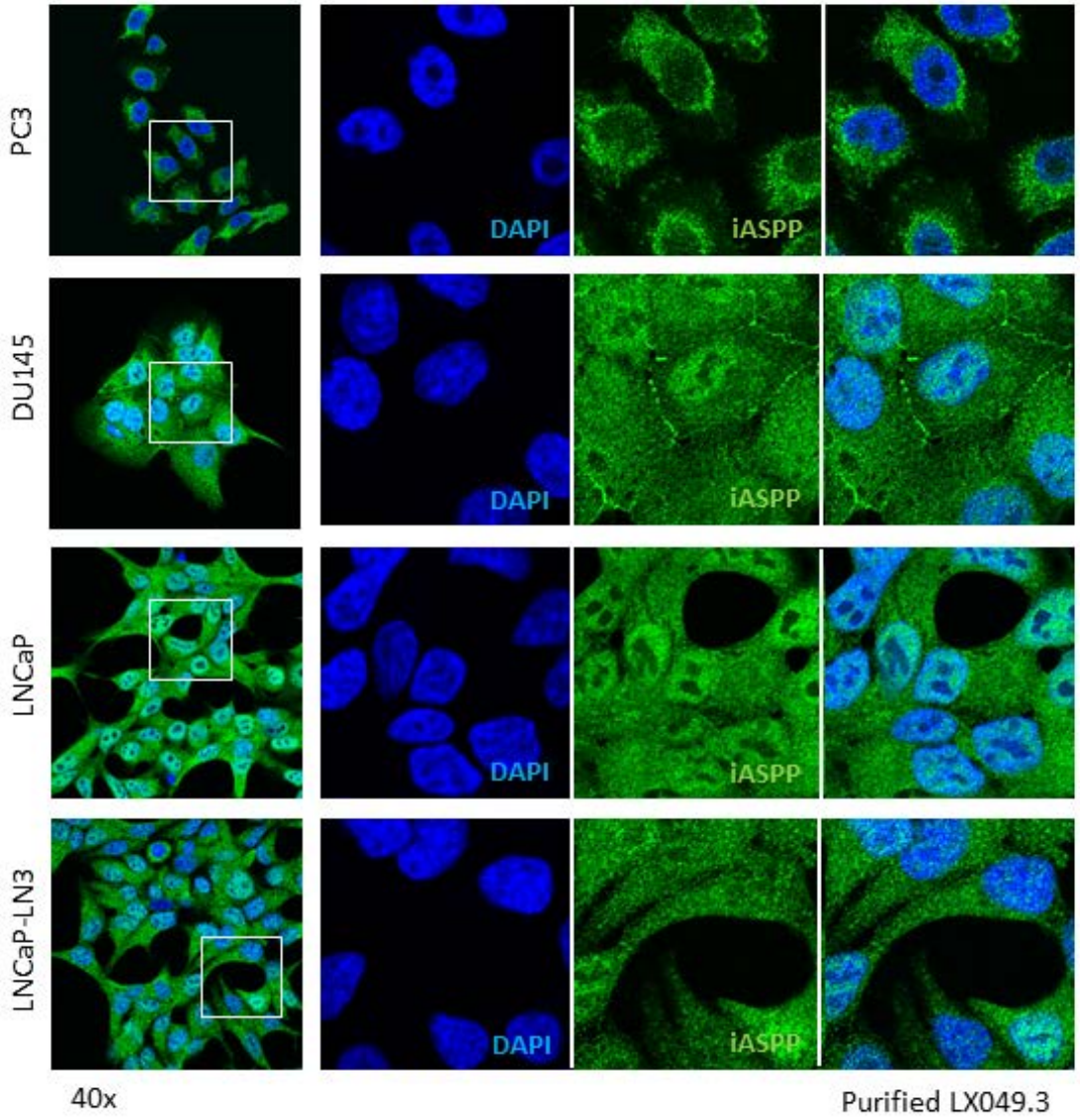

Figure 5.2 iASPP localisation in prostate cancer cell lines

Immunocytochemistry analysis of iASPP expression and localisation in PC3, DU145, LNCaP and LNCaP-LN3 cell lines. All images were taken at 40x magnification. Squares show higher magnification to illustrate detail. Scale bars - 50µm, 10µm.

5.2.2 Loss of iASPP affects the cellular growth pattern of prostate cancer cells

To investigate whether the loss of iASPP function affects the way in which cells grow, endogenous iASPP expression was transiently silenced using siRNA-mediated knockdown in all four cell lines (figure 5.3). The formation of normal cellular growth patterns was found to be disrupted in the cells lacking iASPP expression. The cells grew with less cellular contacts. LNCaP cells normally grow in tight clusters and often overlap one another. These cluster formations were not as tight in the cells treated with iASPP-specific siRNA, with cells growing away from the main group. This was also observed for LNCaP-LN3 cells which have a similar growth pattern. PC3 cells do not form tight cellular contacts, and so the difference in these cells was not as profound. In contrast, the growth pattern of the DU145 cells appeared to be highly affected as a consequence of loss of iASPP expression. These cells are visually more spread out with less cellular contact. These observations suggest the possibility that iASPP may play a role in maintaining the strength and stability of cell-to-cell contacts, or may indeed be involved in signalling at the membrane thereby contributing to the capability of epithelial cells to sense their neighbouring cell. Taken together these observations suggest a novel role, for iASPP as an important membrane-bound protein.

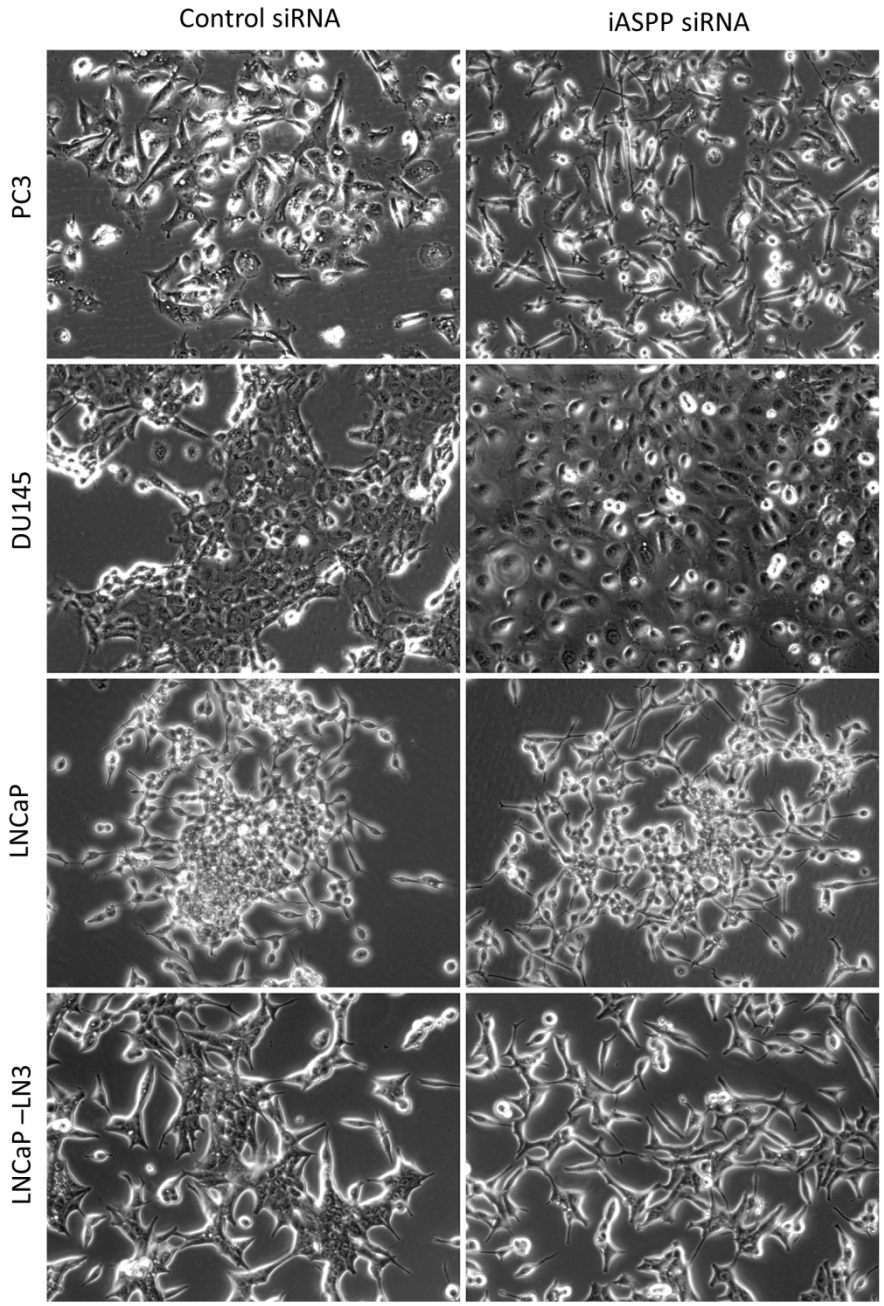

Figure 5.3 Loss of iASPP affects the growth pattern of prostate cancer cells

Cells were treated with either 40nM control siRNA (left panel) or 40nM iASPP-specific siRNA (right panel) and grown for 72 hours. All images were taken at 20x magnification

5.2.3 Loss of iASPP promotes invasion and migration in DU145 cells

Given that iASPP was observed in the membrane in both mouse (figure 3.4) and human prostate epithelial cells (figure 4.9), and a reduction in membranous iASPP was associated with extracapsular extension and invasion of prostate cancer cells through the capsule of men with pT3 disease, we examined whether loss of iASPP function might affect the invasive capacity of the prostate cancer cell lines. Cellular invasiveness was measured using Boyden Chambers. The Boyden chamber is a useful tool to study cell invasion. It consists of a cylindrical cell culture insert nested inside the well of a cell culture plate. The insert contains a polycarbonate membrane at the bottom with a defined pore size. Cells are seeded in the top of the insert in serum-free media, while serum containing 10% FBS is placed in the well below. Invading cells move through the pores toward the FBS chemoattractant below, these cells can then be fixed, stained and quantified. An increase in invasion was observed in all four cell lines treated with iASPP-specific siRNA compared to control siRNA-treated cells. However, the only cell line that showed a statistically significant difference in invasive capacity was DU145 (figure 5.4). DU145 cells treated with iASPP-specific siRNA invaded through the matrigel pores markedly faster than the cells treated with control siRNA ($P < 0.0001$). Even though iASPP-specific siRNA reduces steady state levels of iASPP at all of its cellular locations, a key difference between DU145 cells and the other prostate cancer cell lines is that it is the only cell line with detectable levels of iASPP at the membrane. These results support the hypothesis that iASPP is important for junctional integrity, and that loss of iASPP function might lead to a decrease in cell anchorage or contact dependence resulting in a more invasive phenotype.

