WARWICK MEDICAL SCHOOL

The Role of Visfatin in Prostate Cancer

Snehal Patel

Submitted for consideration of MD March 2011

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DECLARATION

I hereby declare that this thesis and the experiments described herein to be solely the work of the author, except where stated. No part of this thesis has been or is being submitted for a degree at any other university.

Snehal Patel March 2011

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ABBREVIATIONS

5-αR-II	5-α reductase type II
AR	androgen receptor
BMI	body mass index
BPH	benign prostatic hypertrophy
BSA	bovine serum albumin
CaP	prostate cancer
CT	computerised tomography
DHT	dihydroxytestosterone
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
ERK	extracellular signal related kinase
ELISA	enzyme linked immunosorbentassay
FBS	fetal bovine serum
H_2O_2	hydrogen peroxide
HNMPA	Hydroxy(2-naphtyl)methyl]phosphonic acid
IGF-R	insulin like growth factor receptor
IgG	immunoglobulin

IL	interleukin
IR	insulin receptor
IRS	insulin receptor substrate
LHRH	leutinising hormone releasing hormone
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
M-MuLV	moloney murine leukemia virus
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
NMN	nicotinamide mononucleotide
P38	protein 38
PBEF	pre B cell colony enhancing factor
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
PSA	prostate specific antigen
PVDF	polyvinylidene difluoride
RIPA	radioimmunoprecipitation lysis buffer
RNA	ribonucleic acid

RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
SHBG	sex hormone binding globulin
TA	transit amplifying
TEMED	N, N, N', N'- tetramethylethylenediamine
TNF-α	tumour necrosis factor alpha
Tris	tris (hydroxymethyl) aminomethane
Tris-EDTA	tris (hydroxymethyl) aminomethane- ethylenediamine tetraacetic acid
Tris-HCl	tris (hydroxymethyl) aminomethane, pH adjusted with HCl
TURP	trans urethral prostatectomy
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization
WHR	waist to hip ratio

ABSTRACT

The aim of this study was to investigate the role of the adipokine visfatin as a possible molecular mediator between obesity and prostate cancer.

Visfatin, an adipokine that is elevated in obesity and has many proposed roles and has been linked to a variety of cancers. No data pertaining to the role of visfatin in prostate cancer existed and this was an area that this study looked to address. It is suggested that obesity is a significant risk factor for prostate cancer; in particular, aggressive disease and adipokines have been investigated as a link for this hypothesis.

This study presents novel data demonstrating the expression of visfatin in the LNCaP and PC3 cell lines as well as in benign and cancerous prostate tissue at both mRNA and protein level. Furthermore visfatin is shown to have functional roles in auto-regulation and promoting increased cell proliferation in PC3 cells and also showed further effects with respect to cell migration across a wound. These data gave promise to develop the study further and evaluate potential mechanisms of action including common second messenger systems such as MAPK and also other oncologically multifunctional molecules in the forms of MMP-2/-9. We then demonstrated that visfatin up-regulated MAPK phosphorylation and MMP mRNA/protein expression and more importantly MMP-2/-9 zymographic activity. This provided possible mechanisms by which visfatin may mediate a role for obesity driven aggressive prostate cancer.

The study then looked to evaluate NMN (the byproduct of visfatin catalysed biosynthetic activity), as well as the visfatin inhibitor FK866 which is being evaluated as chemotherapeutic agent. Unsurprisingly NMN and FK866 had opposing actions on proliferation and FK866 was naturally proapoptotic. NMN was able to rescue the effect of FK866 on PC3 cell apoptosis. Prior studies have shown that NMN did not affect oncogenes however NMN was found to significantly reduce BAX mRNA expression in PC3 cells.

The findings are consistent with other studies linking visfatin with cancer states. These novel data indicate roles for visfatin in prostate cancer and possible mechanisms linking obesity and prostate cancer.

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1 GENERAL INTRODUCTION

THE PROSTATE GLAND

ANATOMY AND HISTOLOGY

The embryological origin of the prostate gland is from the urogenital sinus and its development in foetal life is driven by androgens. It is part of the male reproductive system and its major role involves the synthesis and storage of seminal fluid. Anatomically it is located deep in the pelvis surrounding the proximal urethra at the base of the bladder and is anterior to the rectum. 70% of the gland is primarily composed of glandular elements with the remaining 30% being made of fibromuscular stroma. The fibromuscular stroma is continuous with the prostatic capsule and is composed of collagen and large amounts of smooth muscle (Wein 2007).

Tubuloalveolar glands make up the majority of the glandular element of the prostate and are lined with simple columnar epithelium. McNeal (McNeal 1981) divided the prostate into 3 distinct zones based on the glandular components; location, embryonic origin and differing pathological lesions.

- The transitional zone is endodermal in origin and surrounds the pre-prostatic (proximal) and prostatic (distal) urethra. It accounts for 5-10% of the prostatic glandular tissue and is the region where the majority of benign prostatic hyperplasia (BPH) arises. Approximately 20% of prostate cancer (CaP) arises from this zone.
- The central zone is a cone shaped region arising circumferentially from around the openings of the ejaculatory ducts. Embryologically speaking it is of Wolffian

duct origin and comprises 25% of the prostatic glandular tissue; this area is immunohistochemically distinct from the remaining prostate gland and is relatively resistant to disease contributing to only 1-5% of CaP.

- The peripheral zone makes up the bulk (70%) of the prostatic glandular tissue, covering the posterior and lateral aspects of the gland. This zone is mesodermal with respect to its embryological origin and is the area where the majority of prostatic adenocarcinomas arise.

Each zone consists of ducts and acini lined by an epithelial bi-layer of basal cells, which lies beneath the secretory luminal cells, and mingles with neuro-endocrine cells. Basal cells are typically androgen receptor (AR) negative and consist of self-renewing stem cells that differentiate into transit amplifying (TA) cells, also AR-negative and are of limited proliferative capacity. The TA cells then clonally expand, differentiate and move up from the basal to luminal layer where they again differentiate to form mature AR-positive luminal cells that are non-proliferative (Litvinov, De Marzo et al. 2003; Uzgare, Xu et al. 2004; Uzgare and Isaacs 2005).

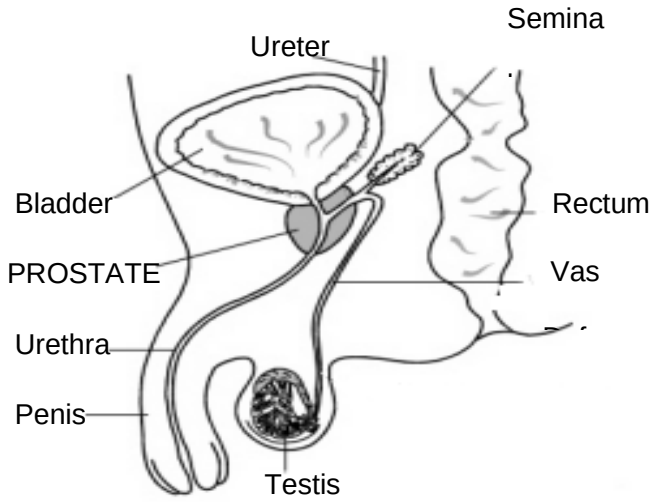

Figure 1.1 Diagram showing the anatomical location of the prostate gland in the human body (Adapted from www.patient.co.uk).

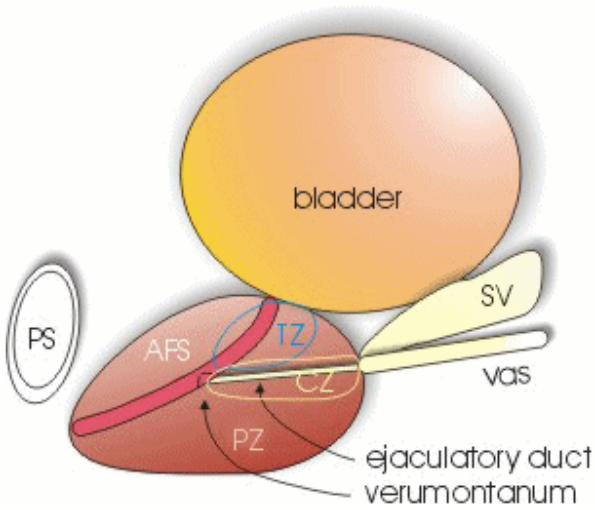

Figure 1.2 Cartoon diagram showing zonal anatomy of the prostate gland (adapted from urologymatch.com). SV- seminal vesicle, PS- pubic symphysis, AFS- anterior fibrous stroma, TZ- transitional zone, CZ- central zone, PZ- peripheral zone

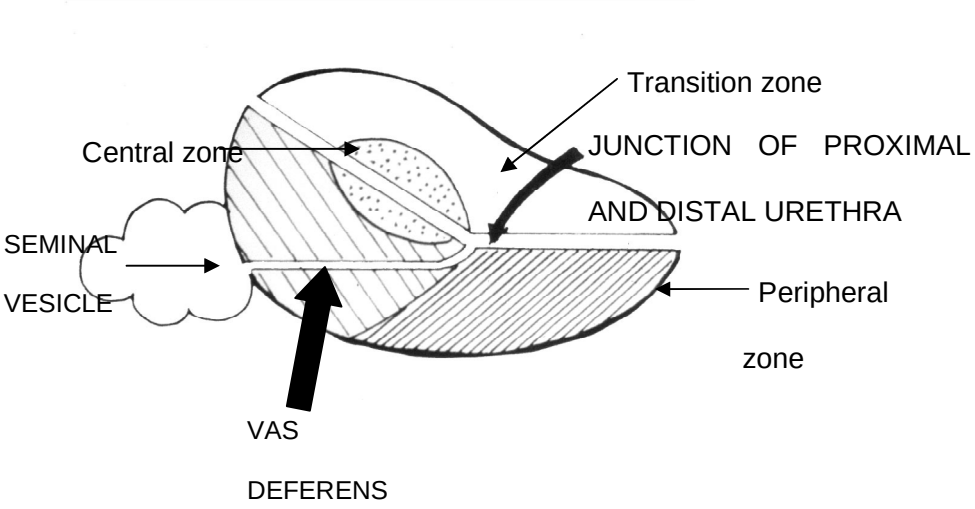

Figure 1.3 Schematic diagram showing the zonal anatomy of the prostate gland (adapted from Lavoipierre (2000)).

NORMAL PHYSIOLOGY

Multiple factors such as steroid hormones, endocrine and peptide growth factors, extracellular matrix factors and epithelial-stromal cell interactions regulate normal prostatic growth. These interactions are complex and still incompletely understood and are still being investigated. The pioneering work by Charles Huggins in the 1930's demonstrating that castration or oestrogen administration resulted in prostate glandular atrophy, which could be reversed by the administration of androgen (Huggins 1940) has laid the foundation for androgen studies in CaP.

ANDROGENS

Testicular Leydig cells are responsible for the production of testosterone under the control of the hypothalamo-pituitary-testicular axis and account for the bulk (95%) of circulating androgens; the remaining 5% of androgens are produced by the adrenal glands and peripheral conversion of oestrogen by aromatase. Testosterone is mostly bound by sex-hormone binding globulins (SHBGs) and circulates in the plasma and

only free testosterone is available for transport into prostate cells by diffusion. Androgens are essential to the normal development of the prostate gland during embryonic development. It is dihydrotestosterone (DHT) which is produced from the metabolic conversion of foetal testosterone by 5α-reductase (located within the urogenital sinus) which drives prostate growth in the foetus.

Testosterone is integral in the post-natal maintenance of normal secretory functions of the prostate gland.

Although testosterone is capable of direct ligand binding to the androgen receptor, 90% is converted to the predominant prostatic isoform, DHT, by 5α reductase type II (5αR-II) within the prostate. In the secretory epithelial cells within which testosterone is able to directly bind via the androgen receptor, 5αR-II does not appear to be present but is present in the basal epithelium and stromal cells. This being said, it does appear that a fraction of the DHT produced by the epithelial and stromal cells is able to diffuse into adjacent secretory epithelial cells and exert a paracrine effect resulting in AR activation. It should be noted that DHT has a much higher affinity for the AR than its precursor testosterone and is therefore a more potent androgen. Androgen binding to the AR results in translocation of the receptor to the cell nucleus where the androgen-AR complex binds to specific DNA binding sites promoting the transcription of various genes and eventually protein synthesis. Therefore, the AR serves a central role in the mediation of androgen responses (Isaacs and Isaacs 2004).

By removing the androgen stimulus from prostatic cells an inactivation of androgen dependent genes results such as prostate specific antigen (PSA) but also key genes involved with apoptosis (Kyprianou and Isaacs 1989). Despite the afore-mentioned importance of androgens for normal prostate development there is no current evidence

that they have direct carcinogenic properties on the prostate despite the fact that androgens are well known to regulate many growth factors and their receptors. In the clinical setting it has been demonstrated that androgen therapy does not lead to an increased risk of CaP (Isbarn, Pinthus et al. 2009).

The traditional paradigm of the androgen receptor pathway as described above, results in a significant delay between ligand binding and the effect. It has however now been shown that androgens can exert a more rapid action via reported membrane receptors that have been detected in CaP tissue (Stathopoulos, Dambaki et al. 2003). Once bound by androgen these membrane receptors cause a rapid induction of calcium mobilisation, resulting in cell signalling pathway activation and alteration in the actin cytoskeleton mediated by PI-3K activation and accompanied by the downstream upregulation of the Rho small GTPases Cdc42, Rac1, RhoA and RhoB (Lieberherr and Grosse 1994; Lyng, Jones et al. 2000; Papakonstanti, Kampa et al. 2003). The same group also showed the greater expression of these androgen membrane receptors in cancerous vs. benign prostate tissue (Stathopoulos, Dambaki et al. 2003). The activation of the membrane receptors has also been shown to have further effects pertaining to apoptosis induction and concomitant reduction in cell proliferation as well as cell motility and adhesion (Hatzoglou, Kampa et al. 2005); the effects observed as a result of activation of these membrane receptors appear to be independent of the nuclear androgen receptor in both nuclear androgen receptor positive and negative cell lines.

On review of the literature membrane androgen receptors have been identified and it is suggested that they play a significant role in apoptotic control via modulation of the actin cytoskeleton and downstream pathways. The physiological role in CaP remains

unclear however the experimental response to testosterone show significant potential for a clinical use based on the following:

- membrane AR is selectively over-expressed in high grade CaP compared to samples benign prostate hyperplasia patients or healthy subjects (Stathopoulos, Dambaki et al. 2003).
- membrane AR ligands (testosterone-BSA conjugates) induced apoptosis of prostate cancer cells in vitro and in vivo (Hatzoglou, Kampa et al. 2005; Kampa, Kogia et al. 2006).
- membrane AR induced apoptosis is independent of nuclear AR status and is still ongoing in the presence of anti-androgens (Hatzoglou, Kampa et al. 2005; Kampa, Kogia et al. 2006).
- Lastly, membrane AR activation potentiates paclitaxel action in hormone sensitive and refractory cell lines and derived xenografts in animals (Kampa, Kogia et al. 2006)

These rapid acting membrane receptors are still the subject of much investigation however further investigation will prove important as they may provide an angle for future CaP treatment targets.

STROMAL-EPITHELIAL INTERACTIONS

Fibromuscular stroma within the prostate is made up of AR-positive smooth muscle cells and fibroblasts. When stimulated by androgens they release various growth factors including basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), transforming growth factor-β (TGF- β) and insulin-like growth factor (IGF) that exert paracrine effects on neighbouring epithelial cells. The above-mentioned proteins bind to their receptors on the TA cells and are participants in cell cycle regulation. The survival

of the AR-positive luminal cells is still dependent on these factors although they do not undergo proliferation in response to androgen-stimulated growth factors. Binding of androgens to the AR in the luminal cells, however, suppresses their proliferation and instead induces differentiation thus regulating their production of various secretory proteins including PSA (Litvinov, De Marzo et al. 2003; Uzgare, Xu et al. 2004; Uzgare and Isaacs 2005).

CARCINOMA OF THE PROSTATE (CAP)

EPIDEMIOLOGY

CaP is the commonest non-cutaneous malignancy in men and the second leading cause of cancer-related death worldwide, accounting for almost 10% of all cancers in men (Parkin, Bray et al. 2001). From the year 2000, CaP became the most frequently diagnosed cancer in men in the U.K, with over 24,000 new diagnoses, with the cancer specific mortality rate reaching 35 per 100,000 in 2001 (Melia 2005). There are marked differences between the incidence of clinically significant disease worldwide however incidental non-clinically relevant disease is still equal. Canada has one of the highest incidence rates and this follows with many western countries including the USA and UK. There are even significant variations between northern and southern countries within Europe, with CaP incidence and mortality being highest in England and Wales, and lowest in Mediterranean countries (Quinn and Babb 2002). The incidences of CaP are increasing, across the world including countries like Japan, China and India, which historically have reported the lowest rates (Melia 2005).

The increased incidence amongst the developed countries is attributable to several factors. The routine examination of transurethral prostatectomy (TURP) histology specimens with improved techniques and rationalised sampling is one such reason and

another being the longer life expectancy of men. The use of serum analysis for PSA as a marker for CaP has also a role to play in the increased incidence particularly in the more developed countries and this is being heavily studied at present with major studies both in Europe and North America (Eckersberger, Finkelstein et al. 2009). Despite our improved ability to detect CaP in the context of an aging population the global increases in CaP incidence suggests that environmental factors are contributing to the pathogenesis of this disease and one cannot simply attribute increased incidence to increased detection.

AETIOLOGY

The specific causes of CaP are unknown. There are many established risk factors associated with the development of cancers and CaP is no exception; age, ethnicity and family history/genetic factors are al implicated. However, there is increasing evidence for the role of other factors such as diet, lifestyle, endogenous hormone profile and obesity in the aetiology of the CaP (Venkateswaran and Klotz 2010)

AGE

CaP is a disease that increases in prevalence with old age. Over 75% of new cases are diagnosed in men over the age of 65 years. Gann (Gann 2002) has studied age-specific incidence and shown that CaP risk begins to rise sharply after the age of 55 and peaks at age 70-74 years. It should be noted that there has been a recent increase in the incidence within the age group of 50-59 and it can be speculated that this rise may well be secondary to the increased use of PSA as an opportunistic screening tool.

ETHNICITY

The highest incidence of CaP is within African American men in the U.S.A. This group has a 60% increased risk of developing CaP, a 2 fold increased risk of developing

distant disease and twice the mortality relative to Caucasians (Hoffman, Gilliland et al. 2001; Jemal, Murray et al. 2003). Countries such as Japan and China have relatively low rates of CaP within the native population. The reason for this is multi-factorial and the genetic role as well as diet and socioeconomic status have been put forward.