To investigate this further, the motility of iASPP-depleted DU145 cells was measured. This was achieved by performing a wound healing assay whereby a scratch through the epithelial monolayer was introduced, and the time taken for the cells to migrate into the scratch and reform a continuous monolayer was measured using time-lapse microscopy (figure 5.5B). This assay concentrates on the movement of the cells alone, whereas the invasion assay evaluates the ability of the cells to move through a substrate such as matrigel embedded in the Boyden chamber, recapitulating the requirements needed to invade through the surrounding stroma. iASPP-specific siRNA-treated cells were significantly more motile and closed the gap far more quickly than cells treated with control siRNA and this was quantified using image J software. Statistical analysis of the results showed the motility difference to be highly significant ($P = 0.0007$) following a reduction in steady state levels of iASPP (figure 5.5C). The movement of the iASPP-specific siRNA-treated cells also appeared altered; they seemed to move in a manic fashion, moving more as individual cells as opposed to the cells treated with control siRNA which moved as a continuous sheet.

In order to demonstrate that iASPP is lost from the membrane after siRNA-mediated knockdown immunocytochemistry was performed on DU145 cells treated with either control or iASPP-specific siRNA (figure 5.5A). iASPP was no longer visibly expressed at the membrane, and only minimal cytoplasmic and nuclear expression of iASPP was seen following iASPP-specific siRNA treatment.

Taken together the data from the evaluation of the growth pattern of the prostate cancer cells, and the invasion and motility assays, suggests that membranous iASPP is important in junctional integrity. iASPP's exact role within the cell membrane has yet to be elucidated. However, these findings, along with data collected in chapter 3 and 4 provide

evidence that iASPP is a multifunctional protein and that it plays a fundamental role in normal prostate homeostasis as well as prostate cancer development, offering novel insights into means by which prostate cancer cells are able to progress to a more aggressive and invasive phenotype.

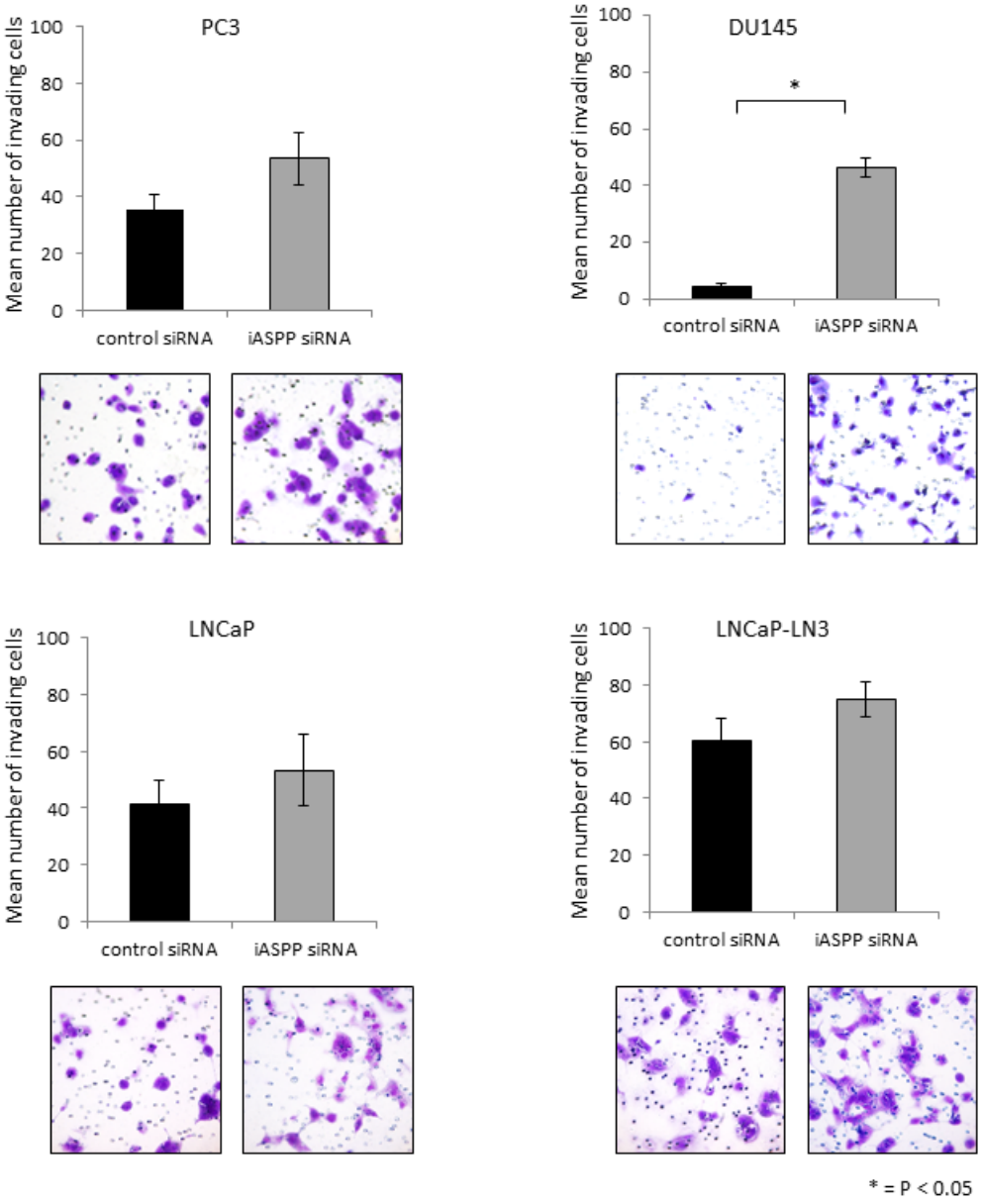

Figure 5.4 Loss of functional iASPP results in increased invasion of DU145 cells

Cells were treated with either control or iASPP-specific siRNA and culture in Boyden chambers. The mean number of cells, for each cell line, that invade through the matrigel filter is shown in the graphs. The experiment was repeated three times. Squares below show a representative image of the stained matrigel filter, all images are taken at 40x magnification. Error bars indicate standard error. P value was calculated using an unpaired t-test.

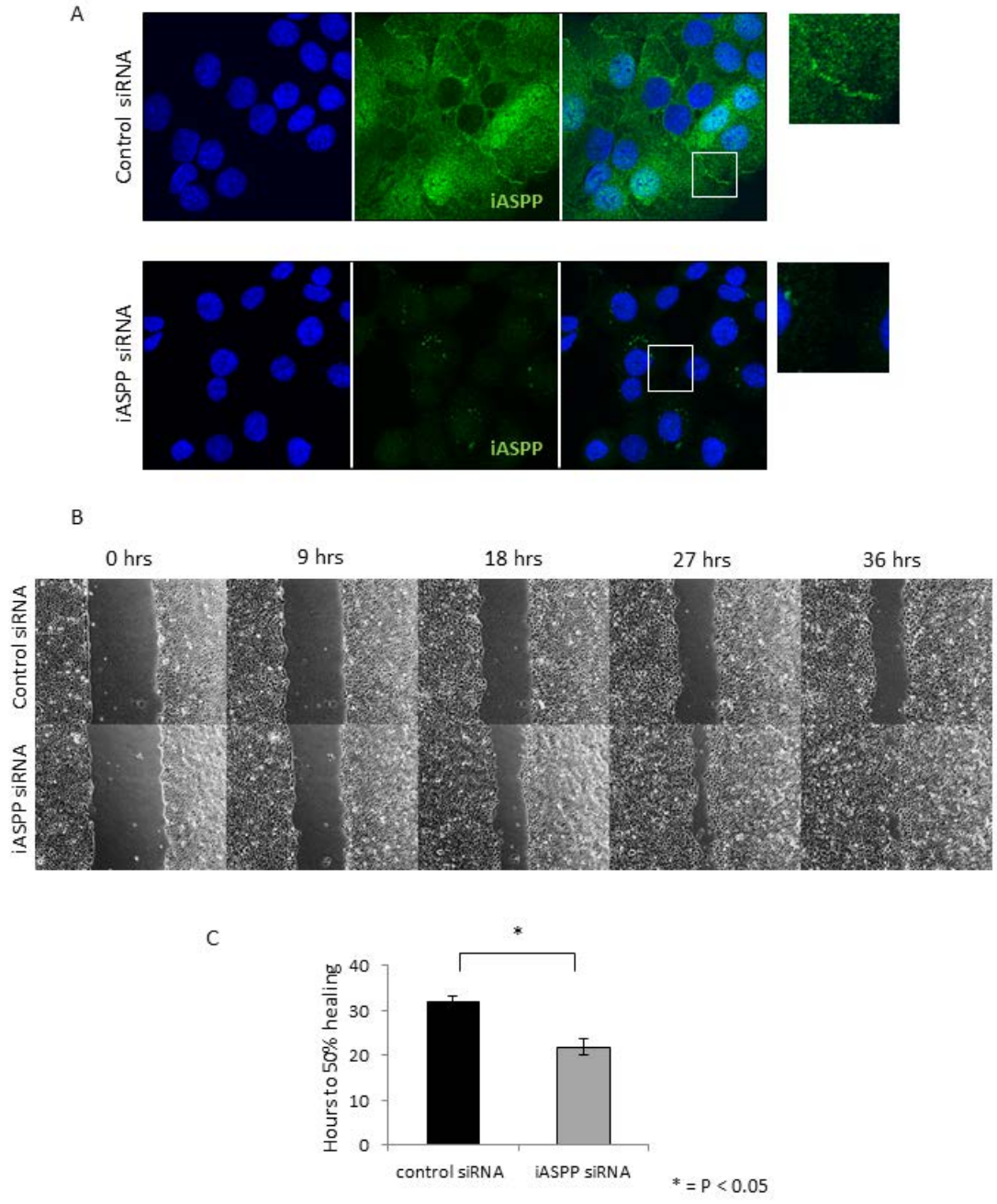

Figure 5.5 Loss of functional iASPP results in increased motility of DU145 cells

(A) Immunocytochemistry of iASPP (green) after siRNA-mediated knockdown of iASPP or treated with control siRNA. All images are taken at 40x magnification. **(B)** Motility assay using DU145 cells treated with control siRNA (top panel) or iASPP-specific siRNA (bottom panel) over a period of 36 hours. **(C)** Image J quantification of the time taken for 50% of the scratch to be covered by migrating cells. Error bars indicate standard error. P value was calculated using a Mann Whitney U test.

5.2.4 Loss of functional iASPP enhances docetaxel-induced apoptosis

Docetaxel is one of the few chemotherapeutic options in the treatment of advanced prostate cancer. It functions in 3 distinct pathways that contribute, directly or indirectly, to apoptosis. The main mode of therapeutic action is the suppression of microtubule assembly and disassembly. Docetaxel binds to free tubulin and promotes the assembly of tubulin into stable microtubules, while simultaneously inhibiting their disassembly. This leads to the production and stabilisation of microtubule bundles without normal function, which results in the inhibition of mitosis. In addition to affecting microtubule structure, docetaxel is thought to work by interfering with 3 of the phases of the cell cycle. It causes cell cycle arrest at S phase, G2 phase, and M phase, causing cells to be unable to progress to the next step, thus triggering apoptosis. Docetaxel also initiates apoptosis by inducing the phosphorylation of the anti-apoptotic Bcl-2 protein, thereby causing its inactivation, allowing apoptosis to occur. Docetaxel has been used in the clinically for around a decade and clinical trials have demonstrated an increase in median overall survival (OS) of 18.9 months in patients treated with a combination of docetaxel and predisone, compared to a median OS of 16.5 months in patients treated with other therapies such as mitoxantrone combined with predisone (Maluf et al., 2012). However, even though docetaxel has shown a level of success in the clinic, there is still a need to improve patient response. The ability to target apoptosis with a combination of drugs could enhance the level of apoptosis and improve the efficacy of the treatment. Nuclear iASPP is an inhibitor of p53-mediated apoptosis, given the fact that many prostate cancers still exhibit wild-type *p53*, inhibition of nuclear iASPP function might facilitate an enhanced apoptotic response.

In order to investigatethe role of iASPP in docetaxel-induced apoptosis, we conducted *in vitro* experiments with siRNA-mediated knock-down of endogenous iASPP, followed by docetaxel treatment, for all four prostate cancer cell lines. 72 hours after treatment with siRNA the cells were treated with DMSO or 50ng of docetaxel for 48 hours. Apoptosis was then determined by propidium iodide (PI) staining. PI is an intercalating fluorescent agent which binds to nucleic acids within the nucleus thereby denoting the quantity of DNA within the cell and identifying which phase of the cell cycle the cell is in. The PI staining was analysed by flow cytometric analysis (figure 5.6) and the number of sub-G1 cells was calculated. Interestingly, there was a significant increase in apoptosis in PC3, DU145 and LNCaP cells after siRNA-mediated knockdown of iASPP alone ($P = 0.014$, $P = 0.008$, $P = 0.038$, respectively). This supports the concepts that iASPP plays a role in cell survival; however as PC3 cells and DU145 cells do not express functional *p53*, this effect is not *p53*-dependent. Docetaxel was seen to induce apoptosis in all 4 cell lines tested. The wild type *p53* cell lines (LNCaP and LNCaP-LN3) showed more resistance to the Docetaxel treatment than PC3 and DU145, however all four cell lines showed a statistically significant increase in sensitivity to the docetaxel treatment after iASPP knockdown (PC3; $P = 0.037$, DU145; $P = 0.036$, LNCaP; $P = 0.032$ and LNCaP-LN3; $P = 0.022$). The effects of the treatment appeared to be more profound in the cells with impaired *p53* function. This is an intriguing observation and concurs with previous reports in the literature (Gan et al., 2011), however the underlining reason for this effect is still not fully understood. Furthermore, different groups have shown conflicting data. Docetaxel has been shown to stabilise TP53 and thereby induce a heightened TP53 response, and so cells with wild-type *p53* may first attempt to repair the damage caused by the drug treatment before they direct a program of cell death. In turn, this causes a lag

in the time taken for cells with wild-type *p53* to commit to apoptosis, compared to the cells with no apoptotic functioning *p53.* This would result in a level of resistance to Docetaxel-induced apoptosis in cells with wild-type *p53*. In contrast, the opposite effect has also been reported whereby cells with functioning TP53 are more sensitive to docetaxel treatment, with docetaxel stabilising TP53, which in turn results in an increased apoptotic response and a faster commitment to apoptosis (Liu et al., 2013a). These conflicting results have caused a degree of controversy as to the true effect of docetaxel on TP53 activity. Our results support the former theory namely that cells with no or impaired TP53 function are more sensitive to docetaxel-induced apoptosis. Our results also demonstrate that inhibiting iASPP function significantly enhances docetaxel-induced cell death. These findings support the work by LiLi Jiang who showed that the over-expression of iASPP contributes to chemoresistance in ovarian cancer by the blocking of mitotic catastrophe. Furthermore, siRNA-mediated silencing of iASPP was shown to enhance paclitaxel-induced cell death (Jiang et al., 2011). Paclitaxel is a member of the taxane family and has a similar mode of action to docetaxel. Nuclear iASPP has also been implicated as being a therapeutic target in the treatment of melanoma. The inhibition of nuclear iASPP in combination with other therapeutics has been shown to alleviate the suppression of wild-type *p53* activity by iASPP, thus reactivating wild-type *p53* function and increasing the efficacy of treatment (Lu et al., 2013). Our results give new insights into the mechanisms involved in chemotherapy resistant prostate cancer, and add prostate cancer to the list of cancers that have been identified as having a level of chemoresistance bestowed upon them by the expression of nuclear iASPP. These findings indicate that patients may benefit from the addition of nuclear iASPP inhibitors in combination with existing chemotherapeutics in the treatment of advanced prostate cancer.

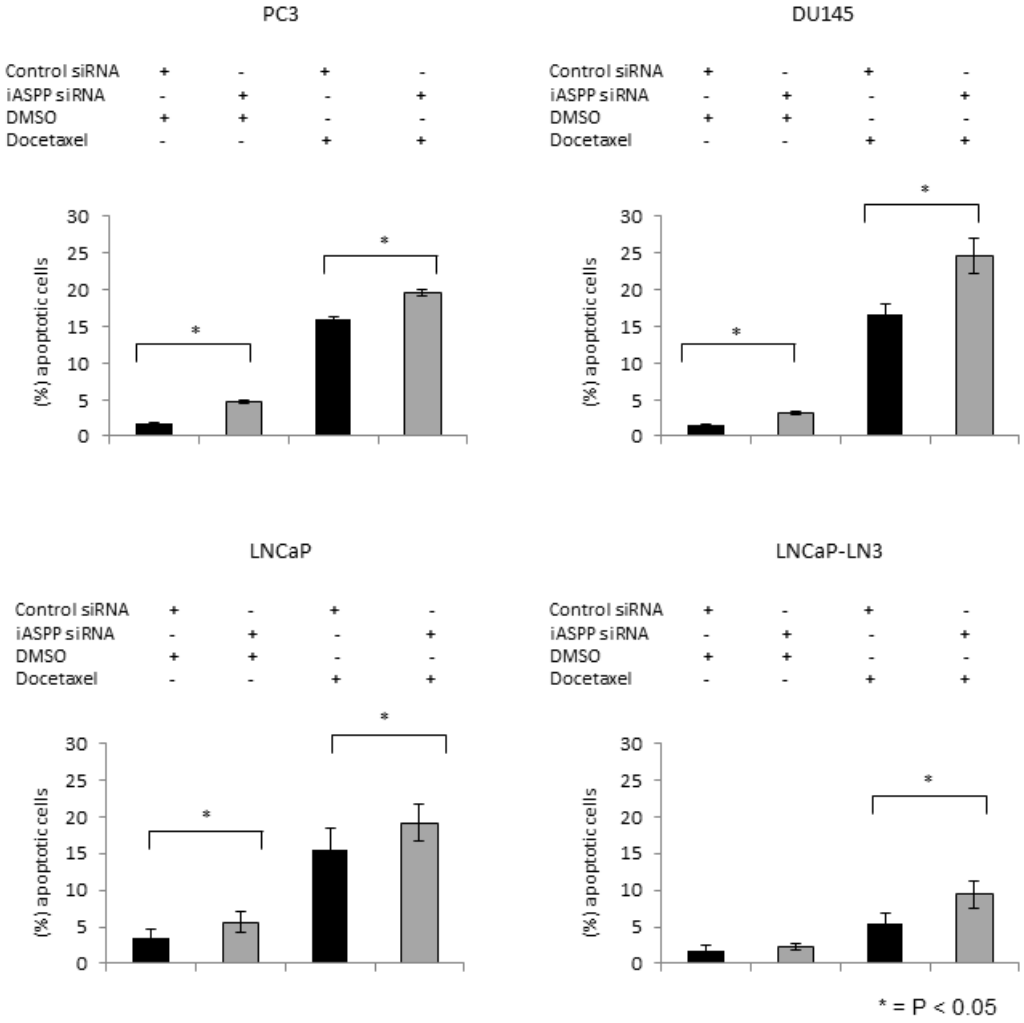

Figure 5.6 Loss of functional iASPP results in enhanced docetaxel-induced apoptosis

Cells in sub-G1 were quantified by PI staining. PC3, DU145, LNCaP and LNCaP-LN3 cells were treated with control or iASPP-specific siRNA for 72 hours followed by 48 hours treatment with either DMSO alone (control) or 50ng docetaxel. The percentage of apoptotic cells was measured using flow cytometry. The experiment was repeated 3 times. Error bars indicate standard error and P values were calculated using a paired t-test.

5.2.5 iASPP cooperates with mutant *p53*

The last decade has seen a plethora of evidence to support the role of iASPP as a co-regulator of wild-type *p53* function. However, whether iASPP is able to influence the activity of mutant *p53* has not been highly studied. In Chapter 4 we demonstrated that nuclear iASPP expression was associated with TP53 expression (a surrogate marker of mutant p53) at the leading edge of pT3 tumours. These tumours have a greater metastatic potential than locally-confined prostate cancers, in part because they contain cells that have gained invasive properties allowing them to invade through, and in some cases out of, the prostate capsule. In the last few years there has been a huge amount of support in favour of the "gain-of-function hypothesis" with regards to *p53* function, given the increasing body of evidence to suggest that mutant *p53* can bestow an advantageous phenotype on cancer cells resulting in increased proliferation, cell survival and metastasis (Freed-Pastor and Prives, 2012). As iASPP has a known relationship with wild-type *p53* and given the fact that it is a putative oncogene, it is not inconceivable that iASPP may play a role in promoting the oncogenic role of a *p53* gain-of-function mutation.