GENETIC FACTORS

Less than 10% of all CaPs are hereditary. The two main characteristics of hereditary CaP are young age of onset, multiple affected first degree relatives and the increased risk is thought to be due to autosomal dominance of rare but highly penetrant genes (Carter, Beaty et al. 1992). Genes such as HPC1, HPCX, and HPC2/ELAC2 have been identified as hereditary CaP genes and they have been found to have genetic locus heterogenecity making them a difficult topic to study.

There are also several low-penetrance genes which can not only alter the way individuals respond to both environmental factors but can also interact with other genes. Two examples of this include vitamin D polymorphisms and 5α-reductase type 2 gene polymorphisms. Both are associated with increased CaP risk and the latter may allow a greater conversion of testosterone to DHT by enhancing the degree of enzymatic activity within the prostate.

ENDOGENOUS HORMONE PROFILE

Higher levels of circulating androgen levels in African-American men as compared to white men (Ross, Bernstein et al. 1986), have been suggested as the cause for their higher incidence of CaP based on the fact that CaP is dependent on androgen growth. Although this would make a compelling hypothesis, there is still much debate about the relationship between circulating androgens and intra-prostatic androgen levels.

IGF-1 levels, which have been shown to have mitogenic and anti-apoptotic effects, are positively correlated with CaP risk (Chan, Stampfer et al. 1998).

DIET

Diet has been long postulated as a risk factor for CaP and especially given the geographical variation in incidence in CaP. One of the best examples of dietary difference is the high calorie and saturated fat diet found in the West. In vitro studies have shown that high saturated fat levels are stimulatory to prostate cell growth compared to a slower growth of androgen sensitive cells that are exposed to low levels of saturated fat (Wang, Corr et al. 1995; Pandalai, Pilat et al. 1996; Ngo, Barnard et al. 2004). Cohort studies in the most part have shown no significant association between dietary fat intake and CaP (Mills, Beeson et al. 1989; Hsing, McLaughlin et al. 1990; Schuurman, van den Brandt et al. 1999), some, however, have found a link between animal fat consumption and CaP risk (Gann, Hennekens et al. 1994; Le Marchand, Kolonel et al. 1994). Postulated mechanisms by which dietary fat may be implicated in CaP are: alteration of serum androgens, IGF-1 levels and free radical or pro-inflammatory fatty acid metabolite production (Sonn, Aronson et al. 2005).

The differences in the dietary consumption of soy products particularly in Asian as compared to Western countries may explain the significant difference in CaP incidence between these geographical areas. Soy contains high levels of phyto-oestrogens (isoflavones) which have weak oestrogenic activity which are suggested to have anti-carcinogenic properties (Adlercreutz 2002).

Elevated dietary calcium has been suggested as a risk factor for CaP (Chan, Giovannucci et al. 1998; Giovannucci, Rimm et al. 1998). The elevated calcium levels

are thought to suppress the levels of vitamin D metabolite 1,25-dihydroxy vitamin D which has anti-proliferative effects (Barreto, Schwartz et al. 2000).

TUMOURIGENESIS: CELL PROLIFERATION, THE CELL CYCLE, APOPTOSIS AND FACTORS CONTROLLING THEIR REGULATION (BCL-2, BAX, MCL-1, BCL2-L1, IGF-R)

Tumourigenesis is a product of dysregulated or deregulated cellular proliferation. This deregulation is the complex product of several successive genetic events that gradually lead to tumour formation. There is a delicate balance between growth factors and apoptotic agents in the regulation of prostate growth (Reynolds and Kyprianou 2006). It is disruption at this level which leads to the loss of apoptosis and the over-expression of factors promoting cell survival and proliferation and subsequent tumour development.

Once cell proliferation is uncontrolled, prostate cancer progression is associated with a variety of growth factors and their respective signaling pathways (Djakiew 2000). Growth factors can be categorized into three distinct groups: traditional growth factors i.e. those that increase cell proliferation e.g. Insulin-like growth factor-1 (IGF-1); negative growth factors i.e. those which inhibit cell proliferation/induce apoptosis e.g. transforming growth factor-β (TGF-β), and angiogenic factors e.g. fibroblast growth factor (FGF).

The human cell cycle broadly consists of four stages: The first three stages combined can be grouped together under the umbrella term of ‘interphase’. This encompasses G1 (gap 1) i.e. the phase when the cell prepares to duplicate its DNA in preparation for cell division; S-phase (synthesis phase) i.e. when the cell duplicates its DNA; G2 (gap 2) i.e. when the cell prepares to divide; The final phase is M-phase during which the cell actually divides and undergoes mitosis; cells that are quiescent are said to be in the G0

phase (Garrett 2001). Disruption of the cell cycle inhibits potential apoptosis which normally takes place if the cycle is arrested (Norbury and Zhivotovsky 2004). This disruption can allow mutations to be transferred to the daughter cells.

2 potential points of control in the cell cycle which are the G1S and G2M check points, and, can be controlled by a sequence of various positive and negative proteins (Garrett 2001). As well as understanding the cell cycle, it is important to evaluate the apoptotic mechanisms as it is another key process which can be altered resulting in tumourogenesis.

APOPTOSIS AND ITS REGULATION

Apoptosis relates to programmed cell death. Apoptosis can be triggered by a multitude of stimuli either intrinsic or extrinsic to the cell, e.g. by ligation of cell surface receptors, by DNA damage, treatment with cytotoxic drugs, or by developmental death signals (Norbury and Zhivotovsky 2004). These diverse signals appear to eventually activate a common cell death pathway leading to programmed cell death or apoptosis.

One of the mechanisms of extrinsic induced apoptosis is the activation of transmembrane death receptors for the ligands of CD95/tumor necrosis factor family via multiprotein complexes and activation of the cysteine aspartyl proteases (caspases) cascade (Ashkenazi and Dixit 1998).

The mitochondria can not only amplify the above process from extrinsic stimuli but also initiate or propagate the death signals intrinsically from stimuli such as DNA damage, oxidative stress and starvation (Kaufmann and Earnshaw 2000). The majority of mechanisms involve the disruption of the mitochondrial inner transmembrane potential (Dy) as well as the so called permeability transition (PT), this in turn leads to the opening of the permeability transition pore (PTP) complex which is located in the

mitochondrial membrane. This also triggers the corresponding release of apoptogenic proteins such as cytochrome c and apoptosis inducing factor (AIF) which can in turn activate caspases. The release of caspases and cytochrome c from the mitochondrial intermembrane space into the cytosol after PTP formation is a crucial event inducing apoptotic cell death (Loeffler and Kroemer 2000). Alternatively mechanisms involving factors released from mitochondria can lead to programmed cell death (Green and Reed 1998).

A distinct evolutionary advantage is gained with apoptosis only allowing undamaged cells to be replicated and is normally triggered when the cells are unable to arrest the cell cycle or institute DNA repair. However, aberrant apoptotic regulatory mechanisms that allow damaged cells to continue replicating are a hallmark of cancer development and progression.

A key apoptotic regulator is TGF-β. In the normal prostate, TGF-β has been shown to inhibit epithelial cell proliferation and promote apoptosis i.e. acts like a tumour suppressor (Bello-DeOcampo and Tindall 2003)..

TGF-β has paracrine like activity upon its release from prostatic stromal cells leading to the inhibition of prostatic cell growth via apoptosis (Wu, Sun et al. 2001; Bhowmick, Coad et al. 2004). TGF-β stimulates a multiple downstream targets – all of which have antiproliferative or apoptotic effects (Reynolds and Kyprianou 2006). TGF-β receptor activation leads to the transcription of BAX, a proapoptotic factor and deactivation of BCL-2, (see below). Thus inhibition of this negative growth factor could lead to tumourigenesis.

Two further examples of classic apoptotic regulators are p53 and BCL-2, and these have been proved to have abnormal function and expression in prostate cancer

progression and the subsequent progression to hormone resistance (Quinn, Henshall et al. 2005).

Tumour suppressor genes and oncogenes are both involved in manipulating the above processes. Tumour suppressor genes are normal genes that allow the cell to enter G0 phase and allow DNA repair, or arrest the cell cycle and carry out apoptosis. Mutations or inactivation in tumour suppressor genes allow development of tumours. Several different tumour suppressor genes have been identified and some of them include p53, BRCA1, BRCA2 (Oesterreich and Fuqua 1999)

An key difference between oncogenes and tumour suppressor genes is that oncogenes result from the *activation* (turning on) of proto-oncogenes. Oncogenes encode proteins that can affect cell proliferations and apoptosis or potentially both.

The BCL family has been previously documented to be involved with prostate carcinogenesis.

BCL-2

The BCL-2 gene family encodes a group of proteins directly involved in regulating apoptosis. They can be either proapoptotic (BCL-2, BCL2-L1 MCL-1) or antiapoptotic (BAX, BAK, BCL-xS, BAD, BID) (Adams and Cory 1998). Reed demonstrated that it was the selective and competitive dimerisation between pairs of pro- and antiapoptotic family members that inferred how the cell will behave following stimuli (Reed 1994).

BCL-2 is not expressed in the normal prostate however the BCL family of genes are commonly expressed in both primary and metastatic CaP (Hughes, Murphy et al. 2005). BCL-2 is one of the oncogenes implicated in the progression to the androgen-independent CaP as well as high grade disease (McDonnell, Troncoso et al. 1992). The adipokine adiponectin has been shown to have anti-apoptotic and cytoprotective effects

on human neuroblastoma cells via regulation of *BCL-2* (and *BAX*) expression (Jung, Lee et al. 2006; Jung, Lee et al. 2006). It is therefore of note that adipokines can have varying effects on BCL-2 expression and by inference oncogene manipulation depending on the cell type. It has also been discovered that adiponectin and leptin interact to modulate the oncogenes p53 and BCL-2 and hence proliferation in PC3 cell lines (Mistry, Digby et al. 2008). Further proof of this concept is noted in that leptin alters the BCL-2:BAX ratio in pancreatic beta cells and thus inhibits apoptosis (Brown and Dunmore 2007). For this reason I sought to identify if visfatin affects multiple members of the BCL-2 family. Multiple oncogenes are continually being discovered.

Many growth factors have been described. Below I use IGF-1 to illustrate an example of growth factors influence on cellular proliferation.

One of the most commonly reported changes associated with CaP are the changes in endocrine growth factor loops. In the advanced stages of prostate cancer, insulin-like growth factor-1 receptor (IGF-1R), and other GFRs have been frequently shown to be over-expressed, and an interaction of these receptors has been increasingly elucidated (Papatsoris, Karamouzis et al. 2005). IGF-1R has also been reported as a novel oncogene in recent times (Werner and Bruchim 2009).

Low levels of IGF-1 production can be demonstrated in nearly all cells (Reynolds and Kyprianou 2006). IGF-1 ligand binding to its corresponding receptor activates signaling pathways that contribute to nearly 50% of cell growth and proliferation (Baserga, Peruzzi et al. 2003).

Androgen stimulation leads to IGF-1 production with a resultant paracrine effect on the surrounding prostatic epithelial cells, leading to increased proliferation (Moschos and Mantzoros 2002; Bogdanos, Karamanolakis et al. 2003). In healthy cells, the IGF-1

pathway is regulated by IGF binding proteins (IGFBPs) (Grimberg and Cohen 2000). The downstream targets of the IGF-1 signaling axis ultimately promote cell survival.

A positive correlation between plasma IGF-1 levels and CaP progression has drawn the conclusion that IGF-1 is an aetiological factor in CaP (Stattin, Rinaldi et al. 2004)

MITOGEN-ACTIVATED PROTEIN KINASES (MAPK) ERK 1/2 AND P38

As summarized in Figure 1.4 a multitude of growth factors, cytokines, and proto-oncogene products impart stimuli through the activation of the small G protein Ras and subsequently of the MAPK network which result in large proportion of key cellular operations for example proliferation, apoptosis and differentiation. Three of the major MAPK include the extracellular-signal regulated kinase (ERK; p42/44 MAPK), p38 MAPK and c-jun N-terminal kinase (JNK; stress activated protein kinase-1 SAPK1).

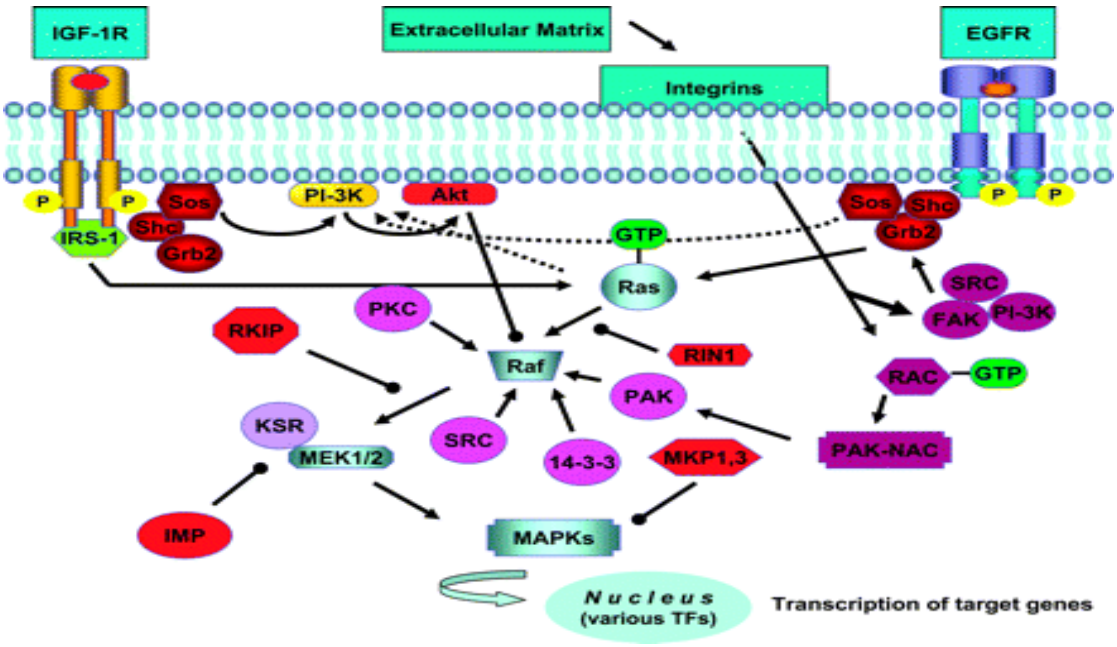

Figure 1.4 Diagram showing the potential second messenger pathways involved. From Papatsoris 2007, (Papatsoris, Karamouzis et al. 2007)

One key observation is that MAPK activity has been demonstrated to be implicated with progression of disease to the more aggressive and advanced hormone-independent state (Uzgare and Isaacs 2004; Oka, Chatani et al. 2005). Importantly, it has been reported that the effect of phosphorylated ERK (pERK) on prostate cancer cells is to enhance proliferation or mitogenesis and is therefore inversely related to apoptosis (Cross TG 2002). It is also possible that the relative activation of ERK1 and ERK2 can have differing effects on prostate carcinogenesis. These observations highlight the importance of ERK activation in this model of disease.

p38 MAPK is mainly activated via external stimuli which in this case would be represented by exogenous visfatin and has been broadly associated with induction of apoptosis (Cross TG 2002). There is however also evidence that activation of p38 may cause increases in cell proliferation in prostate cancer in response to external stimuli, and that its over-expression is implicated in increased cell proliferation (Ricote, Garcia-Tunon et al. 2006). It has been suggested that it is the ratio of p-ERK to p-p38 MAPK that may be useful in identifying the action of CaP or other solid tumours in vivo (Aguirre-Ghiso, Estrada et al. 2003).

ANGIOGENESIS AND DISEASE PROGRESSION

Angiogenesis is the process of developing new blood vessels and is interplay between a variety of activators and inhibitors. Angiogenesis thought to be driven by a hypoxic stimuli and this leads to activation of endothelial-cell proliferation and migration via tyrosine kinase ligands (Carmeliet 2000) e.g. vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). These are counterbalanced by the inhibitors endostatin and angiostatin. Overall it is a balance between the activators and inhibitors of angiogenesis that dictate whether an endothelial cell will become angiogenic.

It has been shown that CaP express VEGF-A and this is a significant mechanism in the disease progression (Yu, Jain et al. 2010). Along with the balance of angiogenic factors and neovascularisation allowing nutrient delivery to invading tumour, the metastatic potential of a tumour is also dependent on features such as degradation of extracellular matrix. Matrix metalloproteinases are a group of agents that are involved in the degradation of the basement membranes. A positive correlation between tumour progression and the expression of the MMPs in tumours has been demonstrated human and animal studies (Stetler-Stevenson 1996; Ellerbroek and Stack 1999). The activity of MMPs is regulated by natural inhibitors called tissue inhibitors of matrix metalloproteinases (TIMP). MMP-2 is inhibited by TIMP-2/-4, while MMP-9 is inhibited by TIMP-1 (Schaefer, Han et al. 1997; Bee, Barnes et al. 2000). An imbalance between these two groups of proteins has been shown to be linked with a variety of different tumours, including prostate (Boag and Young 1994).

MATRIX METALLOPROTEINASES (MMPS)

Matrix metalloproteinases in particular MMP-2 and -9 (gelatinases) are proteases that are involved in the breakdown of the extracellular matrix (ECM) under both physiological and pathological conditions (Saarialho-Kere, Chang et al. 1992; Adya, Tan et al. 2008). More specifically, they degrade type IV collagen, the major structural component of basement membranes and are crucial to the process of cell migration, invasion and metastasis. Both MMP -2 and -9 are known to be highly expressed in prostate cancer tissue (Wilson, Gallagher et al. 2004) and circulating levels of MMP-2 and -9 have also been correlated with bone metastases and tumour grade in prostate cancer patients (Incorvaia, Badalamenti et al. 2007). A dysregulation of MMP biology can lead to a wide range of pathologies including tumour neovascularisation, aneurysms, atherosclerosis, rheumatoid arthritis etc (Woessner 1998).

Dysregulation of MMP activity can have a role in cancer development and progression but it is thought that early expression in a tumour related environment is involved in ECM remodelling which in turn leads to the release of ECM along with membrane-bound growth factors to alter the environment in a fashion which allows the tumour to thrive. MMP-2 and MMP-9, are also known to be key in the regulation of angiogenesis (van Hinsbergh, Engelse et al. 2006), which is a well known component of tumour progression and metastasis. This angiogenic process can arise early prior to any malignant transformation, and has been demonstrated by increased vessel density seen in precancerous lesions (Hanahan and Folkman 1996). The gelatinases have been shown to also activate growth factors, cytokines, and degradation/activation of inhibitors of angiogenesis. For example, MMP-9 can cause the onset of tumour angiogenesis as well as its oppression via the same proteolytic process (Hamano, Zeisberg et al. 2003). In conjunction with this process the gelatinases, which as mentioned above can degrade basement membrane components, allow tumor cells to invade into surrounding tissue. MMPs can contribute to the tumour advancement in several ways and at a variety of stages; they can remove sites of adhesion and aid cell migration, expose novel binding sites, and can directly release chemo-attractants from the ECM (McCawley and Matrisian 2001).

Visfatin has been demonstrated to up-regulate MMP activity in other systems (Adya, Tan et al. 2008). MMPs are upregulated by various stimuli, one of which includes vascular endothelial growth factor (VEGF) (Wang and Keiser 1998). VEGF, a homodimeric glycoprotein, plays an important role in vasculogenesis (Wang and Keiser 1998) and thus an important part of neoplastic growth in supplying oxygen to enlarging solid tumours. With the aforementioned, this study sought to study the possible interplay between visfatin and the pro-angiogenic MMP molecules.

PATHOGENESIS

95% of CaP are adenocarcinomas arising from either the prostatic ductal or acinar epithelial cells. The remaining 5% of cancers include sarcomas and local invasion by transitional cell carcinoma (TCC) of the bladder. 70% of cancers arise in the peripheral zone with 25% arise from the transitional zone and the remainder in the central zone (McNeal, Redwine et al. 1988); CaP is a multifocal disease with more than 85% of prostate adenocarcinomas demonstrating this characteristic. CaP is graded using the Gleason scoring system (Gleason 1966). The Gleason grading system uses a H&E-stain to describe the histological pattern of carcinoma cells. Specifically, the method uses low magnification and characterizes the extent of glandular differentiation and the pattern of growth of the tumor in the prostatic stroma. Five grades were described and these were illustrated in a drawing by Dr Gleason (Figure 1.5). The grading system generates a score from 2 to 10. This is calculated by observing the most common pattern i.e. the primary grade pattern and adding this to the second most common pattern i.e. the secondary grade pattern.

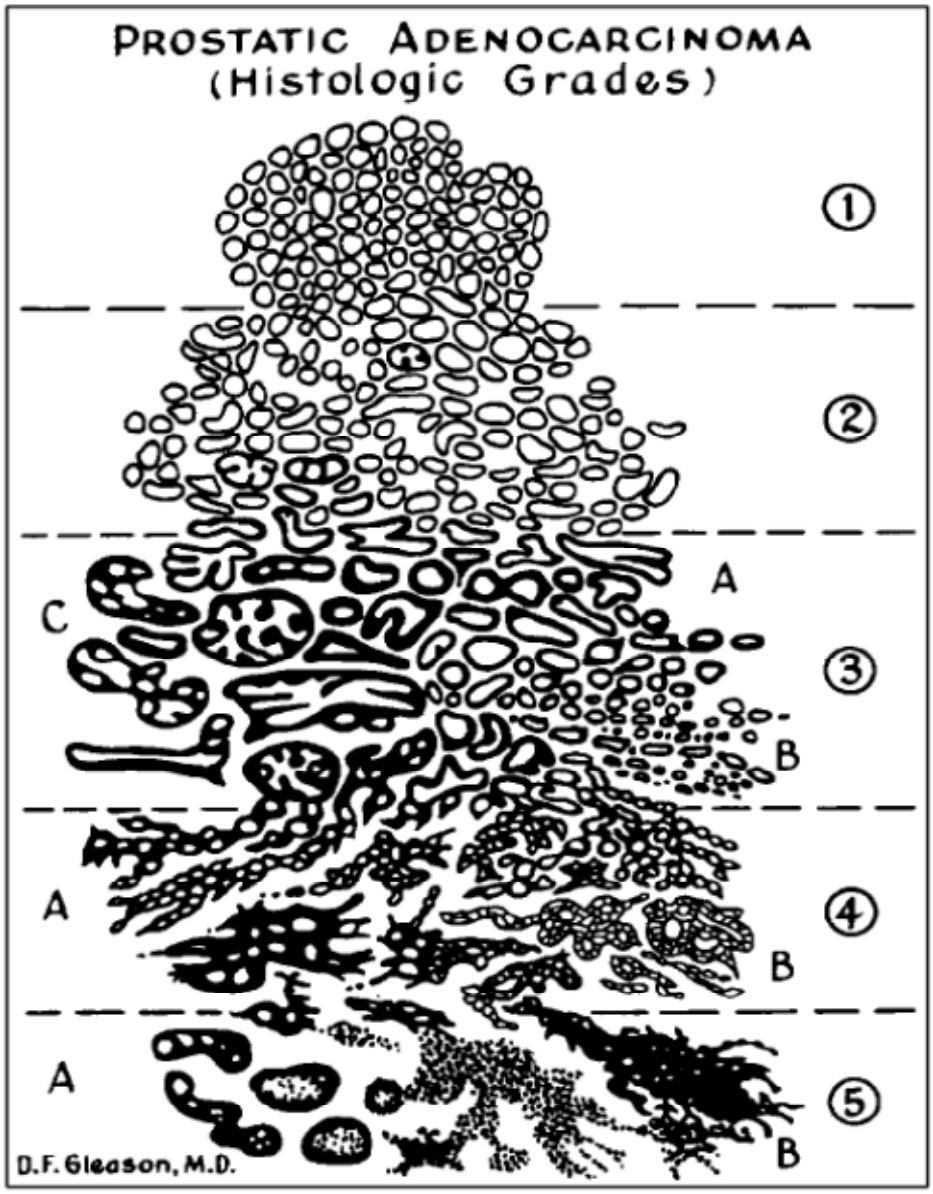

Figure 1.5 Gleason Grading system diagram as drawn by Gleason (Gleason 1966)

The prostate gland has no histological capsule and CaP spread is initially along the course of the autonomic nerves and this is classed as perineural invasion. Local spread can involve any of the surrounding structures (fig 1.1) such as the seminal vesicles, prostatic urethra, corpora of the penis and trigone of the bladder although there is also lymphatic and haematogenous spread to the axial skeleton.

The multistep or multi-hit hypothesis is a well documented process by which a normal prostate cell differentiates into a cancerous phenotype. These steps result in the production of several autocrine growth factors by the CaP cells via AR signalling (Gao, Arnold et al. 2001). Thus, androgens act in an autocrine manner regulating proliferation and survival in androgen-sensitive CaP cells (Litvinov, De Marzo et al. 2003), conversely in healthy normal prostate tissue there is a paracrine, stromal cell dependent androgen pathway (Uzgare and Isaacs 2005). This androgen response was the basis for Huggins theory of castration as method of treatment in prostate cancer (Huggins 1941).

Following the treatment of CaP with anti-androgen therapy all tumours will inevitably become hormone refractory tumours or androgen-independent CaP. This phenomenon is hypothesized to occur through the mutations to independent cell clones as opposed to de-differentiation of the cells. This mechanism has not been elucidated to date but a progression of the multi-hit hypothesis leading to the accumulation of several mutations that confer advantage to this androgen insensitive cell type has been suggested (Uzgare and Isaacs 2005). Frequent genetic changes associated with CaP include somatic loss of alleles on chromosomes 8, 16 and 18; inactivation of PTEN and p53 tumour suppressor genes (chromosomes 10q and 17p, respectively); activation of c-myc and bcl-2 proto-oncogenes.

TREATMENT

Treatment for CaP has evolved over the years and varies dependent on the stage, grade and physiological status of the patient concerned. Local low grade disease can be managed by ‘Active Surveillance’ protocols as well as other more aggressive strategies such as radical prostatectomy (healthy men with a life expectancy of more

than 10 years), external beam radiotherapy, brachytherapy, cryotherapy and high-intensity focused ultrasound (HIFU).

Once CaP has progressed locally or a patient has distant metastasis, whereby treatments such as surgery or radiotherapy can no longer offer a curative option, androgen deprivation becomes the next line of treatment.

The genesis of androgen ablation therapy was primarily thanks to work done by Huggins and Hodges in 1941 when they reported a good response to castration or oestrogen therapy with respect to acid and alkaline phosphatase levels (Huggins 1941). There are several methods that can be employed to cause androgen deprivation ranging from surgical castration to medical hormone manipulation using; LHRH agonists, antiandrogens as well as 5-α reductase inhibitors. Medical manipulation of androgen status results in a reduction of PSA and clinical improvement in more than 70% of patients however due to the often advanced stage of disease in this cohort of patients and progression to the 'androgen independent' growth phase the majority of patients will die within 5 years (Group 2000).

Treatment options for patients reaching this stage of the disease are limited to palliative as well as cytotoxic options and other limited benefit therapy.

OBESITY AND CAP

Obesity, the most significant and growing public health concern, has reached epidemic proportions with a prevalence of 60% or more in some Western societies (Simon 2005). Obesity is well established as a risk factor for type 2 diabetes, cardiovascular disease and cancer. More specifically, visceral obesity is associated with insulin resistance, hyperinsulinaemia and prothrombotic/proinflammatory states as well as cancer.

(Kershaw and Flier 2004). The state of obesity and metabolic dysregulation and its associated conditions; type 2 diabetes, dyslipidaemia, atherosclerosis and others, is termed ‘metabolic syndrome’ (DeFronzo and Ferrannini 1991). Metabolic syndrome is thought to be becoming endemic in populations with a ‘western’ diet and lifestyle, insulin resistance appears to play a central role but its mechanisms are still not fully understood (Ford, Giles et al. 2002).

Multiple studies have been undertaken to try and identify not only if obesity is a risk factor for prostate cancer but also to try and identify whether it plays a specific role in disease progression. With respect to the association between obesity and CaP various studies have been performed. The results for the most part have been inconsistent (Ngo, Barnard et al. 2004) and this may be due to the varying criteria that are used between studies (for example body mass index (BMI) vs. waist to hip ratio (WHR)) . von Hafe et al introduced the concept that visceral fat accumulation as identified on CT scan is a specific risk factor for CaP (von Hafe, Pina et al. 2004).

Both tumour aggressiveness and mortality from prostate cancer correlate positively with body mass index (BMI) (Andersson, Wolk et al. 1997; Calle, Rodriguez et al. 2003). Several studies have also now identified obesity and in particular visceral obesity as a risk factor for prostate cancer (von Hafe, Pina et al. 2004; Freedland, Wen et al. 2008) with evidence suggesting that obesity increases the aggressiveness of the disease (Freedland, Giovannucci et al. 2006; Freedland and Platz 2007). It is therefore reasonably hypothesised that instead of an absolute increase in prostate cancer risk obesity is a risk factor for progression of the so termed latent or clinically insignificant disease group to clinically significant and metastatic CaP (Yip, Heber et al. 1999; Stattin, Soderberg et al. 2001). A meta-analysis has demonstrated a significant positive

correlation with an increased BMI finding a 5% increased risk of prostate cancer per 5 unit rise in BMI (MacInnis and English 2006).

Obesity and the metabolic syndrome have been linked as risk factors to various cancers which include in particular hormone-dependent cancers such as breast, endometrial and prostate (Kelesidis, Kelesidis et al. 2006). There are multiple hypothesized mechanisms for these associations.

One such mechanism is that obesity influences sex steroids, insulin and IGF-1 pathways which are all implicated in the cancer biology (Kaaks and Lukanova 2001). There are multiple reports on the inverse relationship between serum insulin and steroid hormone binding globulin (SHBG) levels (Plymate, Hoop et al. 1990; Strain, Zumoff et al. 1994; Pasquali, Casimirri et al. 1995; Katsuki, Sumida et al. 1996), and further more it has been demonstrated in hepatoma cells that insulin inhibits SHBG production (Plymate, Matej et al. 1988). With a reduction in serum SHBG there is an increase in the level of unbound and therefore active androgen and subsequent risk in the development of androgen dependent cancers. With regards to the risk of CaP, an increase in peripheral aromatization of androgens to oestrogens may also be a contributory factor to this association. Chronic inflammation is also associated with obesity and there is an increase in the levels of acute phase reactants e.g. tumour necrosis factor alpha (TNF-α) and activation of pro-inflammatory signalling (Weisberg, McCann et al. 2003; Wellen and Hotamisligil 2003). It is well documented in the literature that chronic inflammation of the prostate is a risk factor for CaP and thus adds a further possible link between obesity and CaP (Palapattu, Sutcliffe et al. 2005; MacLennan, Eisenberg et al. 2006).

White adipose tissue accumulation is a key step in the development of obesity and has now been well documented as a metabolically active organ rather than a simple store for energy (Kershaw and Flier 2004). Not only is adipose tissue a site for the metabolism of androgens but is also the site for the production of multiple biologically active peptides now termed 'Adipokines'. (Matsuzawa, Funahashi et al. 1999). Although termed adipokines these peptides are produced by numerous other cell types in addition to adipocytes such as stromal cells, macrophages and connective tissue cells, which may also contribute to adipose tissue mass (Frayn, Karpe et al. 2003; Fain, Madan et al. 2004). They may function at local (autocrine/paracrine) and systemic (endocrine) levels, and act as regulators of energy homeostasis as well as contributing to inflammatory and immune processes. Adiponectin and leptin are almost exclusively produced by adipocytes and are the most abundantly circulating adipokines; others include cytokines and growth factors such as tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF) and visfatin/PBEF (Wozniak, Gee et al. 2009).

Serum adipokines have been positively correlated with visceral fat and BMI (Mistry, Digby et al. 2007; Ritchie and Connell 2007), and their influence on cell biology (including apoptosis, proliferation and angiogenesis) is under constant study. CaP cells cultured in the presence of adipocytes demonstrated altered proliferation, differentiation, growth and cytokine expression (Tokuda, Satoh et al. 2003), and lends further support that locally produced and circulating adipokines may be important in the CaP.

Thus, visceral obesity appears to have good rationale as a risk factor for the development of aggressive CaP. Adipokines, represent a potential molecular mechanism for this hypothesis. In particular the paracrine effects of adipokines may be

of relevance in cases of locally advanced CaP where extra-prostatic extension and invasion into the retropubic fat pad occurs, thus resulting in the direct exposure of cancer cells to high concentrations of adipokines (Onuma, Bub et al. 2003).

ADIPOKINES AND PROSTATE CANCER

There has been a significant amount of research into the potential roles that adipokines may play in prostate carcinogenesis, and cancer in general. Two adipokines that have received the great interest with respect to prostate cancer are leptin and adiponectin.

LEPTIN

Leptin is a 16 kDa adipokine first described by Zhang et al. in 1994 (Zhang, Proenca et al. 1994). It is primarily produced by adipocytes of white adipose tissue but also to a lesser extent in other tissues such as the stomach, skeletal tissue and placenta (Zhang, Proenca et al. 1994). Total body fat concentrations are positively correlated with circulating leptin concentrations and serum leptin is known to be elevated in obese individuals (Considine, Sinha et al. 1996). Leptin has been documented to have many functions including; regulation of satiety (Heini, Lara-Castro et al. 1998), energy homeostasis (Jequier 2002), neuroendocrine physiology (Ahima, Prabakaran et al. 1996), and it is also speculated to be important in the development and maintenance of reproductive tissues, including the prostate (Nazian and Cameron 1999).

There have been epidemiological studies evaluating the relationship between obesity, circulating leptin levels and prostate cancer which have been inconclusive. A case control study by Lagiou *et al*. aimed to evaluate the hypothesis that prostate cancer was correlated with circulating leptin levels. The group failed to identify a significant

difference in serum leptin levels between men with prostate cancer and matched healthy controls (Lagiou, Signorello et al. 1998). Conversely a similar study by Chang et al found after adjusting for variables such as age, testosterone levels and BMI, men with high-volume disease had elevated leptin concentrations (Chang, Hursting et al. 2001). Lim et al has shown that the expressed degree of leptin is higher in CaP tissue than in BPH tissue and the group surmised that leptin possibly has an influence on the occurrence of CaP. These positive findings were also corroborated by Stattin *et al*. in a nested case-reference study confirming an association between moderately elevated leptin concentrations and later development of prostate cancer (Stattin, Soderberg et al. 2001). It should be noted that the above studies were all limited by sample size and in particular both the studies by Kim et al and Lagiou et al had small numbers of obese patients included. Another small study by Saglam et al suggested a positive correlation between serum leptin levels, PSA and Gleason score in CaP patients (Saglam, Aydur et al. 2003).

In summary the literature suggests that there may be a correlation between leptin levels, both in serum and tissue, and CaP. The studies need to be reviewed with caution in particular in view of small sample sizes.

In vitro studies have been performed and tend to support a role for leptin in CaP. In androgen-independent prostate cancer cell lines leptin has been shown to increase cellular proliferation (Onuma, Bub et al. 2003). Leptin promotes vascular endothelial cell proliferation and angiogenesis in-vitro and these effects appear to be both direct and indirect (Bouloumie, Drexler et al. 1998; Sierra-Honigmann, Nath et al. 1998; Onuma, Bub et al. 2003). These effects occur via induction of VEGF, transforming growth factor- (TGF)-β1 and basic fibroblast growth factor (bFGF) (Frankenberry,

Somasundar et al. 2004) and it is of importance as these processes are thought to be crucial factors in cancer progression and invasion.

A variety of second messenger systems have been shown to be involved following leptin treatment in vitro such as phosphatidyl-inositol 3-kinase (PI3-K) and c-Jun NH2-terminal kinase (JNK) (Onuma, Bub et al. 2003; Somasundar, Frankenberry et al. 2004). These pathways are of significant interest as they are involved with prostate carcinogenesis and malignant transformation, but also in obesity and the pathology of type 2 diabetes (Miyazaki, Bub et al. 2005).

In conclusion the evidence suggests that leptin may well be a factor in CaP although the evidence is predominantly based on small case controlled studies and in vitro evidence. Further large scale studies are required to determine if there are significant correlations between serum leptin concentrations and CaP and in-vivo studies would help discern whether a significant role in the pathophysiology of CaP exists.

ADIPONECTIN

Adiponectin is also called 30-kDa adipocyte complement-related protein (Acrp30), adipoQ, APM-1 or gelatin-binding protein-28 (GBP28) (Kelesidis, Kelesidis et al. 2006). It is the most abundantly circulating adipokine (Kadowaki and Yamauchi 2005). Circulating levels of adiponectin have been shown to be negatively correlated with obesity (especially central), body mass index, visceral fat accumulation and insulin resistance (Chandran, Phillips et al. 2003). Reductions in plasma adiponectin levels have also been observed in diseases closely related obesity e.g. type 2 diabetes, cardiovascular disease, hypertension and metabolic syndrome; and furthermore

adiponectin has been shown to improve these disorders (Kadowaki and Yamauchi 2005).