To understand whether iASPP could cooperate with mutant *p53* at the leading edge of prostate tumours/ we utilised a technique in which cells were grown in organotypic cultures to create a physiological leading edge *in vitro.* This allows the expression of both iASPP and TP53 arising from mutant *p53* to be simultaneously analysed by immunofluorescence in order to elucidate whether they co-localise. Interestingly, we found that prostate cancer cells expressing large amounts of nuclear mutant TP53 also expressed nuclear iASPP, and these cells were found to be organised at the leading edge (figure 5.7A). To establish whether or not mutant TP53 and iASPP do indeed bind, DU145 cells were used to immunoprecipitate TP53 and probe for iASPP as a binding

partner. Endogenous iASPP was found to bind mutant TP53 in DU145 cells (figure 5.7B). This is the first time to our knowledge that these two proteins have been reported to co-localise, identifying iASPP as a novel binding partner of mutant TP53. These findings reinforce the results illustrated in chapter 4 (figure 4.10) linking nuclear iASPP with mutant TP53 at the leading edge of invasive tumours. Nuclear iASPP may be important in promoting survival and evading apoptosis in a p53-independent manner in these cells, which may be why when iASPP is depleted in DU145 cells they become more sensitive to docetaxel-induced cell death (figure 5.6). Elucidating the exact function of the relationship between iASPP and mutant TP53, and establishing the advantages bestowed on these cells, will be the subject of future research. The immunohistochemical analysis of TP53 expression has been previously shown to significantly add to the current prognostic model of newly diagnosed prostate cancer (Kudahetti et al., 2009). *p53* mutations characterise a small biologically aggressive subgroup with a high risk of progression after radical prostatectomy. TP53 expression has also been shown to be an independent predictor of PSA-recurrence in low- and intermediate-grade cancers. It is noteworthy that the main disadvantage of using TP53 as a stand-alone biomarker is the high rate of false-positivity with different staining protocols giving different levels of sensitivity (Schlomm et al., 2008). The combination of both TP53 and nuclear iASPP could potentially strengthen this model, giving a clearer indication of which patients may progress and ultimately suffer from a more aggressive and invasive disease.

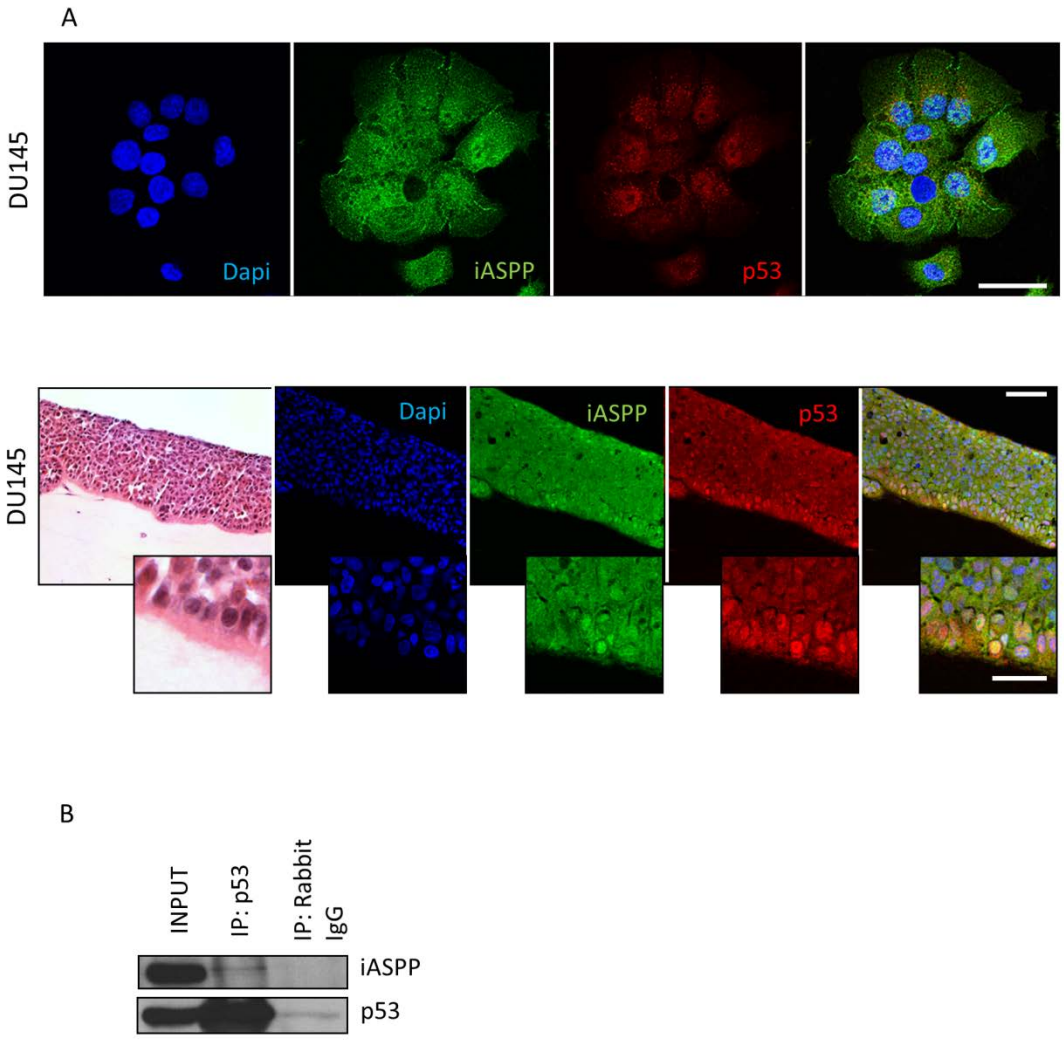

Figure 5.7 iASPP cooperates with mutant TP53

(A) Immunofluorescence of iASPP (green) and TP53 (red) in DU145 cells (top panel) All images were taken at 40x magnification. Haematoxylin and Eosin staining (first box lower panel) and immunofluorescence of iASPP (green) and TP53 (red) in DU145 cells grown as organotypic cultures (lower panel). All images were taken at 20x magnification. Squares illustrate detail. Scale bars - 50µm, 100µm and 50µm. **(B)** Immunoprecipation of TP53 (CM1) and probed with iASPP (LX049.3) and TP53 (DO-1) specific antibodies.

5.2.6 iASPP expression is not regulated by androgens

The prostate gland contains the enzyme 5-alpha-reductase which converts the hormone, testosterone into its more potent form dihydrotestosterone (DHT). DHT is the primary driver of androgen receptor (AR) activation and transcriptional activity, AR is expressed in the luminal secretory cells where it is necessary for epithelial differentiation and secretory protein expression (Cunha et al., 1987). AR is essential for the normal growth, development and maintenance of the prostate gland. It is also responsible for the growth, development and maintenance of prostate tumorigenesis. Understanding the pathways that are under androgen regulation is important in developing prostate cancer treatments strategy and development. Over 8,700 AR binding sites have been identified in the LNCaP prostate cell line (Wang et al., 2009a), highlighting the complex network involved in AR signalling which may potentially be relevant to the initiation and progression of prostate cancer to an aggressive androgen independent phenotype.

Intriguingly, TP53 and AR have been shown to mutually regulate one another (Alimirah et al., 2007, Rokhlin et al., 2005). Both TP53 and AR are transcription factors that independently affect the expression of a wide range of genes. However, a recent study has shown that an alteration in TP53 can impact upon the transcriptional capacity of AR and vice versa. Castration-resistant prostate cancer has an altered pattern of gene expression. One consequence of TP53-inhibition has been shown to be a significant reduction in AR-binding peaks, along with some novel binding not observed in the presence of TP53 (Guseva et al., 2012), These findings suggested that TP53 expression is a modulator of the transition from androgen-dependent to androgen-independent disease. It may be a disadvantage for cancer cells to narrow their androgen driven gene expression until they have accumulated enough genomic mutations for TP53-signalling to no longer be of use.

Given the critical role of the AR in prostate tissue, and the link between AR and TP53, it was intriguing to understand whether AR-signalling could regulate iASPP expression in an androgen dependent manner. To investigate this hypothesis LNCaP cells were cultured in charcoal-stripped FBS for 48 hours, to remove androgens. Cells were then treated with either ethanol alone as a control, bicalutamide, bicalutamide in combination with dihydrotestosterone, or dihydrotestosterone alone. Bicalutamide is an anti-androgen and acts by binding to the AR and preventing its activation and the subsequent up-regulation of androgen-responsive genes. Treating cells with both bicalutamide and dihydrotestosterone should counter any effects on AR signalling and act as a neutralising control. Cells were grown under these conditions for 8 hours, 16, 24 or 36, and these time points were chosen in order to ensure that any early or late fluctuations in iASPP levels, as a result of the addition of androgen would be observed. Overall iASPP expression did not change in response to fluctuations in androgen levels (figure 5.8). In conclusion, despite the relationship between the AR and TP53, *iASPP* does not appear to be an androgen-responsive gene and so consequently would not be expected to be affected by androgen-deprivation therapy. Therefore, in principle, high levels of *iASPP* expression could still exert oncogenic effects during the administration of androgen-ablation therapy, thereby promoting cell survival.

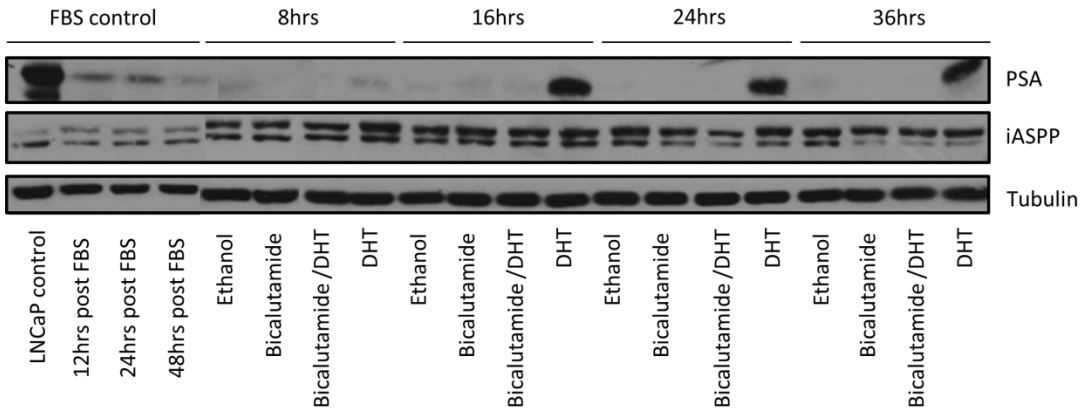

Figure 5.8 iASPP expression is not regulated by androgens

Western blot analysis of LNCaP cells grown in the presence or absence of androgens. PSA was used as a control to confirm androgen-depletion.