A small case controlled study has demonstrated that adiponectin levels were significantly lower in patients with prostate cancer, compared to patients with BPH or controls (Goktas, Yilmaz et al. 2005). The same study also showed a negative association between plasma adiponectin concentrations and Gleason score as well as stage of prostate cancer. Reduced serum adiponectin levels has also been associated with an increased risk of other hormone-dependent cancers such as breast and endometrial (Kelesidis, Kelesidis et al. 2006), and also alarmingly and increased risk of aggressive disease (Miyoshi, Funahashi et al. 2003; Petridou, Mantzoros et al. 2003; Mantzoros, Petridou et al. 2004).

Miyazaki et al demonstrated the presence of adiponectin receptors in the LNCaP-FGC (fast growing colony), DU145, and PC-3 prostate cancer cell lines (Miyazaki, Bub et al. 2005). Adiponectin was also shown to activate the JNK and activator of transcription 3 (STAT3) as common downstream effectors (Miyazaki, Bub et al. 2005). Both JNK and STAT3 play crucial roles in regulating cell proliferation, differentiation, and apoptosis in particular in relation to tumour-genesis (Davis 2000; Levy and Darnell 2002). Mistry et al has also demonstrated the protein distribution of adiponectin receptors both cancerous and benign prostate tissue (Mistry, Digby et al. 2006). The same study also identified that androgens, oestrogen, TNF-α, leptin, and adiponectin, regulate the adiponectin receptors in the LNCaP and PC3 cell lines in a cell specific manner. It is important to note that all of the aforementioned display altered levels in obesity states and are implicated in CaP.

The above findings have demonstrated an early insight into the potential effects and associations of adiponectin in CaP pathology. Further studies are needed to clarify whether adiponectin is significantly associated with prostate cancer biology. It is of great interest and importance that Mistry et al have shown that leptin and adiponectin interact in prostate cancer cell lines to modulate the tumour suppressor and oncogenes p-53 and BCL-2. This highlights the notion that in-vitro studies of one cytokine in isolation need to be interpreted with caution as there is far more likely to be a complex interaction of physiological factors in the true in-vivo setting.

VISFATIN

Visfatin (also known as Pre-B cell colony enhancing factor/PBEF and nicotinamide phosphoribosyltransferase - Nampt) is a 52kDa protein highly expressed in visceral adipose tissue, and in other tissues such as liver, muscle and bone marrow cells. Circulating visfatin levels have been shown to be increased proportionally with visceral fat accumulation (Sandeep, Velmurugan et al. 2007) and pro-inflammatory states (Moschen, Kaser et al. 2007). Since its discovery visfatin has been shown to have a role in multiple cellular processes, such as inflammation and sepsis, and has been reported to have anti-apoptotic effects (Jia, Li et al. 2004). It is of interest that in mammals visfatin has been compartmentalised into intracellular and extracellular (Revollo, Grimm et al. 2007). Thus far the biosynthetic properties of the intracellular form has been well supported by biochemical and structural analysis, yet the functional significance of circulating or extracellular visfatin has yet to be delineated. For the purpose of the thesis the nomenclature used will remain as visfatin. Circulating visfatin plasma concentrations as well as visceral adipose tissue visfatin mRNA expression have been positively correlated with measures of obesity and visceral fat accumulation

(Berndt, Kloting et al. 2005). Visfatin plasma concentrations have also been shown to be markedly elevated in obese subjects (0.037 +/- 0.008 mcg/ml), compared with controls (0.001 +/- 0.000 mcg/ml) (Haider, Schindler et al. 2006)

STRUCTURE OF VISFATIN

The gene, PBEF1, which encodes visfatin was initially detected from a human peripheral blood lymphocyte cDNA library (Samal, Sun et al. 1994). The gene is located on the long arm of chromosome 7 between 7q22.1 and 7q31.33 (Jia, Li et al. 2004) and encodes a polypeptide of 491 amino acids corresponding to a molecular mass of 52kDa (National Center for Biotechnology Information accession number AAA17884). Visfatin lacks a signal sequence in its primary structure and also lacks a caspase I cleavage site (Rongvaux, Shea et al. 2002). The crystal structure of visfatin has been described as a homodimeric protein by Wang et al (Wang, Zhang et al. 2006) and also Kim et al (Kim, Lee et al. 2006) with respect to its enzymatic activity. Visfatin has two active sites at the interface of the dimeric protein. This suggests that dimerisation is essential for the catalytic activity visfatin.

FUNCTIONS OF VISFATIN

Catalytic mechanism:

Visfatin catalyses the rate limiting step in one of the key NAD+ salvage pathways in mammals (Sommer, Garten et al. 2008). It converts nicotinamide to nicotinamide mononucleotide (NMN) which is a key intermediate in nicotinamide adenine dinucleotide (NAD). The catalytic activity was suggested on the grounds of its crystal structure and the similarity between it and other type 2 phosphoribosyltransferases. Both subunits of the visfatin molecule are involved in the catalytic mechanism and is has been postulated that the Phe193 and the Tyr18 from the two subunits are framing

the nicotinamide portion of the NMN molecule. Structural properties confer a specificity to nicotinamide and prevent binding to other negatively charged molecules e.g. nicotinic acid. It is the transfer of a phosphoribosyl residue 5-phosphoribosyl-1 pyrophosphate (prpp) to nicotinamide catalyzed by Nampt to produce NMN, which is finally converted to NAD+ by NMN-adenyltransferase (Nmnat). Importantly, it has been further hypothesized that NMN, a byproduct of extracellular visfatin mediated NAD+ enzymatic reaction exerts an autocrine/paracrine effect on target tissues (Wang, Xu et al. 2009).

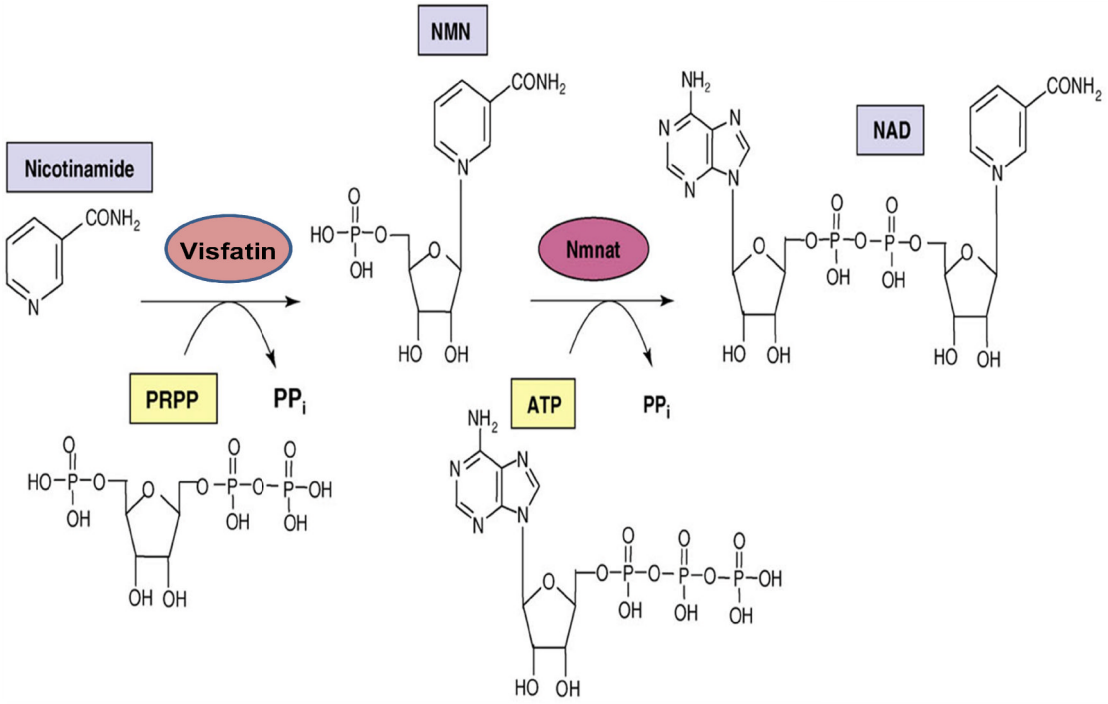

Figure 1.6 Cartoon demonstrating the rate-limiting Step of NAD biosynthesis. Adapted from-Garten *et al*., 2008

NAD^+ is an essential metabolic co-enzyme, levels of which are replenished in part by Nampt. It is often suggested that cancer cells by virtue of their rapid cell multiplication as compared to the cell of origin must have elevated metabolic rates and by inference high NAD^+ turnover rates and therefore it is unsurprising that visfatin expression is up-

regulated in certain cancers (Hufton, Moerkerk et al. 1999; Van Beijnum, Moerkerk et al. 2002). This offers a potential mechanism whereby visfatin may be able to regulate cell proliferation. As demonstrated above NMN is an intermediary product in NAD+ metabolism. NMN has independently been implicated to exert effects on tissue. This has been demonstrated in Nampt$^{+/-}$ mice with insulin secretion defects being corrected by the use of NMN. This is another mechanism by which extracellular visfatin may act on cells i.e. by extracellular production of NMN. Revollo et al has also documented that plasma NMN levels are regulated by e-visfatin independently of intracellular NMN biosynthesis (Revollo, Grimm et al. 2007). This extracellular modulation of NMN by visfatin suggests that NMN also offers a surrogate for visfatin's mechanism of action (Garten, Petzold et al. 2009).

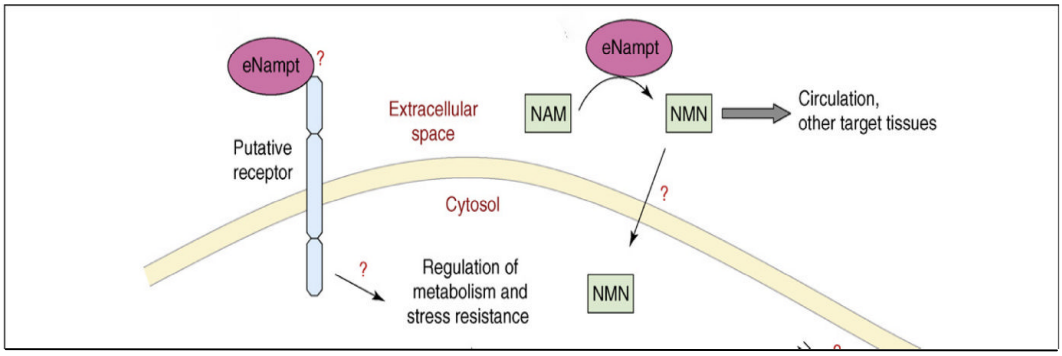

Figure 1.7 Proposed modalities of extracellular visfatin (eNAMPT) action on cells Adapted from-Garten *et al*., 2008.

The NAD+ salvage pathway is essential when the cellular level of NAD+ falls. Examples of situations when NAD+ levels may become depleted would include poly ADP ribosylation during DNA repair and NAD+ dependent protein deacetylase activity (Zhang, Dawson et al. 1994; van der Veer, Nong et al. 2005). Visfatin has also been

shown to regulate the function of the silent information regulator (SIR) 2 orthologue SIRT1/SIRT2α in mammalian cells. SIRT1/SIRT2α is implicated in the transcriptional regulatory activity of a variety of cellular processes, which include stress, cytokine responses, differentiation and metabolism (Revollo, Grimm et al. 2004). Van der Veer *et al.* further demonstrated that visfatin enhanced SIRT1 mediated p53 degradation in human vascular smooth muscle cells which has the effect of delaying the senescence phase and subsequently enhancing cell life (van der Veer, Ho et al. 2007).

Insulin-mimetic function:

In a paper that was subsequently retracted, Fukuhara et al (Fukuhara, Matsuda et al. 2005) suggested there was a role for visfatin as an insulin-mimetic. Subsequently no further studies have reproduced these results; however there has been some data in support of these interesting findings. Xie *et al.* demonstrated insulin-like effects with visfatin in human osteoblasts (Xie, Tang et al. 2007). They found that visfatin increased glucose uptake, stimulating the expression of osteogenic markers at both gene and protein level. It was also shown to cause an increase of mineralisation of osteoblasts similar to that of insulin. They also went on to show that when the insulin receptor (IR) was specifically blocked by HNMPA-(AM) (cell permeable Hydroxy(2-naphtyl)methyl]phosphonic acid) the insulin-like effects were also inhibited. These results were at a visfatin concentration level similar to that initially reported by Fukuhara.

Another group demonstrated, using *in vitro* studies in human SGBS adipocyte cells, that in response to visfatin at 100nmol/L there was a 2 fold increase in glucose uptake by the cells (Moschen, Kaser et al. 2007). Dahl and associates (Dahl, Yndestad et al. 2007) also published further evidence that visfatin acts via the IR. They showed that in

THP cells visfatin mediated secretion of interleukin-8 (IL-8) and tumour necrosis factor-alpha (TNF-α) as well as matrix metalloproteinase-9 (MMP-9) activity was abolished when the IR was inhibited by HNMPA-(AM).

There has been evidence to the contrary and Revollo *et al.* have shown that visfatin does not exert insulin-mimetic effects *in vitro* or *in vivo* but does exhibit NAD biosynthetic activity. It may be that there is a degree of cross talk between visfatin-mediated and insulin signaling pathways in a cell specific manner. An alternative theory is that it is the visfatin mediated NAD synthesis influences insulin signaling. With this evidence it is easy to conclude that visfatin has insulin like effects however further studies are warranted.

Immunomodulatory Functions:

Originally visfatin was termed PBEF and was thought to be a cytokine that increased pre-B-cell colony forming activity (Samal, Sun et al. 1994) and was shown to have a role in neutrophil maturation (Jia, Li et al. 2004). Visfatin has also been shown to be up regulated in psoriasis (Dahl, Yndestad et al. 2007), acute lung injury (Garcia and Moreno Vinasco 2006) and rheumatoid arthritis (Nowell, Richards et al. 2006; Otero, Lago et al. 2006) which are all conditions associated with inflammation. Visfatin has been demonstrated to activate human leucocytes and induce cytokine production (Moschen, Kaser et al. 2007).

Apoptosis was earlier discussed on page 32. Interestingly visfatin has been shown to be a cytokine with a role in inhibiting apoptosis. Jia *et al*. have demonstrated that in human neutrophils, visfatin inhibits apoptosis in response to multiple inflammatory stimuli (Jia, Li et al. 2004).

In patients with inflammatory bowel disease, plasma levels of visfatin have been shown to be elevated and the gene expression has been significantly elevated in colonic tissue with inflammatory bowel disease compared to control.

Recently Oki *et al.* have demonstrated that serum visfatin is independently correlated with C reactive protein (CRP) and IL-6 in a cohort of Japanese Americans however there was no correlation between visfatin and markers of insulin sensitivity (Oki, Yamane et al. 2007).

In the afore-mentioned studies it remains unclear whether it is the NAD biosynthetic property of visfatin, either in isolation, in conjunction with or separable from the proposed cytokine-like activity of circulating visfatin that drives these reported inflammatory effects. These results infer that visfatin enzymatic activity is required for the secretion of pro-inflammatory cytokines, however the question as to whether visfatin acts as an inflammatory cytokine in its own right remains largely unanswered. More recently, Li *et al.* published data showing that extracellular visfatin is protective in macrophages in the face of endoplasmic reticulum stress-induced apoptosis. This was demonstrated to be via non-enzymatic activity and this triggered IL-6 secretion and consequentially activates the pro-survival pathways (Li, Zhang et al. 2008). In conclusion it appears that visfatin may mediate inflammatory cytokines and is therefore has an immunomodulatory function although the exact mechanism remains unclear.

VISFATIN AND ANGIOGENESIS

Angiogenesis is a crucial step in the progression of cancer and adipokines have been shown to play a significant role in dysregulated angiogenesis (Lau, Schillabeer et al. 1996; Mohamed-Ali, Pinkney et al. 1998). Visfatin has been recently shown to induce and up-regulate VEGF secretion and matrix metalloproteinases 2-&-9 (MMP-2/9)

gelatinolytic activity, with angiogenesis occurring *via* the PI3K/Akt and MAPK signaling pathways in human endothelial cells (Adya, Tan et al. 2008). The latter cascades are crucial in cancer progression. Additionally, the same group has shown a role for the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) signaling in visfatin-induced up-regulation of MMP-2 and –9 activity (Adya, Tan et al. 2008). NF-kB is a protein complex that is involved with DNA transcription. These observations are pertinent given that NF-kB plays a vital role in anti-apoptosis, proliferation and progression of CaP *via* MMP-9 activation (Suh and Rabson 2004).

VISFATIN AND CARCINOGENESIS

Visfatin has been described as a biosynthesiser of NMN which is a precursor for Nicotinamide adenine dinucleotide (NAD+), a key metabolic coenzyme. Visfatin up-regulation has been described in colorectal cancer cells, which have a high metabolic rate and thus high NAD+ turnover (Hufton, Moerkerk et al. 1999; Van Beijnum, Moerkerk et al. 2002). Visfatin expression has also been demonstrated in tumour tissues (Bae, Kim et al. 2006), and has been implicated in chemo-resistance in breast cancer patients (Folgueira, Carraro et al. 2005). Its role as an inhibitor of apoptosis in cancer has not been elucidated; however, it is of interest and importance that inhibition of visfatin by FK-866 induces apoptosis in tumours (Drevs, Loser et al. 2003; Hasmann and Schemainda 2003; Khan, Tao et al. 2006; Kim, Lee et al. 2006).

More recently another visfatin inhibitor, CHS-828, has been identified and causes cell growth inhibition in multiple tumour types but the mechanism for its action is unknown (Ravaud, Cerny et al. 2005; Olesen, Christensen et al. 2008).

IN VITRO CAP MODELS

Primary prostate epithelial cells have a finite life span and then senesce, making the establishment of primary prostate cancer lines from tissue very difficult. Two commonly published in vitro models are the LNCaP and PC3 human CaP cell lines. LNCaP cells are androgen-receptor positive and PSA-secreting, which can be extrapolated to represent an early stage or androgen sensitive model of CaP (Horoszewicz, Leong et al. 1983) whilst PC3 cells exhibit low testosterone-5-alpha reductase activities, are non-PSA-secreting and are androgen receptor negative and may provide a model for advanced, androgen-insensitive CaP (Kaighn, Narayan et al. 1979). It should be noted that these two cell types are from different origin and one cell type does not develop into the other.

HYPOTHESIS AND AIMS

The association between obesity and CaP remains poorly understood, but evidence suggests that obesity may adversely affect the risk of developing high grade disease. Adipokines may contribute to the molecular basis for a link between obesity and CaP, in particular, adiponectin and visfatin. The following broad hypothesis will be investigated in this thesis:

- Visfatin is involved in the pathophysiology of CaP

AIMS

1. To demonstrate Visfatin in human prostate tissue and LNCaP and PC3 human CaP cell lines.

2. To determine the effects of and visfatin on LNCaP and PC3 CaP cell proliferation both alone and in combination with other factors.

3. To investigate the effect of visfatin on the expression of key tumour suppressor genes and oncogenes involved in CaP development and progression.

4. To identify the intracellular pathways activated by visfatin in CaP cells

5. To demonstrate a role for FK866 in regulation of prostate cancer growth

6. To measure levels of visfatin in human plasma as a potential biomarker.

CHAPTER 2

MATERIAL AND METHODS

CHAPTER 2: MATERIALS AND METHODS

MATERIALS

EQUIPMENT

'Mr. Frosty' cryo-freezing unit: Fisher Scientific, Loughborough, UK.

Bio-Rad IQ5 iCycler Real-Time PCR Detection System: Bio-Rad Laboratories Inc. California, USA

Bio-Rad mini Protean II Gel system: Bio-Rad Laboratories, Hemel Hempstead, UK.

Cell culture flasks: BD Biosciences, Bedford, MA, USA

Digital microscope camera system: Olympus, Tokyo, Japan

DMRE laser-scanning confocal microscope: Leica, Milton Keynes, UK.

ELISA plate reader: EL800, Bio-Tek Instruments, Inc., Winooski, VT, USA

Fuji medical X-ray film: Fuji Photo Film Company, Tokyo, Japan.

Haemocytometer: Paul Marienfeld GmbH & Co. KG, Germany

Hot Block Techne TC-312: Geneflow

NanoDrop spectrophotometer: Labtech International, Ringmer, UK.

Roche Light Cycler™ system: Roche Molecular Biochemicals, Mannheim, Germany.

Spectrophotometer (Beckman DU640: Beckman Instruments Inc, California, USA.

BIOCHEMICAL REAGENTS AND KITS:

4'-6-diamidino-2-phenylindole (DAPI) nuclear marker: Invitrogen, Paisley, UK

Alexa Fluor® 488: Invitrogen, Paisley, UK

10X protein running buffer: Department of Biological Sciences, University of Warwick – Media Preparation.

10X protein transfer buffer: Department of Biological Sciences, University of Warwick – Media Preparation.

30% Acrylamide: Bisacrylamide (37.5:1): GENEFLOW, Fradley, UK.

Ammonium persulphate (APS): Sigma-Aldrich, Gwellingham, UK.

Bio-Rad DC Protein Assay: Bio-Rad Laboratories, Bio-Rad Laboratories Inc. California, USA

Bovine serum albumin (BSA): Sigma-Aldrich, Gwellingham, UK.

CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) kit: Promega, UK.

Cellular DNA fragmentation ELISA: Roche, Mannheim, Germany.

Complete mini protease inhibitor cocktail: Roche, Manheim, Germany

DNA ladder: Invitrogen, Paisley, UK.

ECL+: GE Healthcare, Amersham, UK.

Ethidium Bromide: Sigma-Aldrich, Gwellingham, UK.

Foetal Calf Serum (FCS): Invitrogen, Paisley, UK.

geNorm™ ™ Housekeeping Gene Selection Kit: Primer Design Ltd, Southampton

Human VEGF duoset ELISA kit: R&D systems, Abingdon, UK

Human Visfatin ELISA: Phoenix Secretomics, California, USA

Light Cycler DNA Master SYBR® Green I: Roche, Manheim, Germany.

$MgCl_2$: Biogene, Kimbolton, UK.

Moloney Murine Leukaemia Virus Reverse Transcriptase (MMLV): Fermentas, York, UK.

N, N, N', N'- tetramethylethylenediamine (TEMED): Sigma-Aldrich, Gwellingham, UK.

OptiMax Wash Buffer: BioGenex, San Ramon, CA, USA.

Penicillin: Department of Biological Sciences, University of Warwick – Media Preparation.

pH 7.8 Tris-EDTA buffer: Sigma-Aldrich, Gwellingham, UK.

Phosphate Buffered Solution (PBS): Department of Biological Sciences, University of Warwick – Media Preparation.

Phophotase Inhibitor Set 2, CalBiochem (Merck Chemicals Ltd), Nottingham, UK

PMSF: Sigma-Aldrich, Gwellingham, UK.

Poly-D-Lysine (Sigma, P7280-5MG)

Polyvinylidene difluoride (PVDF) membranes: Millipore, Billerica, MA, USA.

Precision Plus Protein Standard: Bio-Rad Laboratories, Hemel Hempstead, UK.

PrimerDesign Ltd Precision Reverse Transcription kit: Primer Design Ltd, Southampton

Protease Inhibitor Cocktail: Sigma-Aldrich, Gwellingham, UK.

Protein gel running buffer: Department of Biological Sciences, University of Warwick – Media Preparation

QIAquick Gel Extraction Kit: Qiagen, Crawley, UK.

Radioimmunoprecipitation lysis buffer (RIPA): Upstate, Lake Placid, NY, USA.

Reaction Buffer: Fermentas, York, UK.

RNase-free DNAse (Promega, Madison, WI, U.S.A)

SDS: Bio-Rad Laboratories Inc. California, USA.

SDS-sample buffer: Sigma-Aldrich, Gwellingham, UK.

Streptomycin: Department of Biological Sciences, University of Warwick – Media Preparation.

SYBR® Green: Roche, Mannheim, Germany

TBE agarose gel running buffer- Department of Biological Sciences, University of Warwick – Media Preparation.

TE (Tris-HCL/EDTA) buffer- Department of Biological Sciences, University of Warwick – Media Preparation.

Tris Buffered Saline (TBS) containing 0.1% TIen-20: Department of Biological Sciences, University of Warwick – Media Preparation.

Tris-HCL [tris (hydroxymethyl) aminomethane, pH adjusted with HCl]: Sigma-Aldrich, Gwellingham, UK.

Trypsin solution: Department of Biological Sciences, University of Warwick – Media Preparation.

β-mercaptoethanol: Sigma-Aldrich, Gwellingham, UK.

PEPTIDES AND INHIBITORS:

Camptothesin: BioVision, California, USA.

Fk866: Axon Medchem, Groningen, The Netherlands.

Human Insulin: Sigma-Aldrich, Gwellingham, UK

Human Recombinant Visfatin: Axxora Ltd, Nottingham, UK (ALX-201-336)

Human VEGF peptide: Calbiochem, UK.

NMN: Sigma Aldrich, Gwellingham UK

ANTIBODIES:

All secondary antibodies were purchased from DakoCytomation Ltd (Cambridge, UK)

Anti-Rabbit horseradish peroxidase-conjugated Ig (P0450): Dako, Ely, UK.

Human β-actin antibody (4967): Cell Signalling Technology, Beverly, MA, USA.

MMP-2 antibody (ab2462), MMP-9 antibody (ab7299): ABCAM, Cambridge, UK.

Visfatin antibody: Universal Biologicals Cambridge, A300-778A

Human Phospho $ERK_{1/2}$ (9106S) and p38 (4631) MAPK antibodies: Cell Signalling Technology, Boston, MA, USA.

Human total $ERK_{1/2}$ (4695) and p38 (*9212)* MAPK antibodies: Cell Signalling Technology, Boston, MA, USA.

CELL CULTURE MEDIA AND CELL LINES

Cell Culture Media (Department of Biological Sciences, University of Warwick – Media Preparation supplemented as follows).

Hams-F12 (Kaighn's modification) (F12K)

F12K growth medium		F12K starvation medium	
+Foetal Calf Serum	10%		
+Penicillin/Strep	100 IU-100µg/ml	+Penicillin/Strep	100 IU/100µg/ml
+L-Glutamine (200mM)	5ml	+ L-Glutamine (200mM)	5ml

RPMI 1640

RPMI growth medium		RPMI starvation medium	
+FCS	10%		
+L-Glutamine (200mM)	5ml	+ L-Glutamine (200mM)	5ml
+Penicillin/Strep	100 IU-100µg/ml	+Penicillin/Strep	100 IU-100µg/ml

PC3/ LNCAP FREEZING MEDIA

Dimethylsulfoxide (DMSO)	5 %
+FCS	95%

PROSTATE CANCER CELL LINES

PC3 cells: American Type Culture Collection (ATCC), Manassas, VA, U.S.A.

LNCaP cells: American Type Culture Collection (ATCC), Manassas, VA, U.S.A.

Normal and tumour prostate tissue cDNA: BioChain Institute, Hayward, CA, U.S.A.

SOFTWARE

Scion Image™: Scion Corporation, Maryland, U.S.A

ADLab software: ADI instruments, UK.

EXPO 32 ADC software: Beckman Coulter, UK.

Gel Pro image analysis: Gel Pro 4.5, Media Cybernetics, USA.

GraphPad Prism 4: GraphPad Software, California, USA.

Image-Pro Plus software: Media Cybernetics Inc, Bethesda, USA.

METHODS

CELL CULTURE

LNCaP and PC3 cells were cultured, as per manufacturer's instructions, in RPMI and F12K media, respectively. Both media were supplemented with 10% FCS and cultured in 75 cm^2 cell culture flasks. Flasks were incubated at 37 °C and 5% CO_2, with 100 µg/ml penicillin G and 100 µg/ml streptomycin sulphate. The cells were seeded at 3 x 10^6 cells per flask and maintained in 175cm^3 vented tissue culture flasks in a humidified incubator at 37^oC in 5% CO_2 and 95% air atmosphere and routinely passaged at approximately 80% confluence (determined by microscopic inspection). Cells were seeded onto 6/12/96-well plates and, cultured to pre-confluence prior to treatments.

SUBCULTURE OF ADHERENT CELLS:

Culture medium from the flask was carefully aspirated and the cells were washed with PBS. 2 ml of Trypsin EDTA solution was added to the flask (T75 cm^2) and incubated at 37ºC for between 3-5 minutes or until the cells had detached. 8ml of fresh culture medium was used to block further trypsinisation. A small amount of this cell solution was taken and added into a new flask with fresh medium for culture.

MAINTAINING CELLS:

Following cellular attachment at 48 hours 15ml of the pre-incubated medium was added to the flask (T75 cm^2). The culture medium was refreshed every 2-3 days.

PRESERVATION OF CELLS:

Cells were prepared as described above as for splitting and then centrifuged at 1000rpm for 5 minutes. The cells were then re-suspended with cryo-protective culture solution (5% DMSO, 95% FBS). 1 ml of the cell suspension was pipetted into a cryogenic vial and stored at -80ºC. After 48 hours the cells were placed in liquid nitrogen.

CELL COUNTS USING HAEMOCYTOMETER

A haemocytometer was used to determine the cell density (cells/ml) to allow accurate plating of cells for optimising experiments. The haemocytometer was cleaned with a 70% ethanol solution and a cover-slip placed. The correct position of the coverslip was noted by the presence of Newton's rings, which are 'rainbow-like' interference patterns, visible on holding the haemocytometer up to light. The cell suspension was agitated and 100µl taken and added to the haemocytometer chamber. Low magnification microscopy was then used to view the haemocytometer. The number of unstained cells in at least two 1mm^2 areas (more if low count was noted) were counted. Cells within the

left and upper border markings were included whilst those in the right and bottom markings were excluded. The calculation was as follows:

Total number of viable cells = Total cell volume (ml) x 10^4.

TOTAL RIBONUCLEIC ACID (RNA) EXTRACTION AND COMPLEMENTARY DEOXYRIBONUCLEIC ACID (CDNA) SYNTHESIS

RNA extraction was carried out using Qiagen RNeasy™ Plus Mini-Kit as per manufacturer's guidelines. RNA samples were treated with RNase-free DNAse to eliminate genomic DNA contamination and the concentration quantified using the NanoDrop spectrophotometer and reverse-transcribed into cDNA using 1.0µg RNAA primer design first strand cDNA synthesis kit (using random nonamer primers) was used to reverse transcribe the RNA as follows:

Annealing step: the following was added to a 500µl eppendorf and heated to 65ºC for 5 mins using a heat block and then immediately cooled in ice without allowing them to cool first:

RNA	0.5-1µg
RT Primer	1.0µl
dNTP mix 10mM	1.0µl
RNAse/DNAse free water	to a total 10µl

Extension Step: 10µl of a master-mix containing the following was added to the samples and then mixed thoroughly before incubating at 42ºC for 60 mins. The subsequent cDNA samples were then stored at -20ºC until use.

Moloney Murine Leukaemia Virus (MMLV) 5X buffer	4µl

RNAse/DNAse free water	5.2µl
Moloney Murine Leukaemia Virus (MMLV) Reverse Transcriptase	0.8µl

REAL TIME POLYMERASE CHAIN REACTION (RT-PCR)

For in depth RT-PCR protocols see Chapter 3 and 5.

Figure 2.1 Primer Sequences

Gene/product size	Sense primer	Antisense primer
B-Actin (249bp)	5'-AAGAGAGGCATCCTCACCCT-3'	5'-TACATGGCTGGGGTCTTGAA-3'
GAPDH (185bp)	5'-GAGTCAACGGATTTGGTCGT-3′	5'-GACAAGCTTCCCGTTCTCAG-3′
B-Actin, GAPDH, 18S, YWHAZ, UBC, B2MG*	NOT SUPPLIED BY MANUFACTURER[1]	NOT SUPPLIED BY MANUFACTURER[1]
Insulin Receptor (INSR) (97bp)*	5'-AGAGCAGGAGCGTCATCAG-3'	5'-CCCACTGTGAAGGAGAGAAATG-3'
Insulin like	5'-	5'-

growth factor receptor (IGFR) (98bp)*	TTAACAGATGGAAAGAACCTCATTG-3’	CCAGACCAATCAAGCCAGATG-3’
MMP2 (179 bp)	5′-TGGCAAGTACGGCTTCTGTC -3′	5′-TTCTTGTCGCGGTCGTAGTC-3′
MMP9 (200 bp)	5′-TGCGCTACCACCTCGAACTT-3′	5′-GATGCCATTGACGTCGTCCT-3′
Visfatin (228bp)	5’-AAGAGACTGCTGGCATAGGA-3’	5’-ACCACAGATACAGGCACTGA -3’
P53 (102bp)*	5’-GTGGAGTATTTGGATGACAGAAAC-3’	5’-GTAGTTGTAGTGGATGGTGGTAC-3’
BCL2 (88bp)*	5’-GAGGTCACGGGGGCTAATT-3’	5’-GAGGCTGGGCACATTTCTG-3’
BAX (101bp)*	5’-ATGGAGCTGCAGAGGATGAT-3’	5’-CAGTTGAAGTTGCCGTCAGA-3’
BCL2L1 (84bp)*	5’-	5’-

	CACTTACCTGAATGACCACCTAG-3'	GCATTGTTCCCATAGAGTTCCA-3'
MCL1 (104bp)*	5'-CTGATTGTTCTGCTCCCTCTAC-3'	5'-GTTTCACAGTGCCAAAATCTAAAAG-3'

* Pre-validated primers purchased from PrimerDesign Ltd. [1]Sequences not supplied for housekeeping genes used in 'GeNorm™' analysis and subsequent RT-PCR from PrimerDesign Ltd.

'GENORM™' ANALYSIS FOR OPTIMISATION OF HOUSE KEEPING GENES

To help optimise the results from my gene expression data it was important to identify suitable housekeeping genes as a fixed point of reference. It is commonly accepted that there is no one universal gene or genes that can be used in all cases. This is based on a concept and software initially developed by Vandesompele et al (Vandesompele, De Preter et al. 2002). We looked to identify the most suitable housekeeping genes for use in the cell model I adopted for my project. cDNA was prepared as described above with n=3 samples including basal and treated cells. 6 commonly selected housekeeping genes were selected and provided by PrimerDesign Ltd as part of the geNorm™ kit. These were; B-Actin (ACTB), GAPDH, YWHAZ, 18S, UBC and B2MG. RT-PCR was subsequently using the above protocol on the Biorad I-cycler as described previously.

The data was then analysed using the supplied geNorm™ software. The aim of the software is to firstly assess the gene-stability and rank the selected housekeeping

genes and secondly use a normalization factor calculation based on the geometric mean of multiple control genes to assess the optimal number of housekeeping genes to generate the best quality data.

The concept is built on the principle that the expression ratio of two ideal internal control genes is identical in all samples, irrespective of the conditions or cell type. Vandesompele developed a calculation whereby for every control gene the pairwise variation with all other control genes as the standard deviation of the logarithmically transformed expression ratios, and defined the internal control gene-stability measure *M* as the average pairwise variation of a particular gene with all other control genes.(Vandesompele, De Preter et al. 2002) Genes with the lowest *M* values have the most stable expression. To manage the calculations, they have developed a Visual Basic Application (VBA) for Microsoft Excel - termed geNorm. In addition, the systematic variation was calculated as the pairwise variation, *V,* for repeated RT-PCR experiments on the same gene, reflecting the inherent machine, enzymatic and pipette variation. (Vandesompele, De Preter et al. 2002) It is also widely accepted that to optomise expression levels accurately, normalization with multiple housekeeping genes instead of one is required. Consequently Vandesompele et al developed a normalization factor based on the expression levels of the best-performing housekeeping genes. A combination of these two concepts allows a second data set to be generated again using the software programme to calculate the ideal number of housekeeping genes to accurately quantify data

Practically speaking CT values were transformed into relative quantification data using the ΔCT method. Following this for each data point the equation $2^{(-\text{deltaCt})}$ is applied, hence allowing all data to be expressed relative to the expression of the least expressed gene. The data is then inserted into an excel spreadsheet with an inbuilt

applet. The software allows for automated analysis and produces 2 charts displayed below. Figure 2.2 shows which of the 6 genes are most stably expressed in my system. In this case B-Actin (ACTB) and YWHAZ were the most stable and 18S the least stable. Figure 2.3 reveals the ideal number of housekeeping genes against which to normalise data based on a 'pair-wise variation' or 'V score'. The manufacturers suggest a V score of <0.15 as a cut off for optimal normalisation but stress this is only a guide. For the purpose of the experiments undertaken 2 housekeeping genes were used as standard.