5.3 Discussion

The use of *in vitro* models is invaluable in the attempt to further our understanding of the cellular mechanisms underlying the processes of malignant transformation and cancer progression. *In vitro* systems make it possible to validate observations taken from the clinic including patient data and patient samples, along with *in vivo* animal models. iASPP has been identified as having a fundamental oncogenic role in the progression of numerous cancers. Through the use of *in vitro* studies we have been able to advance our understanding of the role of iASPP in prostate cancer tumorigenesis.

To investigate iASPP function in prostate cancer we utilised the well-known prostate cancer cell lines PC3, DU145, LNCaP, and a derivative of LNCaP, LNCaP-LN3. iASPP was found to be expressed in all four prostate cancer cell lines, although the levels of expression were variable depending on *p53* status. Cell lines with impaired *p53* function had higher levels of iASPP expression in comparison to cell lines with wild-type *p53*, suggesting the presence of a feedback loop regulating both the amounts and activity of TP53 (Laska et al., 2010). The cells with no *p53* or mutant *p53* expression appeared to have lost this regulation, resulting in an increase in iASPP expression. iASPP localisation also varied in these cell lines, with iASPP being confined to the cytoplasm in PC3 cells, expressed in both the cytoplasm and nucleus in LNCaP and LNCaP-LN3 cells, and found in the cytoplasm, nucleus and membrane in DU145 cells. Given our previous data clearly illustrating iASPP localisation in the membrane of mouse and human prostate epithelial cells, DU145 cells provided a good model in which to study the consequences of loss of membranous iASPP localisation, particularly as this appears to be a significant step in human prostate cancer progression.

Endogenous iASPP was transiently reduced using iASPP-specific siRNA in these four prostate cancer cell lines. Loss of iASPP function affected the cell's normal growth pattern and caused them to grow in a more scattered formation. It is noteworthy that this scattered pattern could be partly due to increased apoptosis, resulting in gaps in the normal growth formations, however the difference observed is unlikely to be due to this alone. This affect was observed to be most profound in the DU145 cells, suggesting that the loss of membranous iASPP impacts on cell-to-cell contacts. However, it is not clear at this stage whether loss of iASPP results in a loss of contact strength and stability, or a loss of cellular communication, compromising the ability of neighbouring cells to sense each other. To investigate the effects of iASPP-depletion further, we assessed the motility and invasive potential of prostate cancer cells after siRNA knock-down. We observed a significant increase in the number of invading DU145 cells after siRNA-mediated knock-down of iASPP compared to control, as well as an increase in motility. These findings complement the observations in chapter 4 suggesting that membranous iASPP may play a tumour-suppressive role at the junctions of normal benign cells. However, during malignant transformation of prostate epithelial cells, membranous expression may become down-regulated thereby allowing these cells to invade through the surrounding stroma.

One of the most characterised functions of iASPP is its role as a co-regulator of p53-mediated apoptosis. Elevated iASPP expression has been reported to attenuate apoptosis-inducing therapeutics, resulting in a chemoresistant phenotype. To assess whether iASPP contributes to chemoresistance in prostate cancer cells, the chemotherapy drug docetaxel was used for *in vitro* experiments. Docetaxel is a therapy used for the treatment of castrate resistant prostate cancer, and has shown a level of success in the clinic as it can

prolong a patient's life for a few months. Nevertheless, a number of patients exhibit resistance to this treatment. Cells treated with iASPP-specific siRNA were significantly more sensitive to docetaxel-induced apoptosis than cells treated with control siRNA. Hence the inhibition of nuclear iASPP, in combination with the administration of docetaxel, could enhance the patient response to docetaxel and may prevent resistance in a subset of patients. Caution would clearly have to be taken to ensure that the inhibition only acted upon nuclear iASPP and not membranous iASPP. The prescription of cyclin B1 inhibitors could overcome this problem. The cyclin B1/cdk complex phosphorylates iASPP, resulting in its activation and nuclear translocation. Only when iASPP is present in the nucleus can it inhibit *p53* transcriptional activity. Consequently, if iASPP is unable to undergo activation, it cannot convey its chemoresistant properties. Interestingly docetaxel-induced apoptosis was further enhanced in the cell lines with no apoptotic-functioning *p53*, therefore iASPP is exerting its anti-apoptotic function in a p53-independent manner, the details of which are not yet understood.

One of the main issues investigated in this thesis is whether iASPP cooperates with mutant *p53*. In recent years the evidence for the "*p53* gain-of-function theory" has strengthened, demonstrating that mutant *p53* is far more than just a dominant negative mutation. The evidence provided in chapter 4 supports the hypothesis that there is a cooperative relationship between nuclear iASPP and mutant *p53*, and that nuclear iASPP promotes mutant *p53* function at the leading edge of invasive tumours. To confirm this we utilised an *in vitro* model that recapitulated the leading edge. Nuclear iASPP was found to co-localise with mutant TP53. To investigate this further, we immunoprecipated mutant TP53 and confirmed that iASPP is a binding partner. This finding is the first report to our knowledge to suggest that iASPP may regulate mutant *p53* gain-of-function

at the leading of invasive cancer. TP53 immunohistochemical detection has been shown to have prognostic value, directly correlating with the malignant potential of the tumour and prognosis of the disease, and thereby providing insight with regards to which subset of patients with newly diagnosed prostate cancer may go on to develop more aggressive invasive disease (Schlomm et al., 2008, Saidi et al., 2011). Nonetheless, it is not used as a reliable biomarker as many studies have demonstrated a high number of false positives due to overly-sensitive detection techniques. We have shown that increased nuclear iASPP correlates with TP53 accumulation and is an independent prognostic marker in non organ-confined disease, suggesting that the detection of a combination of both nuclear iASPP and TP53 in combination could serve as a more reliable prognostic marker.

These results shed new light on the molecular mechanisms behind iASPP expression and localisation in prostate cancer. iASPP evidently plays a role in prostate cancer progression, although its function is complex, with cellular localisation being a key factor. We have shown that iASPP is a protein with many cellular localisations, participating in a number of different cellular functions. It would appear that though its role as purely an oncogene is questionable. The development of nuclear iASPP inhibitors could have a positive contribution to patient care, although caution would have be taken to ensure that membranous iASPP function was not compromised.

Chapter 6: Conclusions and future work

6.1 iASPP is a mediator of prostate homeostasis

In this study evidence has been presented suggesting that that the program of differentiation within the prostate epithelium is altered in iASPP$^{\Delta 8/\Delta 8}$ mice lacking functional iASPP. At a gross morphological level the absence of functional iASPP resulted in a smaller organ, although no obvious architectural lobular or glandular abnormalities were observed. iASPP-deficient mice were also found to have a significant decrease in basal cell TP63 expression, coupled with an increase in Ck19 expression. Moreover, they were found to have a significant decrease in the expression of the neuroendocrine marker synaptophysin. Finally, both cell proliferation and apoptosis were found to be reduced in the prostate of the iASPP$^{\Delta 8/\Delta 8}$ mouse. Taken together these results provide evidence that deficiency of functional iASPP affects cell lineage maintenance within prostate epithelium, as well as the rate of cellular turnover. Overall these results suggest that mice lacking functional iASPP may suffer from premature cellular senescence due to alterations in TP63 mediated differentiation.

The basal cell population of prostate epithelium is responsible for cell lineage regeneration. Androgen-ablation and regeneration studies have shown that the TP63-positive basal cells give rise to a population of terminal differentiated luminal cells, thereby restoring the prostate epithelial tissue (Pignon et al., 2013). *p63* has been shown to be essential for maintaining the highly proliferative basal cell compartment, and is rapidly degraded when basal cells are induced to differentiate (Yang et al., 1999a). iASPP has been shown to be an important co-regulator of *p63*, as it inhibits its' transcriptional activity of genes involved in the differentiation program, such as envoplakin in mouse keratinocytes, while maintaining the expression of basal cell markers such as Ck14.

These findings were the first indications of iASPP's role in maintaining the proliferative potential of the basal layer in epithelium (Notari et al., 2011). Our results in prostate support these initial findings; however we have also demonstrated that a depletion of the basal cell lineage occurs in the prostate epithelium of the iASPP$^{\Delta 8/\Delta 8}$ mouse lacking functional iASPP. This depletion was coupled with a significant increase in Ck19-positive intermediate cells. These cells have the capacity to regenerate tissue, although their proliferative capability is less than that of basal cells. This shift in cell lineage may explain the decrease in proliferation observed in the prostate epithelium of these mice. iASPP has been implicated in the prevention of premature cellular senescence through its regulation of *p63*. The iASPP$^{\Delta 8/\Delta 8}$ mice were found to have a reduced number of cells undergoing apoptosis, and this could be due to an increase in cells undergoing senescence. However, more work needs to be carried out to confirm this hypothesis. A summary of the findings presented in this thesis regarding mouse prostate development are illustrated in figure 6.1. This work clearly shows that iASPP is required for normal prostate organogenesis by maintaining the TP63-posisitve cell lineage, thus maintaining normal prostate homeostasis.