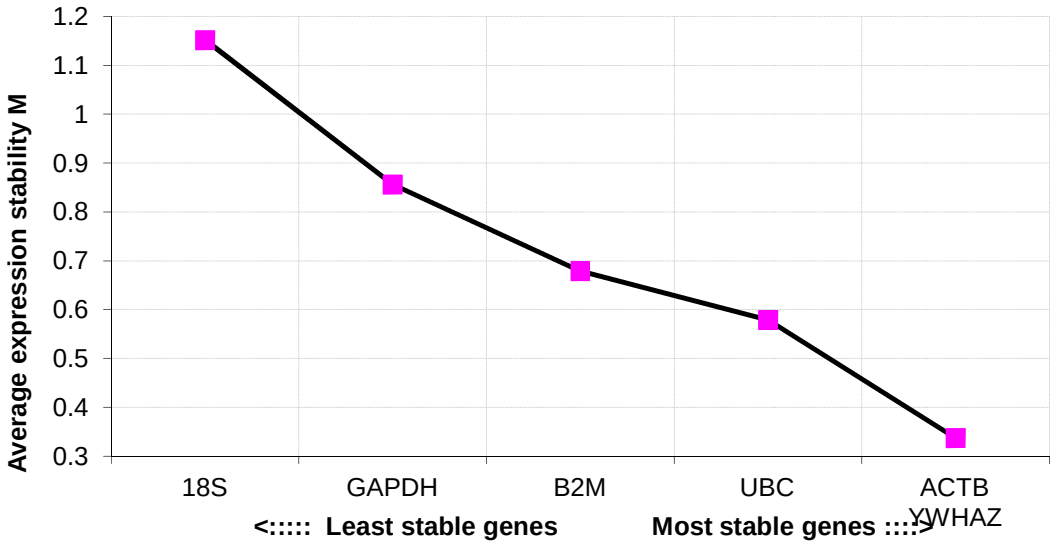

Figure 2.2 Average expression stability values of remaining control genes

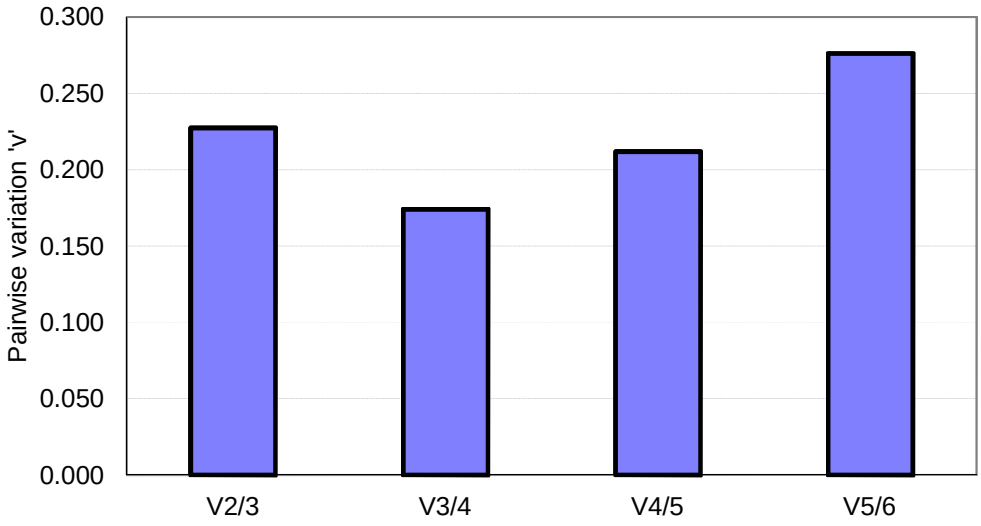

Figure 2.3 Determination of the optimal number of control genes for normalisation

(Pairwise variation (Vn/n + 1) analysis between the normalization factors NFn and NFn + 1 to determine the number of control genes required for accurate normalization (arrowhead = optimal number of control genes for normalization) (Vandesompele, De Preter et al. 2002)

WESTERN BLOTTING

MAKING SDS-PAGE GEL

Using the Bio-Rad mini Protean II Gel system, 8% or 10% SDS-PAGE gel was made up, as per the manufacturer's protocol. The resolving gel was prepared using 30% Acrylamide: Bisacrylamide (37.5:1), 1.5M Tris-HCL [tris (hydroxymethyl) aminomethane, pH adjusted with HCl], pH 8.0) pH 8.8, 10% SDS, deionised water, 10% ammonium persulphate (APS) and N, N, N', N'- tetramethylethylenediamine (TEMED). The mixture was carefully added into the casting system and allowed to set at room temperature for approximately 30 minutes.

The stacking gel was identically prepared aside from 1.5 M Tris-HCl, pH 8.8 being substituted by 0.5 M Tris-HCl, pH 6.8. The mixture was then poured on top of the pre-set resolving gel and a comb used for well formation. The gel was allowed to set at room temperature for a further 20 minutes. The gel was placed into the electrophoresis running system and copious protein gel running buffer was added to the tank.

SAMPLE PREPARATION AND WESTERN BLOTTING TECHNIQUE

Western blotting was used to assess protein levels in cell lysates from both treated and untreated prostate cancer cell lines. All protein extraction was carried out on ice. Protein lysates were prepared by lysing cells from tissue culture plates in radioimmunoprecipitation lysis buffer (RIPA) containing (containing; 0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA and 2µl of Protease Inhibitor Cocktail and 2µl of Phosphatase Inhibitor). Protein concentrations were determined colourimetrically using the Biorad DC Protein Assay Method which is a modification of the Lowry method (Lowry, Rosebrough et al. 1951)

Protein lysates were mixed with equal amounts of sodium dodecylsulphate (SDS)-sample buffer (5M urea, 0.17M SDS, 0.4M dithiothreitol, and 50mM Tris-HCl, pH 8.0) and placed in a boiling water bath for 5 min, and then gently allowed to cool. Protein lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes using a protocol of; 100 V for one hour in a transfer buffer containing 20mM Tris, 150mM glycine, and 20% methanol. To prevent non-specific binding the membranes were ‘blocked’ by incubating in tris buffered saline containing 0.1% Tween-20 (TBS-T) and 5% BSA for one hour at room temperature. The PVDF membranes were incubated with the relevant primary antibodies for at least 12 hours at 4°C. Following overnight incubation, membranes were washed for 1 hour

with TBS-T before addition and incubation with the required secondary antibody for one hour at room temperature. Antibody complexes were demonstrated using chemiluminescence ECL+ and autoradiography. Where necessary the appropriate positive controls were used. The densities were measured using a scanning densitometer coupled to scanning software.

STATISTICAL ANALYSIS

All of the data in the present study are expressed as mean ± SEM. Parametric comparisons between groups were made by ANOVA. If significance ($P<0.05$) was present, a post hoc Tukeys multiple-comparison test was performed. All statistical analysis was performed using Graph Pad software (version 4.0) unless stated otherwise in individual results chapters.

The ANOVA test was chosen based on the notion that performing multiple two-sample t-tests would most likely result in type 1 error. ANOVA has also been shown to be an established data analysis tool for RT-PCR (Pfaffl, Horgan et al. 2002; Brunner, Yakovlev et al. 2004). It should be noted that parametric tests rely on the assumption that the data is normally distributed. However, to prove or disprove this notion, particularly in the context of gene expression data, can be difficult. On the other hand, the use of non-parametric tests based on ranks (Mann-Whitney, Kruskal-Wallis, etc.) suffer a significant reduction in power relative to parametric tests (t-tests, ANOVA, etc.). Based on the above, it was the decision of the author to use the established test, ANOVA, on the basis of Gaussian distribution of data..

CHAPTER 3

THE IDENTIFICATION OF INTRACELLULAR VISFATIN IN PROSTATE CANCER CELL LINES AND TISSUE

CHAPTER 3 THE IDENTIFICATION OF INTRACELLULAR VISFATIN IN PROSTATE CANCER CELL LINES AND TISSUE

INTRODUCTION

Obesity is well established as a risk factor for type 2 diabetes, cardiovascular disease and cancer (Kershaw and Flier 2004). Specifically, visceral obesity is associated with insulin resistance, hyperinsulinaemia and prothrombotic/proinflammatory states (Kershaw and Flier 2004), and has also been identified as a risk factor for prostate cancer (von Hafe, Pina et al. 2004). Evidence also suggests that the presence of obesity increases the aggressiveness of the disease (Freedland and Platz 2007).

As commented on earlier adipose tissue has been documented as a metabolic organ and not just a fat store. This encompasses the notion that adipose tissue is also the site for the production of multiple biologically active peptides now termed ‘adipokines’ (Matsuzawa, Funahashi et al. 1999). Adipokines which include adiponectin, leptin, TNF-α and IL-6 are not only produced by adipocytes but also by other stromal cells (Frayn, Karpe et al. 2003; Fain, Madan et al. 2004). Amongst the newly discovered adipokines is Visfatin and this is the adipokine of interest to my thesis. Circulating visfatin plasma concentrations as well as visceral adipose tissue visfatin mRNA expression correlated with measures of obesity and visceral fat accumulation. (Berndt, Kloting et al. 2005). Visfatin plasma concentrations have also been shown to be markedly elevated in obese subjects ((0.037 +/- 0.008 mcg/ml) mean +/- SD),

compared with controls ((0.001 +/- 0.000 mcg/ml mean +/- SD) (Haider, Schindler et al. 2006).

Visfatin has gained momentum as having a more multifunctional physiological role with its identification as nicotinamide phosphoribosyltransferase (NMPRTase), an enzyme involved in NAD^+ biosynthesis (Rongvaux, Shea et al. 2002). NAD^+ is an essential metabolic co-enzyme and visfatin in its capacity as NMPRTase replenishes cellular NAD^+ by converting free nicotinamide (NM) to nicotinamide mononucleotide (NMN). Intracellular visfatin expression has already been demonstrated in normal and inflamed tissues (Bae, Kim et al. 2006). Cancer cells by virtue of their rapid cell division have a naturally high NAD^+ turnover and unsurprisingly therefore visfatin expression has been shown to be up-regulated in certain cancers (Hufton, Moerkerk et al. 1999; Van Beijnum, Moerkerk et al. 2002).

It is of note and importance that in obesity it has been reported that there are elevated circulating visfatin levels in serum. However, there has been no study to date evaluating the association between the level of exogenous or extracellular visfatin and its impact on intracellular visfatin levels. This is important given the documented over-expression of visfatin in certain cancers, most recently for example ovarian cancer (Shackelford, Bui et al. 2010).

HYPOTHESIS

Visfatin is present intra-cellularly and can be identified in both in-vitro and in-vivo models of prostate cancer.

AIMS

- To identify and investigate the expression of visfatin in human prostate cancer cell lines and tissue.
- To identify if visfatin plays a role in visfatin gene regulation.

METHODS

Methods were as outlined in the methods chapter. Following from work initiated by Tina Mistry previously in the group, I looked to confirm her preliminary data with respect to immunohistochemistry as a starting point to my thesis and overall hypothesis. Therefore all experiments were performed as stated by Ms Mistry and completed in this part of the study; subjects were identified and archived tissue used.

Following ethical approval, paraffin embedded sections (3μm thickness) were cut from archived prostate tissue obtained from patients undergoing transurethral prostatectomy (n = 13; mean 75.1 years; range 57 – 92; SEM ± 2.6). Sections were floated onto 3-aminopropyltriethoxy-silicane (APES) coated slides. All patients had histologically diagnosed prostatic adenocarcinoma, and cut sections had foci of benign and malignant tissue.

Gene expression was outlined using standard qualitative and quantitative PCR techniques as described in the literature (Mistry, Digby et al. 2008).

Immunocytochemistry and immunohistochemistry were used as detailed below to identify intracellular visfatin using cell lines and human tissue respectively. This was a well established technique both in the group and in the literature (Mistry, Digby et al. 2006; Wang, Hasan et al. 2011).

IMMUNOCYTOCHEMISTRY AND CONFOCAL ANALYSIS

COVERSLIP PREPARATION

Coverslips were prepared by placing them in a beaker with concentrated acetic acid for 30 mins. Then washed with distilled water and placed in 70% ethanol for 30 mins. Then washed again in distilled water. They were then placed in a petri dish containing 200µl of APES and 10mls acetone in a fumigation hood for 5 mins until the acetone had evaporated. Wash with distilled water thoroughly and then air dry on styrofoam blocks. Once dry, place in 6 or 12 well plates as needed. Immediately prior to use they are exposed to ultra violet (UV) light for 30mins to sterilize.

POLY-D-LYSINE COATING

Poly-D-Lysine (5mg) was diluted in 5ml of sterile PBS and stored in 500µl aliquots at -20ºC. Each aliquot was further diluted 1:100 with PBS prior to use. When using a 12-well plate, 100µl was added per well. The plate was incubated for 15mins and then washed with PBS. Cells were cultured onto the poly-D-lysine-coated glass cover slips for 48-hours.

FIXATION

The media was aspirated and the plate washed with sterile PBS. 400µl of 4% paraformaldehyde (PFA) in PBS was added to each coverslip and left for 30mins. The PFA was removed and the slips washed with PBS several times.

PERMEABILIZATION, BLOCKING AND ANTIBODY INCUBATION

Non specific binding was inhibited by incubating cells with 3% BSA in PBS-0.01%-Triton x100 for 1 hour at room temperature. Cells were incubated with 100µL primary rabbit anti-human visfatin antibody diluted in PBS-0.1% TRITON x100 (1:100) overnight

at 4ºC. A blank with no antibody was used to check for non specific binding. After washing with PBS-0.05%-Tween, cells were incubated in the dark with secondary conjugated donkey anti-rabbit antibody, Alexa Fluor® 488. Following further washes cover slips were mounted on slides with glycerol containing DAPI. Images were acquired using a Leica SP2 confocal laser scanning microscope system linked to a Leica DM RE7 upright microscope and an X40 oil immersion lens. Settings for aperture, laser line and filters were set using the conditions detailed in Mistry et al (Mistry, Digby et al. 2006). Images were acquired using an argon laser (excitation, 488 nm; emission, UV excitation and a BP 385-470 nm emission filter for DAPI). The confocal aperture was set to a pinhole diameter of 1 Airy unit.

IMMUNOHISTOCHEMISTRY

The paraffin embedded sections collected as described above were floated onto APES coated slides. All patients had histologically diagnosed prostatic adenocarcinoma, and cut sections had foci of both benign and malignant tissue. Each section of prostate tissue had foci of both benign and adenocarcinomatous prostate glands. The benign glands have tall columnar epithelial cells with well circumscribed lumens, whereas the malignant glands are distinguishable by their variation in size, shape, prominent nuclei and occlusive lumens.

Optimax Wash Buffer was used for all washes and dilutions. An immunoperoxidase-based detection system (VECTASTAIN Elite ABC Kit (Universal), Vector Laboratories, Peterborough, U.K.) and diaminobenzidine tetrahydrochloride (DAB) visualization agent (Menarini Diagnostics, Florence, Italy) were used. All incubations were carried out at room temperature.

To revive the antigen heat-induction was used by incubating the mounted sections in pH 7.8 Tris–EDTA buffer for 80 seconds in a pressure cooker. Endogenous peroxidise activity was inhibited by incubating in 1.5% hydrogen peroxide (H_2o_2) solution for 20 mins. Then sections were blocked with BSA for 20 mins. Rabbit anti-human visfatin primary antibody was used for 1 hour (1:200). Sections were washed with buffer for 10 minutes and incubated with secondary antibody for 30 minutes. Further wash was done with buffer for 10 mins. Sections were incubated with the Avidin: Biotinylated Enzyme Complex for 30 mins and then washed with buffer for 5 minutes. DAB was applied for 5 minutes and rinsed with de-ionised water. Sections were counterstained for 1 minute in Mayers Haematoxylin, then blued in Scott's tap water, dehydrated, cleared and mounted. They were then examined under a microscope.

REAL TIME POLYMERASE CHAIN REACTION (RT-PCR)

Quantitative RT-PCR was performed using the Roche Light Cycler™ system. Reaction mixtures contained SYBR® Green and 1.5µl cDNA. PCR conditions consisted of denaturation at 95°C for 1 min followed by 40 cycles of 95°C for 1 s, 59°C for 10 s and 72°C for 15 s, followed by melting curve analysis. Quantitative amounts of visfatin were standardised against β-actin/GAPDH. For each sample, ∆CP (crossing point) values were calculated as the CP of the target gene minus the CP of the β-actin gene. The RNA levels were expressed as a ratio, using comparative "delta-delta method" for comparing relative expression results between treatments in real-time PCR (Pfaffl *et al*., 2001). and changes in gene expression expressed as x-fold over basal.

10µl of the PCR products were then electrophoresed on a 1% agarose gel and ethidium bromide used to identify the products. A 1kb DNA ladder was used to estimate the band sizes. RNA was assayed from n ≥3 independent biological replicates. RT negatives were used throughout the study.

RESULTS

VISFATIN MRNA EXPRESSION IN PROSTATE CANCER CELL LINES AND HUMAN PROSTATE

Using RT-PCR, visfatin mRNA was detected in both cell lines used in this study, with agarose gel electrophoresis yielding an expected 249 base pair product. Visfatin gene expression was also present in cDNA prepared from mRNA obtained from normal and malignant human prostate tissue (Figure 3.1); sequencing analysis confirmed the identity of these products.

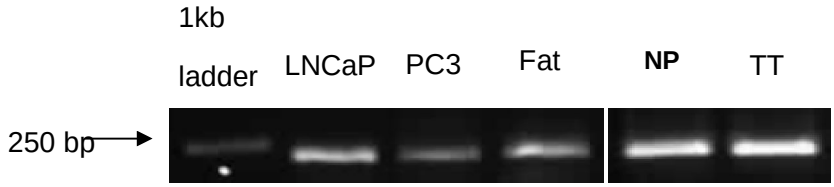

Figure 3.1 Visfatin mRNA expression in the LNCaP (LN) and PC3 (PC) prostate cancer cell lines. Human fat (HF) cDNA was used as a positive control. Visfatin mRNA was also detected in normal prostate tissue (NP) and prostate adenocarcinoma tumour tissue (TT) cDNA. A no template negative control was also performed using water instead of cDNA and an RT was performed to confirm absence of genomic contamination.

VISFATIN PROTEIN EXPRESSION IN PROSTATE CANCER CELL LINES

Expression of visfatin protein was demonstrated in LNCaP and PC3 cells using both immunocytochemistry and confocal analysis. The expression of visfatin in both cell types showed a predominantly cytoplasmic distribution, although there was also

evidence of nuclear staining in LNCaP cells (Figure 3.2 Arrow). Nuclear and cytoplasmic staining was seen in all cells with a qualitative ratio of approximately 1:4.

VISFATIN PROTEIN EXPRESSION IN PROSTATE TISSUE.

Immunohistochemistry was used to study visfatin protein expression in human benign and malignant prostate tissue (Figure 3.3). Visfatin staining was detected in both benign and malignant tissue, with glandular epithelial cells showing high levels of expression. Interestingly, visfatin demonstrated both nuclear and cytoplasmic staining in both normal and malignant cells. Qualitative analysis revealed a ratio of 1:5 (nuclear:cytoplasmic).

REGULATION OF VISFATIN MRNA BY EXOGENOUS VISFATIN

Real time PCR was used to identify if exogenous visfatin plays a role in regulating visfatin mRNA expression. PC3 cells showed down regulation in mRNA expression in response to treatment with exogenous visfatin at dose ranges greater than 50ng/ml at 4 hours (figure 3.4a) and this appeared to be sustained at 24 hours although only statistically significant at 200ng/ml at this time point (figure 3.4b). As visfatin appeared to have no functional effect in LNCaP cells (see chapter 4) no further studies were carried out using the LNCaP cell line with respect to visfatin.

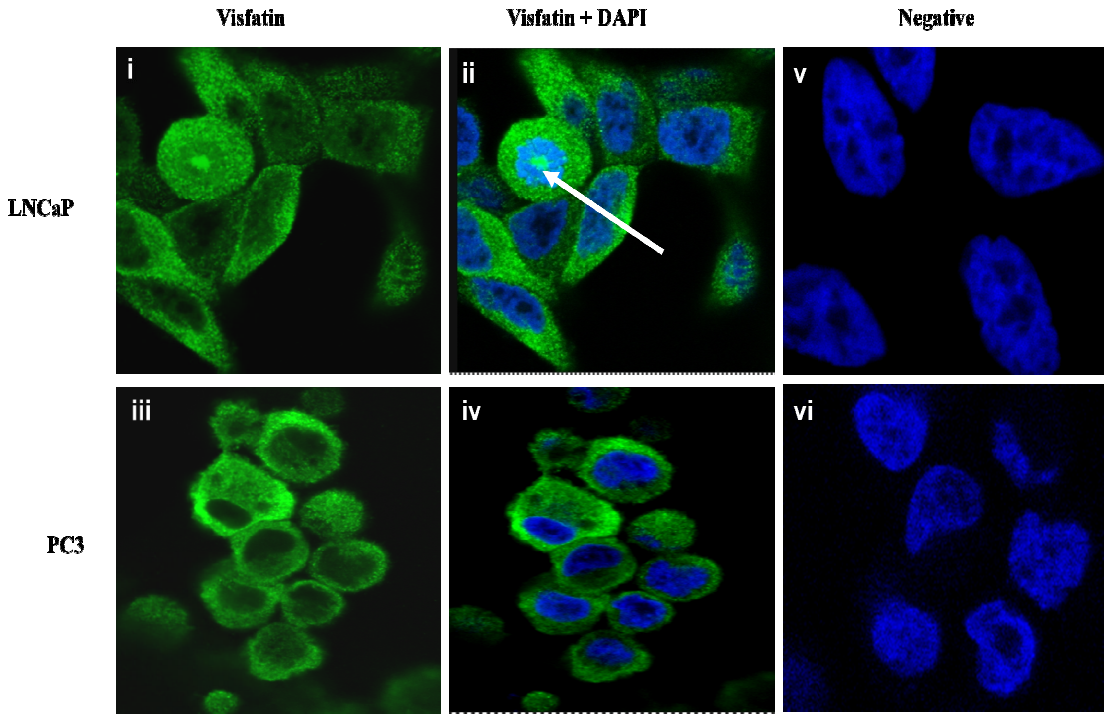

Figure 3.2 Immunocytochemical and confocal analysis were used to study visfatin protein expression in LNCaP and PC3 prostate cancer cell lines. Visfatin protein was detected in the cytoplasm of both cell lines (green), and following co-visualization with the DAPI nuclear marker (blue), nuclear staining for visfatin was also detected in LNCaP cells (arrowed).

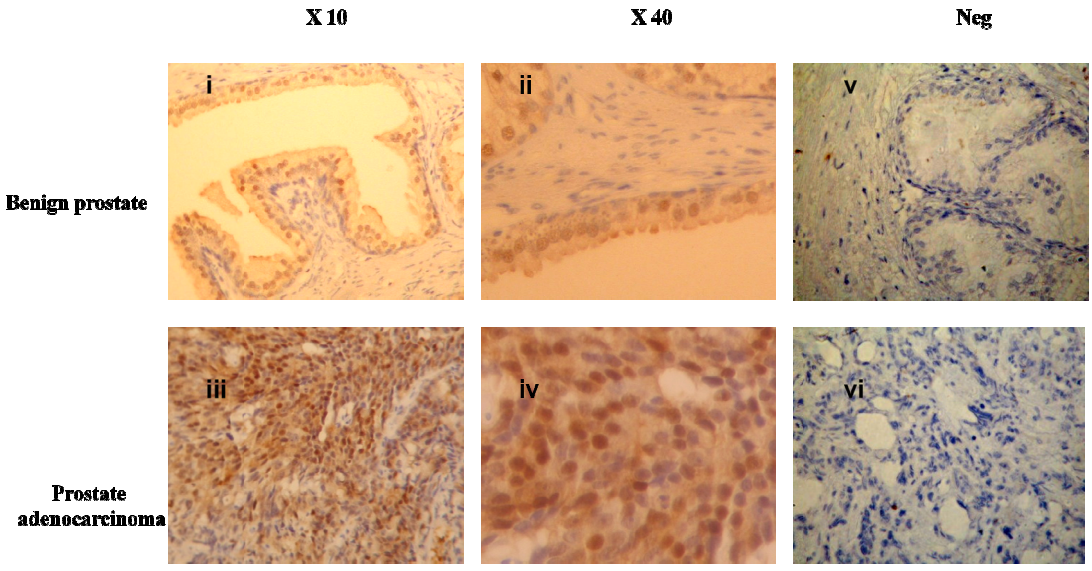

Figure 3.3 Immunohistochemical analysis of benign and adenocarcinomatous human prostate cancer tissue. Visfatin expression was detected in the glandular epithelial cells of both benign (i, ii) and malignant tissue (iii, iv), with evidence of cytoplasmic and nuclear staining. Visfatin stained brown.

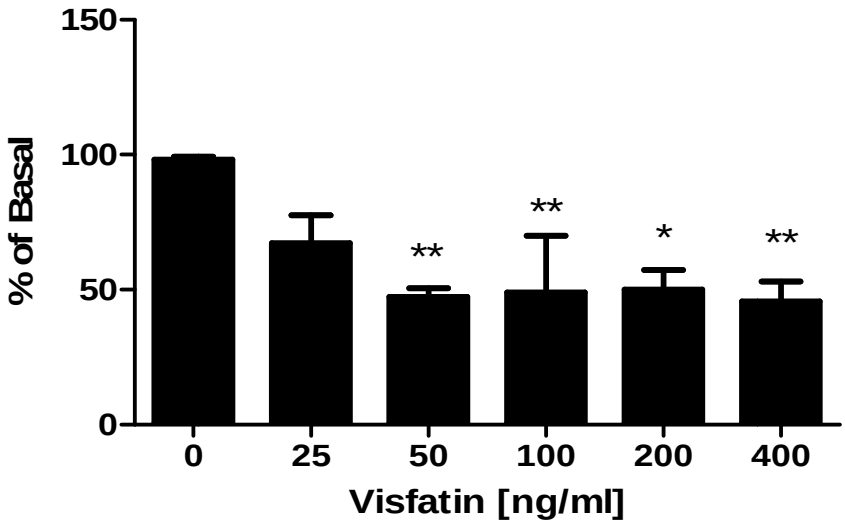

Figure 3.4a Effect of Visfatin on PC3 cell gene activation using real time PCR: Effect of visfatin [0– 400ng/ml] on PC3 cell Visfatin RNA expression at 4 hours (fig 3.4a). Data are expressed as relative to basal proliferation; n = 3; *p<0.05, ** p < 0.01.

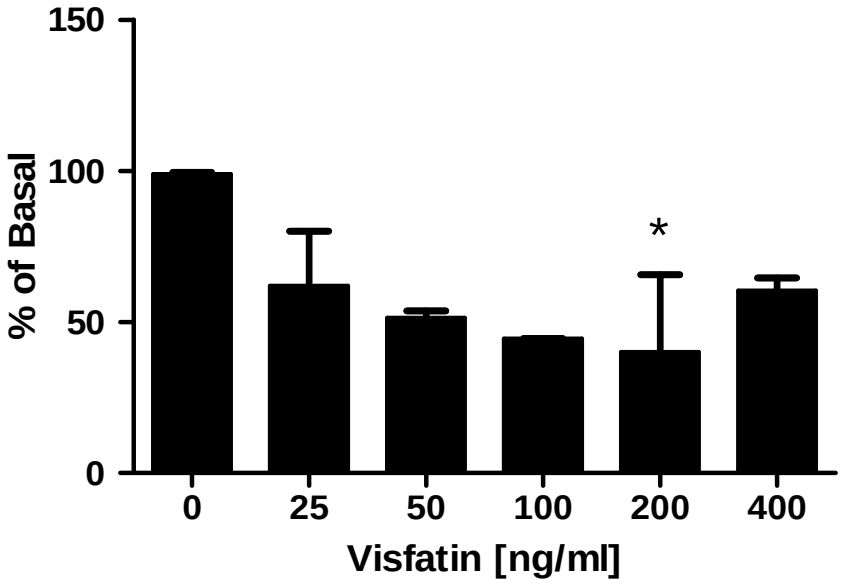

Figure 3.4b Effect Of Visfatin on PC3 cell gene activation using real time PCR: Effect of visfatin [0– 400ng/ml] on PC3 cell Visfatin RNA expression at 24 hours. Data are expressed as relative to basal proliferation; n = 3; * $p < 0.01$.

DISCUSSION

This initial part of the study was undertaken as a starting point to determine if there was a detectable level of visfatin present in prostate cancer cells, and therefore could visfatin be involved in the pathogenesis or aetiology of prostate cancer. Observations in this area could therefore potentially lend support to visfatin as a mechanism in the association between obesity and prostate cancer. Although Berndt et al (Berndt, Kloting et al. 2005) has shown an inverse relationship between plasma visfatin levels and levels in the subcutaneous fat suggesting visfatin auto-regulation there is no data on the correlation in prostate cancer cells given the description of both an intracellular and extracellular version of this interesting molecule.

The result of this study demonstrates the novel expression of visfatin in LNCaP and PC3 cell lines and human prostate tissue. It also describes the novel role of visfatin auto-regulation in PC3 prostate cancer cell lines.

These data in prostate cells are consistent with previous findings of visfatin expression in other normal tissues such as lymphocytes, bone marrow, liver and muscle (Samal, Sun et al. 1994) and also malignant tissues such as breast and colorectal cancer (Hufton, Moerkerk et al. 1999; Van Beijnum, Moerkerk et al. 2002; Folgueira, Carraro et al. 2005). In my study, immunocytochemistry and immunohistochemical analysis of prostate cancer cell lines and tissue, demonstrated cytoplasmic and nuclear staining for visfatin. This is in keeping with the literature which has shown in the PC-12 (adrenal) and Swiss 3T3 (fibroblast) cell lines the identification of visfatin in both nuclear and cytoplasmic compartments and this is thought to be dependent on the stage of the cell cycle (Kitani, Okuno et al. 2003). Immunohistochemical analysis did not suggest any difference between cancerous and benign sections of tissue with respect to visfatin staining. This was purely a qualitative rather than quantitative analysis and further studies would be required to confirm this observation. This could be performed in the future but would require a greater volume of tissue collection and new ethical approval and analysis using techniques such as western blotting in the first instance.

Visfatin has been shown to act as a novel inhibitor of apoptosis in response to a variety of inflammatory stimuli (Jia, Li et al. 2004). This is of significance with regards to prostate cancer as low grade, chronic inflammation has been proposed as a risk factor for prostate carcinogenesis (Palapattu, Sutcliffe et al. 2005); obesity itself is also recognized as being a state of chronic low-grade inflammation (Visser, Bouter et al. 1999). The significance of these findings are supported by discovery of the function of visfatin as NMPRTase (Rongvaux, Shea et al. 2002). Of even more importance is the

finding that inhibition of visfatin by FK866 results in depletion of intracellular NAD^+ and the induction of apoptosis in tumour cells (Drevs, Loser et al. 2003; Hasmann and Schemainda 2003; Khan, Tao et al. 2006). An interesting finding by the group Samal et al 1994 was that the 3' untranslated region of the mRNA has been shown to have multiple TATT and message-destabilizing sequences that are usually found in the labile mRNA of cytokines and oncogenes (Samal, Sun et al. 1994).

Visfatin expression and its subsequent activity may well represent a physiological response to meet the increased demand for NAD in proliferating cells. It should also be considered that increased visfatin expression in cancer cells with a high turnover rate correlates to a pathological state and provides cancer cells with the capacity to fuel its high metabolic rate.

I have commented on the compartmentalisation of visfatin into intra and extracellular forms and this is corroborated by the detection of the peptide in the circulation. This has raised the question that visfatin may function as a possible cytokine-like secreted protein (Rongvaux, Shea et al. 2002) as well as its well documented enzymatic action. This is still a hotly contested issue however and despite visfatin, under the guise of PBEF, having been documented to have mRNA encoding the 52 kDa polypeptide it lacks the typical signal sequence for secretion which makes it surprising that visfatin secretion has been demonstrated from activated lymphocytes and HeLa cells (Samal et al. 1994). One hypothesis regarding this surprising finding is that high circulating levels of visfatin in obesity are secondary to visfatin release during adipocyte necrosis/apoptosis, which occurs in obese states (Rongvaux, Shea et al. 2002; Stephens and Vidal-Puig 2006). The presence of circulating visfatin is however undisputed and the mechanism of its action remains unknown and may be via an undiscovered receptor. It is interesting therefore that although obesity and elevated

levels of various adipokines including visfatin are postulated to increase prostate cancer risk high extracellular visfatin appears to have a negative feedback effect on visfatin mRNA production in the prostate. This may simply be a protective mechanism or suggest that intracellular visfatin has a less important role than extracellular visfatin. Recent studies have suggested that this is not the case (Wang, Hasan et al. 2011). It should also be heavily considered that an isolated effect does not represent the holistic in-vivo environment.

CONCLUSION

This initial study provides novel data identifying visfatin in prostate cell lines and tissue including prostate cancer. Concurrent high circulating visfatin, as occurs in obesity, combined with increased intracellular prostatic visfatin expression may represent a possible mechanism whereby these two disease states may be associated although the above data implies each mechanism may exert separate effects that combine to increase risk. The remainder of this thesis will look to identify the role that visfatin has in prostate cancer biology and identify mechanisms of action.

CHAPTER 4

FUNCTIONAL EFFECTS OF VISFATIN ON PROSTATE CANCER CELLS LINES

CHAPTER 4 FUNCTIONAL EFFECTS OF VISFATIN ON PROSTATE CANCER CELL LINES

INTRODUCTION

It has been well documented that numerous adipokines can exert varying effects on prostate cancer cell models Mistry, Digby et al. 2007). Prior to this study, there were no published data with respect to visfatin and its effects in prostate cancer cells or tissue. Visfatin has been documented to have elevated serum level in obese patients and it would therefore be reasonable to postulate that it is a contributory factor in leading on to aggressive prostate cancer (Haider, Schindler et al. 2006). In stark contrast the serum levels of adiponectin (which is the most abundantly circulating adipokine) are inversely correlated with visceral obesity (Arita, Kihara et al. 1999), adiponectin levels also appear to be lower in prostate cancer patients, with levels being inversely proportional to Gleason score (Freedland 2005; Goktas, Yilmaz et al. 2005). Interestingly, adiponectin has been shown in its high molecular weight form (HMW) to inhibit the proliferation of androgen dependent (LNCaP-FGC (fast growing colony)) and androgen-independent (DU145 and PC-3) prostate cancer cell lines at sub-physiological concentrations (Bub, Miyazaki et al. 2006).

The adipokine leptin is another molecule that has been studied in prostate pathology. Leptin circulates in plasma levels that are proportional to visceral fat accumulation (Considine, Sinha et al. 1996), and they have also been demonstrated to be significantly higher in men with high grade, advanced prostate cancer (Chang, Hursting et al. 2001; Saglam, Aydur et al. 2003). As well as the plasma correlation leptin expression has also been observed in prostate cancer cell lines and human tissue

(Cioffi, Shafer et al. 1996; Onuma, Bub et al. 2003). Leptin is also known to have a proliferative effect in DU145 and PC-3 prostate cancer cells but not in androgen-dependent LNCaP-FGC cells (Onuma, Bub et al. 2003; Somasundar, Yu et al. 2003; Somasundar, Frankenberry et al. 2004).

During in vitro studies it is easy to fixate on solitary interactions of one cytokine either elevating or decreasing the basal level and one should remain constantly aware that there are multiple factors in-vivo that can account for changes in homeostasis. An example of this is shown in the opposing actions and properties of adiponectin and leptin. It has been demonstrated in breast cancer patients, where the serum leptin:adiponectin ratio was found to be higher in cancer patients compared to controls and displayed a positive correlation with tumour size (Chen, Chung et al. 2005). Subsequently Mistry et al (Mistry, Digby et al. 2008) also demonstrated that leptin and adiponectin interact causing a decrease in prostate cancer cell proliferation via the regulation of p53 and bcl-2 gene expression. The group concluded that high leptin and low adiponectin levels may be important in leading obesity-related prostate cancer progression.

In human endothelial cells as well as vascular smooth muscle cells, incubation with exogenous visfatin has been shown to stimulate cell proliferation (Adya, Tan et al. 2008; Wang, Xu et al. 2009). More recently it has also been shown to induce proliferation in MCF-7 cells (human breast cancer cells) (Kim, Kim et al. 2010).

As described in the introduction, visfatin had also been identified as nicotinamide phosphoribosyltransferase - Nampt, and this provides further intonation for the multifunctional role for visfatin. It has been described as a vital rate limiting enzyme in

nicotinamide adenine dinucleotide (NAD^+) biosynthesis in particular the conversion of nicotinamide to nicotinamide mononucleotide (NMN) (Rongvaux, Shea et al. 2002).

Thus far we have discussed the possible rationale for examining cell proliferation on relation to visfatin. With respect to neoplastic states there is a state of flux between dysregulated cell proliferation and apoptosis.

Apoptosis is an important physiological phenomenon that is a regimentally controlled process of programmed cell death. It is involved in normal development and cell biology and disruption to this process is highly significant in tumourogenesis (Kerr, Wyllie et al. 1972; Thompson 1995). Any potential roles of visfatin as an inhibitor of apoptosis in cancer are still as yet unclear and not defined; the compound (e)-n-[4-(1-benzoylpiperidin-4-yl) butyl]-3-(pyridin-3-yl) acrylamide (FK866) is a long established very selective non-competitive inhibitor of visfatin (Hasmann and Schemainda 2003). The compound interferes with NAD+ production and hence total cell reduction of NAD+ concentration. FK866 leads to an effect on pyridine nucleotide biosynthesis i.e. the induction process and then disrupts the mitochondrial membrane potential i.e. the effector process and finally leads to irreversible degradation via cellular caspases (Holen, Saltz et al. 2008). It is therefore of great interest and importance that inhibition of visfatin by FK866 induces apoptosis in tumours (Drevs, Loser et al. 2003; Hasmann and Schemainda 2003; Khan, Tao et al. 2006; Kim, Lee et al. 2006). During this study FK866 has been used to help evaluate the effect of visfatin in this prostate cancer model. Given this potential therapeutic target clinical trials are currently in progress to explore the role of FK866. (Holen, Saltz et al. 2008).