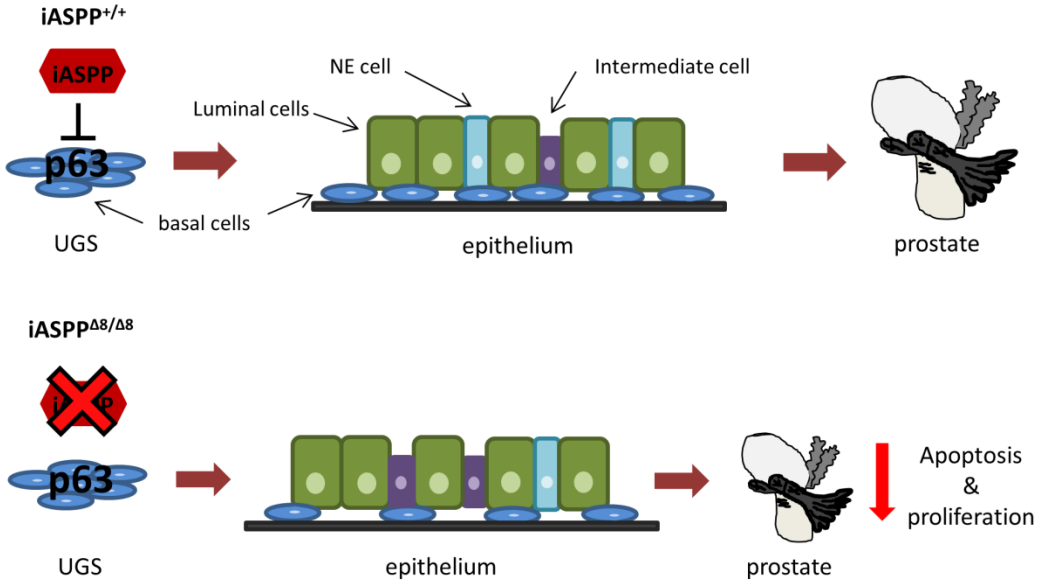

Figure 6.1 iASPP inhibits *p63* and thus maintains the TP63-positive basal cell lineage

Loss of iASPP induces early differentiation causing a reduction in TP63-positive cells (blue), an increase in Ck19 intermediate cells (purple) and a decrease in neuroendocrine cells (turquoise). In turn, these alterations result in a decrease in both apoptosis and proliferation within prostate epithelial cells, causing an overall reduction in organ size.

In order to study these findings further we have established a technique to culture primary mouse prostate cells and to grow prostate spheroids. Our evaluation of the iASPP$^{\Delta 8/\Delta 8}$ mice has clearly demonstrated that these mice develop a structurally normal prostate, and so by studying prostate spheroid formations, the dynamics of tissue regeneration can be followed. This allows the results presented above to be further validated along with the opportunity to study the effects of loss of iASPP on differentiation, proliferation and apoptotic processes in more detail. The evaluation of spheroid formation also allows the contribution of iASPP in early cell fate decision to be

studied. iASPP has been shown to be expressed in the basal cell compartment of the prostate epithelium and it is from this cellular compartment that both luminal and NE cells are thought to arise. iASPP$^{\Delta 8/\Delta 8}$ mice exhibit a significantly reduced level of synaptophysin expression suggesting a decrease in NE cell number. When the NE cell lineage was first identified it was thought that these cells originated from the neural crest (Aumuller et al., 1999). However, a number of studies have now shown that they originate from a common progenitor cell (Noordzij et al., 1995, Uzgare et al., 2004, Pignon et al., 2013). The pathway that controls the differentiation of normal NE cells is unclear. It is thought that the increased NE cells in cancer are not true NE cells, but are cells that have undergone a process of transdifferentiation resulting in the expression of NE markers such as serotonin and Chromogranin A (Yuan et al., 2007). This process is thought to occur as a consequence of the up-regulation of developmental genes such as *snail* (McKeithen et al., 2010). *Snail* is up-regulated in the transition to an EMT-like state, and it may therefore be that NE differentiation (NED) is a consequence of the progression to an invasive cancer phenotype. This population of NE cells have been studied in far greater detail than the "normal" population of NE cells. It is known that luminal and NE cells can develop from *p63*$^{-/-}$ UGS, whereas basal cells cannot (Kurita et al., 2004). However, in normal development the UGS consists of purely TP63-positive progenitor cells which have been shown to ultimately give rise to both luminal cells and NE cells (Signoretti et al., 2005). This suggests that once TP63 expression is down-regulated in basal cells, they are able to commit to either a luminal or a NE cell lineage, however the precise factors that drives this cell fate decision are unknown. Given iASPP's evident regulation of *p63*, it may be that iASPP contributes to cell fate decision through is regulation of TP63 regulated differentiation genes. In the future it would be

fascinating to investigate the ratio of each cell lineage in the iASPP$^{\Delta 8/\Delta 8}$ spheroids compared with wild-type spheroids in order to confirm iASPP's role in NE differentiation within the prostate epithelium.

Using transgenic mice that are born with the iASPP protein deleted is not without restraints. These mice are not born according to Mendelian segregation frequency, resulting in fewer mice available to study. Furthermore these mice have cardiac defects resulting in a reduced lifespan which again has to be factored into the experimental design of *in vivo* studies. Therefore, in order to further our knowledge regarding the implications of iASPP loss in the context of normal regeneration and homeostasis, cells taken from conditional iASPP$^{\Delta 8/\Delta 8}$ mice can be used.

In our transgenic mouse model iASPP expression is controlled by the activation of the Cre recombinase enzyme in the presence of 4-hydroytamoxifen (4-OHT). The addition of 4-OHT activates the *Cre recombinase* promoter, causing the Cre to excise exons 8 to 13, which are flanked by 2 LoxP sites. This system therefore allows the deletion of iASPP in a spatio-temporally controlled manner and overcomes any embryonic influences iASPP loss may have, resulting in higher numbers of mice available to study. The mice are born healthy and develop normally. They can be injected with 4-OHT just 4 days before sacrifice in order to ensure complete deletion of the *iASPP* gene and the degradation of any residual iASPP protein. The cells can then be harvested from mice that carry the *Cre recombinase* gene and mice that do not, or from mice that both carry the *Cre recombinase* gene, but where only half of the cohort have been treated with 4-OHT and half have been treated with oil as a control. These cells can be grown to form prostate spheroids and the growth, differentiation and level of apoptosis between the two cell populations can be studied in detail.

We have optimised a technique for future studies using wild-type cells (figure 6.1A) Spheroid structures grew with both Ck5 and Ck8 positivity, demonstrating the potential presence of both basal and luminal cells. In a number of spheroids a lumen was observed, suggesting the development of an acinus-like structure. iASPP could be seen in the nucleus of a number of the outer cells (figure 6.1B), which suggests the co-localisation of iASPP with TP63, as the majority of the outer cells are TP63 positive cells. This finding complements the observations recorded in the mouse prostate tissue regarding TP63 expression, as well as previous studies showing the link between iASPP and TP63 expression in the skin (Notari et al., 2011). To complete the optimisation of this technique, the deletion of *iASPP* in the iASPP-conditional knockout mice ($iASPP^{flox/flox;}$ $Cre^{+}ER^{T}$ mice) was confirmed. When these mice are treated with 4-OHT the deletion of *iASPP* should be global, affecting all the cells of the body. To ensure that the 4-OHT treatment successfully deleted *iASPP* from the prostate, a pair of mice were injected with a single dose of 4-OHT 4 days before sacrifice. The mice were selected based on their genotype; one mouse was positive for the *Cre recombinase* and one mouse was not (figure 6.1C). The deletion was successful with only a very low level of iASPP being detected via western blot. The number of doses of 4-OHT could be increased to optimise the level of deletion.

This model has the potential to be a powerful tool as the cells have the same genetic alterations as the transgenic mice. This gives us the opportunity to link tissue phenotypes with *in vitro*-based cell systems, and to study the underlying molecular mechanism behind the expression and localisation of numerous proteins.

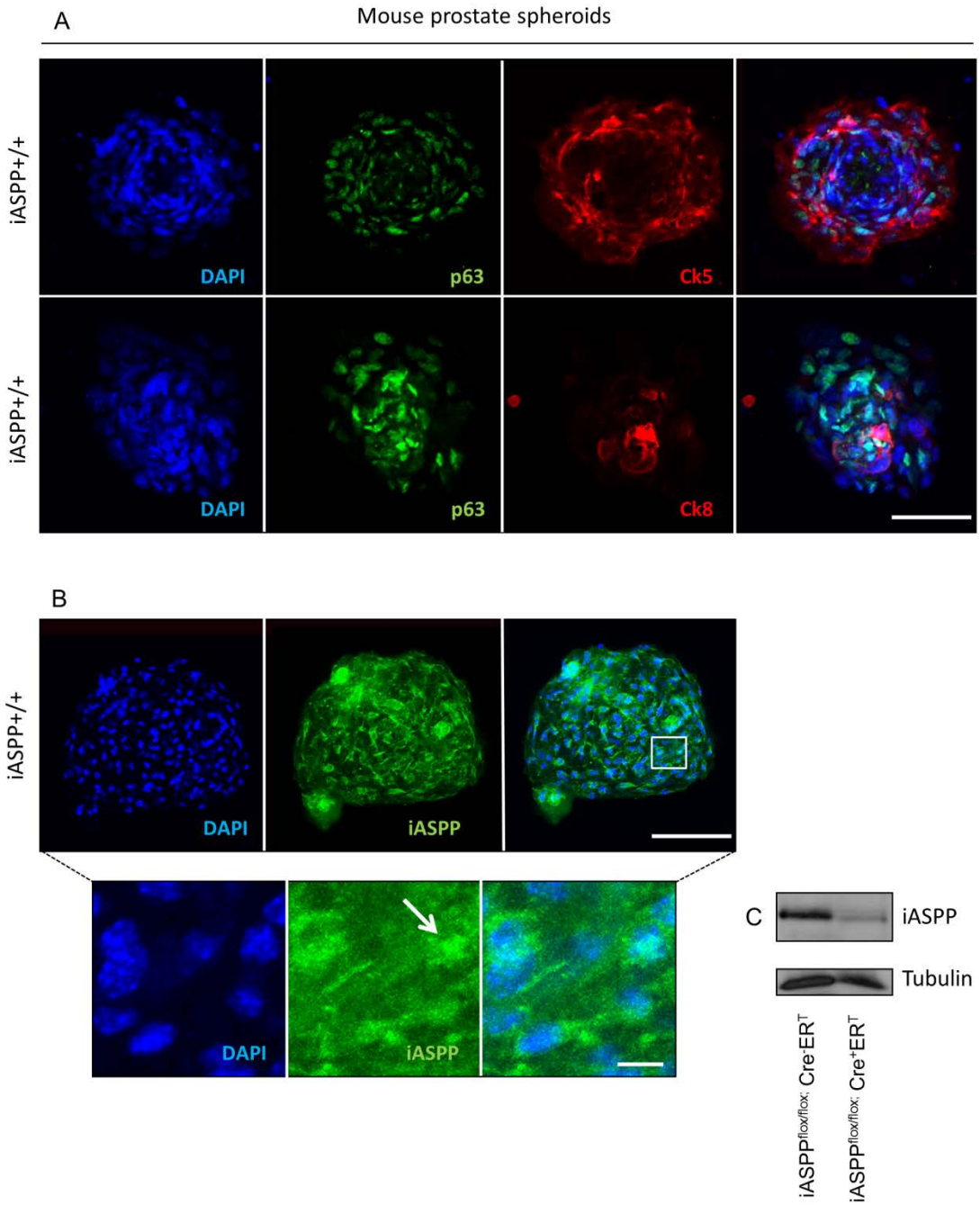

Figure 6.2 iASPP loss can be studied in primary mouse cell cultures

(A) Immunofluorescence staining of the cell lineage markers in mouse prostate spheroids [(upper panel: TP63 (green) Ck5 (red)] [(lower panel) TP63 (green) Ck8 (red)] Scale bar - 100µm. **(B)** Immunofluorescence staining of iASPP (green) in mouse prostate spheroids. Square shows higher magnification to illustrate detail. Arrow shows nuclear iASPP expression. Nuclei were counterstainied with DAPI. Scale bar - 200µm, 10µm. **(C)** Western blot analysis to confirm the depletion of iASPP protein after 4-OHT treatment.

6.2 Is iASPP a tumour suppressor or oncogene?

The observed effects of iASPP-deficiency on the program of differentiation in the mouse prostate epithelium raises the question as to whether a loss of iASPP function would have a cyto-protective role in cancer development and progression. It would be intriguing to cross the *iASPP*$^{\Delta 8/\Delta 8}$ mice with a prostate tumour-forming mouse strain, such as the *PTEN/p53* knockout mouse, in order to assess the rate of tumour formation. We would hypothesise that these mice might be less likely to form tumours in the first instance, due to *iASPP*'s role as a putative oncogene. However, due to the shift in cell lineage, with a reduction in TP63-positive cells and an increase in Ck19-positive cells, this might not be the case. There is evidence to suggest that the Ck19-positive intermediate cells may also be the cancer-initiating cells due to the fact that they have undergone more rounds of division, and so have a higher probability of incorporating genetic mutations (Schalken and van Leenders, 2003). By carrying out this experiment, the implications of increasing the pool of intermediate cells in a cancer model could also be investigated. One point that is noteworthy is that during development iASPP exerts its affects through the regulation of *p63* and a consequence of iASPP loss is a reduction in TP63-positive cells. Moreover, in prostate cancer TP63 expression is lost. It is therefore a possibility that iASPP$^{-/-}$ mice may be more susceptible to cancer initiation. *iASPP* could be playing a tumour-suppressive role in this setting through its interaction with *p63*. Upon the loss of TP63 function iASPP may contribute to cancer progression through its regulation of *p53*. Inhibiting its ability to drive apoptosis and so taking up its well documented role as an oncogene.

Following the discovery of iASPP in 1999 the main focus of research has been on its role as a putative oncogene and its interaction with TP53. To date a number of studies have

clearly demonstrated that elevated levels of both nuclear and cytoplasmic iASPP are associated with cancer progression and in a number of cases poor clinical outcome (Liu et al., 2012, Jiang et al., 2011). Our study provides evidence that high iASPP expression also plays a detrimental role in prostate cancer progression. However, to add a further level of complexity iASPP was found not just to regulate the p53 family of proteins, but was also unexpectedly identified as a novel tumour suppressor at the cell membrane. This finding is intriguing as it clearly shows that iASPP has a different role depending on its cellular localisation and its protein interactions. This would not be the first time an oncogene has been identified as having tumour suppressive properties, or vice versa. There have been many genes that have been identified as playing a protective and/or a detrimental role depending on cellular context. For example, *NOTCH* has been shown to have an oncogenic function in lymphocytes and mammary tissue, driving aberrant gene expression and subsequent outgrowth of tumorigenic clones. In contrast a growth-suppressive role for *NOTCH* has been described in head and neck squamous cell carcinoma (HNSCC) and in skin tumours through the induction of differentiation and cell cycle arrest (Lobry et al., 2011). This highlights the intriguing dual-role of a single signaling pathway. Indeed, depending on the cellular context, Notch may promote stem cell maintenance or induce terminal differentiation. *P63* has also been identified as having a dual role, for example in HNSCC *p63* is up-regulated, which would suggest an oncogenic function (Rocco et al., 2006). In contrast it has been shown to be lost or reduced in other cancers, such as mammary and prostate adenocarcinomas, which would point to more of a tumour suppressive role for *p63*. *C-myc* has also been shown to have both an oncogenic and a tumour suppressive role in leukemia *C-Myc* stimulates cell proliferation but also regulates apoptosis, senescence, cell competition and cell

differentiation. C-Myc induces transcription by forming heterodimers with Max and then directly binding DNA at E-box sequences. Conversely, transcription repression depends primarily on the inhibitory interaction of c-Myc/Max with Miz-1 at DNA initiator elements. However, recently a distinct mechanism of *c-Myc* gene regulation, in which c-Myc interacts with the retinoic acid receptor α (RARα) and is recruited to RAR DNA binding sequences (RAREs), was described. In leukemia cells this c-Myc/RARα complex functions either as an activator or repressor of RARα-dependent targets through a phosphorylation switch. (Uribesalgo et al., 2012). This finding highlights the impact of post-translational modifications on protein behaviour. Another protein that has been shown to have both oncogenic and tumour suppressive functions within the same cell type is SnoN. In the nucleus, SnoN can bind the Smad complex with transcription factors and repress TGF-β-induced inhibition of proliferation, thus acting as an oncoprotein. Independently of the Smad interactions SnoN can interact with the promyelocytic leukemia protein in PML nuclear bodies, resulting in stabilization of TP53 expression. This leads to premature senescence and defines an anti-oncogenic role for SnoN (Lamouille and Derynck, 2009). Proteins with dual functions are intriguing to study and challenging to target therapeutically. So how do we deal conceptually with a protein that can function both as an oncoprotein and as a tumour suppressor? If the oncogenic and anti-oncogenic functions result from different mechanisms that can be uncoupled, then other components of the pathway could be targeted to prevent the downstream oncogenic interactions from occurring thereby tipping the balance in favour of tumour suppressive activity.

In the case of iASPP it has already been shown that the protein is held in an inactivate state in the cytoplasm as the N-terminus of one iASPP protein binds to the C-terminus of

another, resulting in dimerization and the masking of its nuclear localisation signal. During mitosis iASPP is phosphorylated by the cyclin B1/cdk complex, which in turn unmasks its nuclear localisation signal, allowing its translocation into the nucleus where it can interact with its nuclear binding partners such as TP53 and TP63. (Lu et al., 2013). Therefore, in cancers that have an elevated level of iASPP expression, cyclin B1 could potentially be targeted. Conversely, in the case of membranous iASPP, the mechanisms that control its junctional localisation are still unknown. Whether its' loss at the membrane is a consequence of the down-regulation of other membrane-bound components has yet to be elucidated.

In the future, work needs to be carried out to identify junctional binding partners of iASPP and to assess the dynamics and kinetics of junction formation. In order to do this we plan to use the DU145 cell line to evaluate a panel of junctional markers such as desmoplakin, E-cadherin, β-catenin and the ZO family together with immunofluorescence techniques to establish whether any of these proteins co-localise with iASPP. We also plan to study the formation of junctions in response to calcium-switching. This would reveal the dynamics involved in junction formation, and whether iASPP recruits other proteins, or whether iASPP is recruited by other proteins to the cell junction. Finally, if we are successful in identifying iASPP's membrane bound binding partners, we will assess the implications for its binding proteins when iASPP expression is reduced at the membrane, in order to understand whether its' loss is sufficient to disrupt the formation of its associated junctional complex.

The preliminary data collected throughout this study of the iASPP$^{\Delta 8/\Delta 8}$ mouse prostate, as well as the human prostate tissue sections, has shown some interesting findings. Using *in vitro* and *in vivo* studies in the future these findings can be validated and our hypothesis

regarding the oncogenic role of *iASPP* can be tested. Figure 6.3 illustrates the cancer-related conclusions found in this study along with the associations revealed by studying iASPP expression in its different cellular locations.

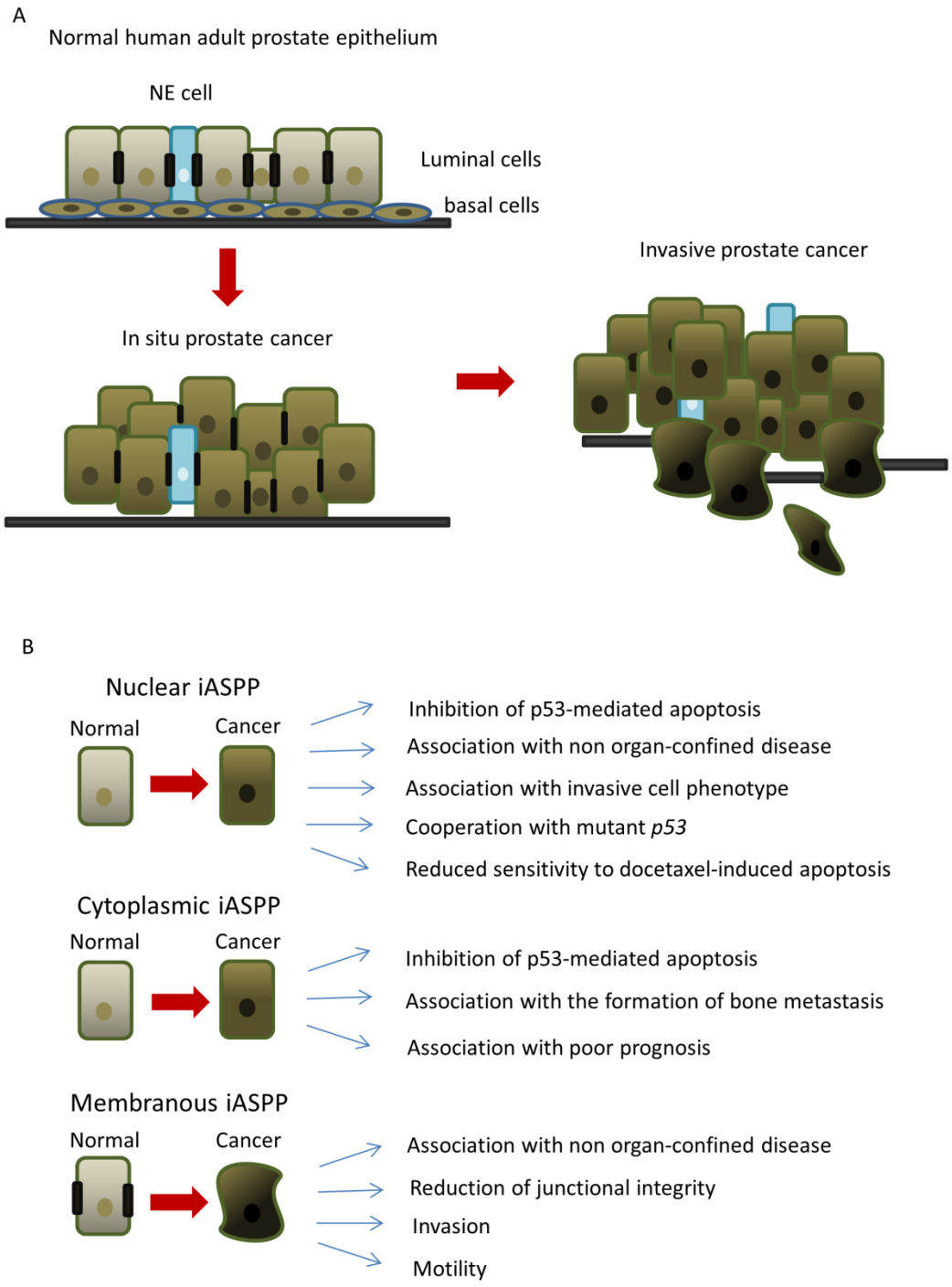

Figure 6.3 A model for the changes in iASPP expression associated with the progression of prostate cancer

(A) In normal prostate epithelium iASPP is expressed in the nucleus, cytoplasm and at the cell membrane (brown). As the development of in situ cancer foci occurs iASPP expression increases in the cytoplasm and the nucleus and this is coupled with a reduction in expression at the membrane. As the cancer progresses to an invasive phenotype, iASPP expression is increased further in both the nucleus and cytoplasm, coupled with the loss of membranous iASPP. **(B)** The outcomes associated with the changes in iASPP localisation in prostate cancer cells.

6.3 iASPP and mutant p53: a consequence or a partnership

To date, iASPP has been implicated as being a co-regulator of wild-type *p53* function. However, its ability to bind and co-regulate mutant *p53* has not been well studied. Numerous reports have demonstrated that many of the hotspot *p53* mutations, such as R175H and R273H, have gain-of-function properties, promoting proliferation, cell survival and invasiveness (Freed-Pastor and Prives, 2012). In this study we provide evidence to implicate iASPP has having a cooperative relationship with mutant TP53. Cells at the leading edge of pT3 tumours were found to often exhibit high levels of TP53 expression. Using consecutive sections we were able to show a reciprocal level of nuclear iASPP expression in the same areas of the tumour as those with high TP53 expression, suggesting some level of interaction. The function of this interaction is still unclear, however we speculate that iASPP enhances the ability of *p53* gain-of-function mutants to direct the transcription of genes associated with cell survival and metastasis (figure 6.4). In support of this hypothesis, DU145 cells grown as organotypic cultures showed an elevated expression of nuclear iASPP in cells with high TP53 expression at the leading edge. To confirm that the mutation in the *p53* gene did not prevent binding between the two proteins, and that the levels of iASPP were not a consequence of a compensatory mechanism to counteract high levels of TP53, resulting in no actual interaction, TP53 was immunoprecipated in order to probe for iASPP. Mutant TP53 was found to bind endogenous iASPP in this cell line. DU145 cells have different mutations of *p53* on each allele, one being the mutation of P223L and the other a mutation of V274F. These mutations have been shown to exert a gain-of-function on TP53 (Gurova et al., 2003). Although, it is noteworthy that iASPP may not bind all mutant TP53 or regulate all *p53*

mutations, this hypothesis has yet to been tested. It may depend directly on the particular *p53* mutation exhibited in the cells.

Immunohistochemical analysis of high TP53 expression in patient samples has been shown to have prognostic significance. Studies have shown that TP53-positivity can predict poor prognosis and the probability of the cancer spreading outside the confines of the prostate capsule (Kudahetti et al., 2009). This could be an invaluable tool as there are few predictive tools currently available in the clinic to predict whether a patient will suffer from non-aggressive or aggressive prostate cancer. However, TP53 expression is not currently used as a diagnostic tool due to a high rate of false-positive results (Schlomm et al., 2008). This study has shown that demonstration of elevated iASPP expression also has prognostic value, and that TP53 and iASPP appear to have a synergistic relationship. Therefore, the combination of both iASPP and TP53 detection could have clinical value.

In the future we plan to express different *p53* mutants in PC3 cells which are *p53*-null, in order to determine whether iASPP preferentially associates with particular *p53* mutations. This study has also provided evidence to indicate that high levels of nuclear iASPP are associated with the invasive properties bestowed on cells which harbor gain-of-function mutations in the *p53* gene. In the future, more work is needed to further understand the advantages imparted on these cells by the up-regulation of nuclear iASPP, and to evaluate any differences in mutant *p53*-driven transcriptional activity in cells with high and low levels of nuclear iASPP expression.

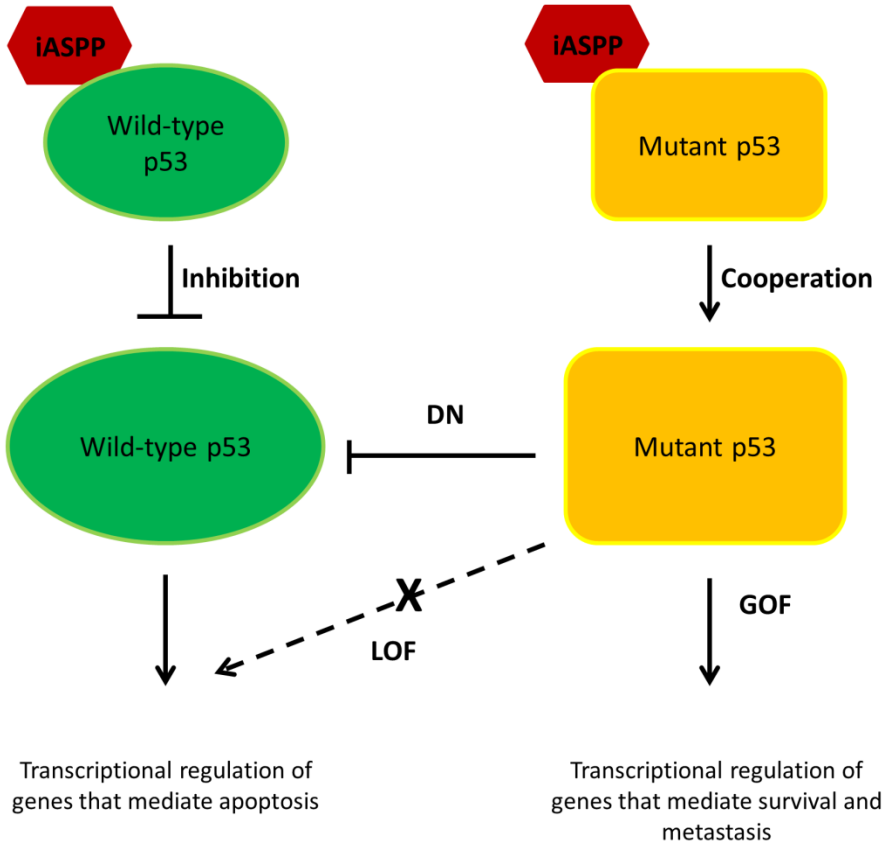

Figure 6.4 iASPP enhances gain of function mutant *p53*

iASPP (red) binds to wild-type TP53 (green), thereby inhibiting the transcription of genes associated with apoptosis. In the context of mutant *p53* (pink), iASPP binds in a cooperative manner, enhancing its gain of function (GOF) properties. Mutant *p53* is also able to act as a dominant-negative (DN) towards wild-type *p53* causing loss of wild-type *p53* function (LOF).

6.4 iASPP: a tiger or a pussycat?

Prostate cancer is one of the major causes of morbidity and mortality in the Western world. Current diagnostic and prognostic markers, such as PSA, clinical/radiological staging and Gleason sum scores, have their own limitations in the wider context of disease treatment and prediction of outcome. One of the main challenges faced by prostate cancer researchers and clinicians is the ability to distinguish between non-aggressive and aggressive localised disease and thereby risk-stratify patients for aggressive treatment. There is therefore a pressing need for novel and powerful biomarkers at the protein level that are associated with aggression, in order to give a clear insight into the clinical course the disease is expected to take. There is also a need to further understand the molecular functions of these proteins in order to develop new targeted therapies. The over-riding question we wanted to address in this thesis, was whether iASPP is a suitable biomarker of aggressive prostate cancer, and if so, would patients benefit from its' inhibition? The evidence presented herein demonstrates the complexity of iASPP function. Nuclear and cytoplasmic iASPP expression is clearly associated with the progression of prostate cancer to a more aggressive phenotype. Interestingly, iASPP was also found to not be androgen responsive, and so consequently androgen ablation therapy would not be expected to influence its' function. This in itself is a fundamental observation, as iASPP may be promoting cell survival and so contributing to androgen-independent disease. If iASPP's role in the cell was to purely regulate the apoptotic activity of the p53-family of genes, it's inhibition would be a definite possibility in order to increase therapy efficacy.

The loss of iASPP function did not have any structural effects on the mouse prostate gland, and so developing an iASPP inhibitor could increase the level of apoptosis without

having a profound effect on normal tissue architecture. However, when iASPP's role at the membrane is factored in, the outcome could be very different. By inhibiting membranous iASPP, cells may favour a more invasive phenotype. It may be that by inhibiting cyclin B1 nuclear iASPP activation could also be reduced, without effecting membrane expression. Caution should however be taken as iASPP is known to have a number of binding partners, and the function of these interactions is still unknown, therefore inhibition of iASPP may have detrimental effects on other signalling pathways.

The last decade has revealed a great deal about the multifaceted iASPP protein and its role in normal embryonic development and cancer progression. Its' increase in expression is observed in many cancer types and clearly has prognostic value in the case of prostate cancer. iASPP is a protein that is able to offer cancer cells the ability to survive while they accumulate enough genetic mutations to allow them to invade through an ever-changing environment. iASPP is evidently an interesting candidate as a prostate cancer biomarker, particularly in combination with TP53. Future work will undoubtedly reveal more functional aspects of this protein, and maybe in time we will see its expression pattern being used as a prognostic tool in the fight against aggressive prostate cancer.

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