HYPOTHESIS

Visfatin has functional effects on prostate cancer and these can be demonstrated using in vitro models.

AIMS

- To identify if visfatin has proliferative effects on both LNCaP and PC3 cell lines
- To identify if visfatin is anti-apoptotic in LNCaP and PC3 cells
- To identify if visfatin alters the cellular migration of prostate cancer cells

METHODS

To assess cellular proliferation a MTS based proliferation assay was performed as outlined below. This method is well established both in the group and in the literature (Mistry, Digby et al. 2008; Wang, Hasan et al. 2011). A DNA fragmentation assay was used a marker of late stage apoptosis to elucidate apoptosis. This was chosen as it was consistent with the literature to date in particular with reference to the inhibitory molecule FK866 (Hasmann and Schemainda 2003). As surrogate for cellular 'invasion' a wound scratch assay was performed. This assay was used in part due to limitations with time and funding, however it has been well documented as a valid assay in CaP models (Hermani, De Servi et al. 2006; Gkika, Flourakis et al. 2010)

All of the data in the present study are expressed as mean± SEM. Parametric comparisons between groups were made by ANOVA in keeping with published work from the group (Mistry, Digby et al. 2008; Patel, Mistry et al. 2010). The test was chosen based on its established use, as well as the notion that performing multiple two-

sample t-tests would be most likely result in type 1 error. For this reason, ANOVAs are useful in comparing two, three or more means. If significance (P<0.05) was present, a post hoc Tukeys multiple-comparison test was performed. All statistical analysis was performed using Graph Pad software (version 4.0)

PROLIFERATION ASSAY

10,000 cells per well of LNCaP and PC3 cells were uniformly seeded onto 96-well plates, cultured for 48 hours and incubated with serum-free media for 24 hours before treatment with visfatin (0-400 ng/ml) and IGF-1 (10 ng/ml) for 24 or 48 hours. Cell proliferation was assayed using CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay according to manufacturer's instructions by adding 20µl of 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium MTS reagent was to 100µl of fresh culture medium in each well. This is an MTS-based assay that quantifies the amount of formazan produced by metabolically active cells as measured by absorbance at 490nm using a spectrophotometric plate reader following an incubation period of 2 hours, which is directly proportional to the number of living cells in culture (according to product literature). The percentage of the absorbance was calculated against untreated cells.

This assay was used as it had already been well established within the group and has been commonly used in this cell model as a surrogate for cell proliferation.

APOPTOSIS ASSAY

DNA Fragmentation ELISA (Roche):

A photometric enzyme linked immunosorbent assay (ELISA) was used for the detection of 5'-Bromo-2'-deoxy-uridine (BrdU)-labelled DNA fragments in cell lysates as an alternative to the [^{3}H]-thymidine assay. BrdU is used as a metabolic labelling agent by

the nuclear DNA of target cells. This can then be easily detected by using a monoclonal antibody directed against the BrdU in an ELISA.

10000 cells were cultured to 80% confluent in 96 well plates. Following serum depletion they were then batch labelled with BrdU [10µM]. After overnight incubation at 37ºC all BrdU containing media wash aspirated and washed off with PBS.

BrdU labelled cells were then incubated with visfatin [0-400ng/ml], NMN [100µM], or FK866 [10µM] or a combination for 24 hours. At the end of the incubation all supernatant is removed and 200µl of incubating solution was added and incubated for 30 mins to lyse the cells. The cells were then centrifuged and the supernatant containing LMW-DNA of the cellular lysate was analyzed for apoptosis ELISA. Camptothesin [2µM] was used as a positive control. To adsorb the Anti-DNA antibody onto the wells of the 96 well plate 100µl of anti-DNA coating solution was added to each well and left to incubate at 37ºC for 1 hour. After washing the plate to remove the coating solution, NSB was prevented by adding 200µl of incubation solution and left for 30 mins and then washed. 100 µl of the supernatant of the cell lysates was added to the microplate allowing BrdU-labeled DNA fragments in the sample bind to the immobilized Anti-DNA antibody. This incubation was left for 90 mins at room temperature after covering the plate. The plate was then washed several times with wash solution. The final wash solution was then left in situ prior to irradiation in a microwave oven for 5 mins to allow denaturing of the immunocomplexed BrdU-labeled DNA-fragments. 100µl of Anti-BrdU antibody peroxidase conjugate (Anti-BrdU-POD) was then added and incubated for 90 mins which reacts with the BrdU-labeled DNA to form an immunocomplex. After this step the plate was washed and 100µl of substrate solution was added to each well incubated in the dark for 20 mins before adding 20µl of stop solution and incubating for a further minute and then reading the plate at 450nm.

The absorbance was then calculated as values relative to basal and analyzed using graphpad software for statistical significance.

WOUND SCRATCH ASSAY

A complicated and highly methodical process is required for tissue wound healing. The processes required can include infiltration of inflammatory cells which is needed to eradicate any necrotic tissue from the site, followed by a mechanism which increases vascularization mediated via angiogenic factors, and increased cell proliferation and extracellular matrix deposition. A method of estimating the migration as well as proliferation of a cell type that has been used throughout the literature is the wound healing assay and is a technique that can employ different cells and culture conditions. The assays generally require the growing of a confluent cell monolayer. A small area within the monolayer is then disrupted by scratching a line through the layer. The open gap is then inspected microscopically over a defined time as the cells migrate in and fill the ‘wound’. This “healing” can take from several hours to over a day depending on the cell type, conditions, and the extent of the “wounded” region. The cells must be nearly confluent (~90%) for this to work and all steps done in a sterile fashion. The protocol used was similar to that described by Denker 2002 (Denker and Barber 2002).

PROTOCOL

Cells were cultured to confluence or near (>90%) confluence in 12 well plates. They were then washed with PBS and serum depleted overnight.

On the day of the assay:

Using a sterile 200µl pipette tip, a ‘wound’ was scratched on to the plate and the cells were rinsed (very gently as sheets of the cells may lift off if you are not careful) with PBS and replaced with 1.5 ml of media containing any additives (visfatin, FK866,

NMN). Photos were taken via the microscope at time points 6, 12 and 24 hours at 10x and 40x magnification. Measurements were taken in micrometers of the width of the wound at each time point and compared to basal. All experiments were carried out to n=3.

RESULTS

VISFATIN REGULATION OF PROSTATE CANCER CELL PROLIFERATION

Incubation of cells with visfatin alone resulted in a significant concentration-dependent increase in proliferation after 24-hours incubation in PC3 cells compared to control, ($p<0.001$ at 200 and 400 ng/ml) (Figures 4.1a/4.1b). In contrast treatment with visfatin alone had no significant effect on LNCaP cell proliferation at either 24 or 48 hours (Figures 4.2a/4.2b) compared to control. Given that there was no functional proliferative outcome in the LNCaP cell line no further studies were conducted in this cell line. IGF was used as a positive control to optimise the assay and the results are seen in Fig 4.3a and 4.3b in both PC3 and LNCaP cells respectively.

EFFECTS OF VISFATIN INCUBATION ON PC3 CELL MIGRATION

Visfatin treatment alone resulted in a significant reduction of the wound distance at 24 hours as compared to basal ($p<0.001$ at 400 ng/ml). NMN also had a significant decrease in wound distance but less so than visfatin alone ($p<0.001$ at 100µm). 10µm FK866 partially inhibited the action of visfatin although there was still closure of the wound distance. The addition of NMN further negated the effect of the FK866 on inhibition of cell migration but it still did not revert to the same wound distance as visfatin treatment alone. (Figure 4.4)

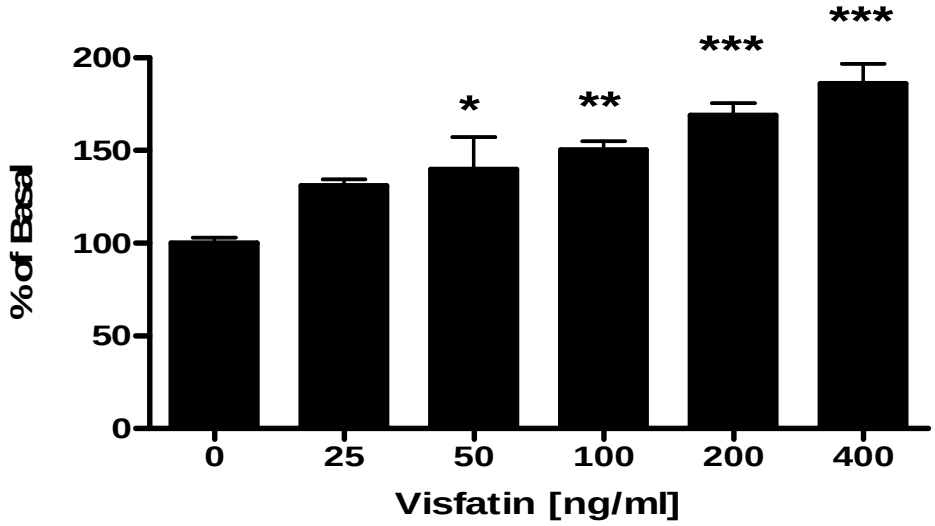

Figure 4.1a Effect of visfatin on PC3 cell proliferation at 24 hours. Effect of visfatin alone [0– 400ng/ml] on PC3 cell proliferation. Data are expressed as relative to basal proliferation; n = 4 for all experiments, *p<0.05, **p<0.01, *** p < 0.001.

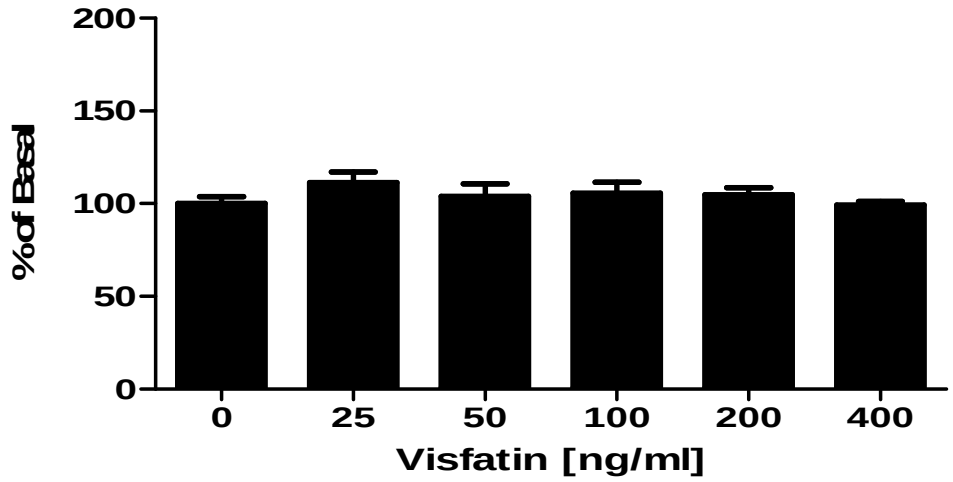

Figure 4.1b Effect of visfatin on PC3 cell proliferation at 48 hours. Effect of visfatin alone [0– 400ng/ml] on PC3 cell proliferation. Data are expressed as relative to basal proliferation; n = 4 for all experiments.

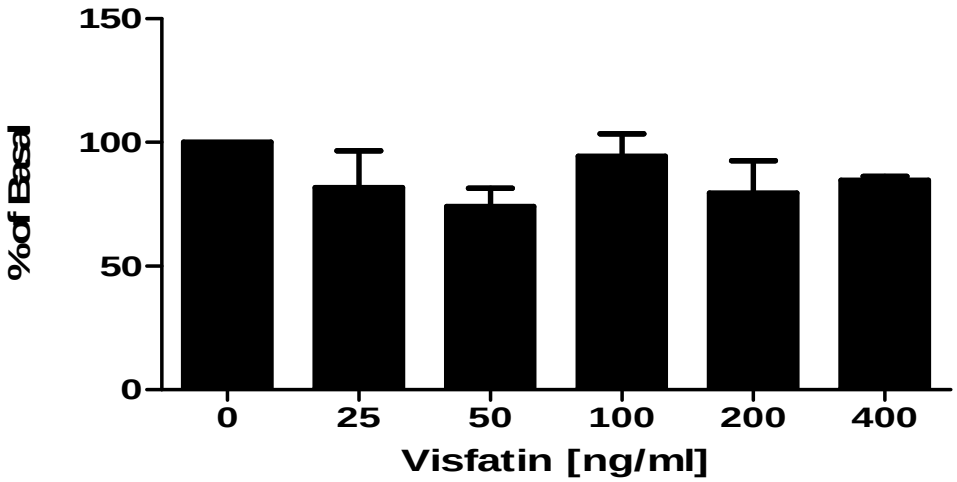

Figure 4.2a Effect of visfatin on LNCaP cell proliferation at 24 hours. Effect of visfatin alone [0– 400ng/ml] on LNCaP cell proliferation. Data are expressed as relative to basal proliferation; n = 4 for all experiments.

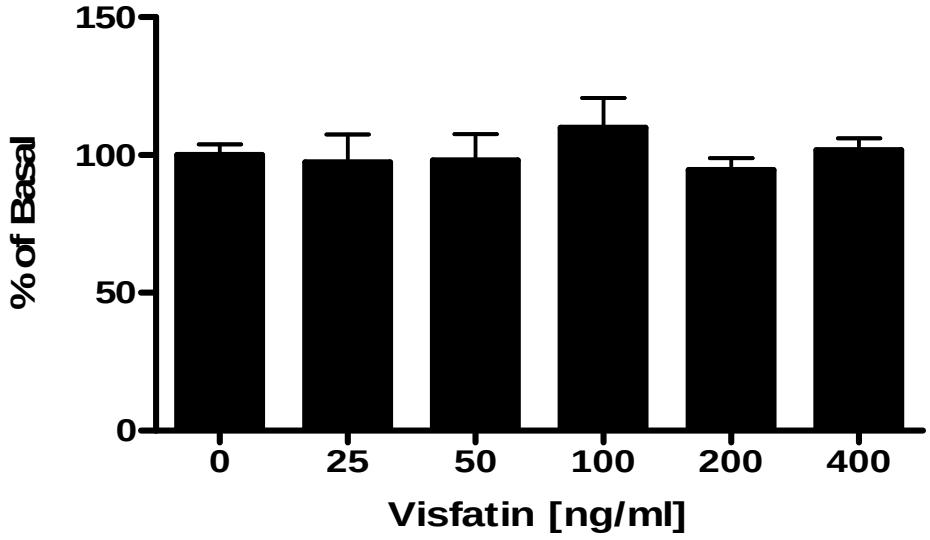

Figure 4.2b Effect of visfatin on LNCaP cell proliferation at 48 hours. Effect of visfatin alone [0– 400ng/ml] on LNCaP cell proliferation. Data are expressed as relative to basal proliferation; n = 4 for all experiments.

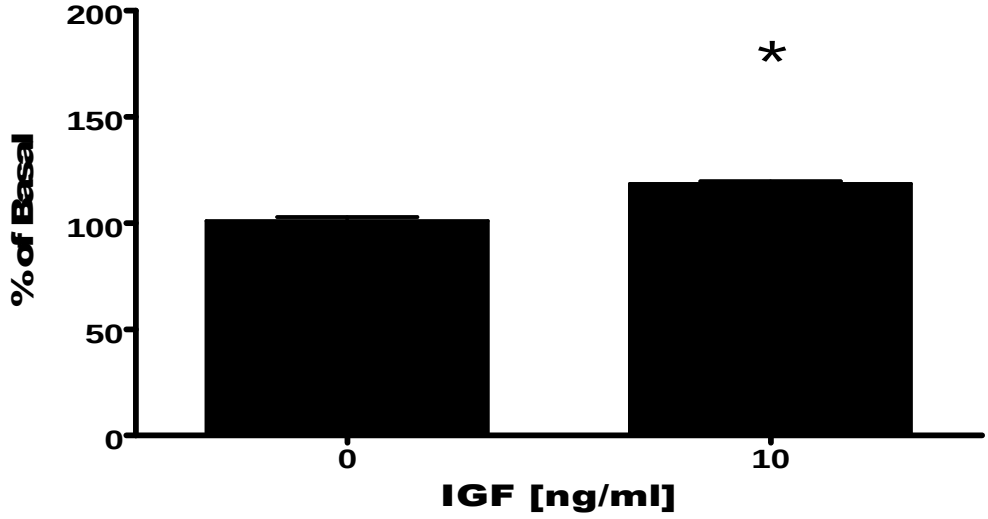

Figure 4.3a Effect of IGF on PC3 cell proliferation. Effect of IGF alone [0–10ng/ml] on PC3 cell proliferation. 24 hour data are expressed as relative to basal proliferation; n = 4 for all experiments, * $p < 0.05$.

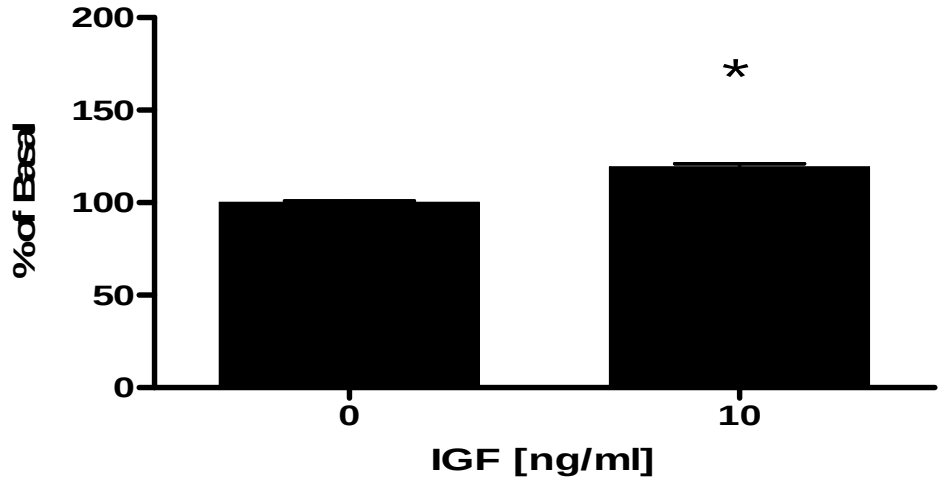

Figure 4.3b Effect of IGF on LNCaP cell proliferation. Effect of IGF alone [0–10ng/ml] on LNCaP cell proliferation. 24 hour data are expressed as relative to basal proliferation; n = 4 for all experiments, * $p < 0.05$.

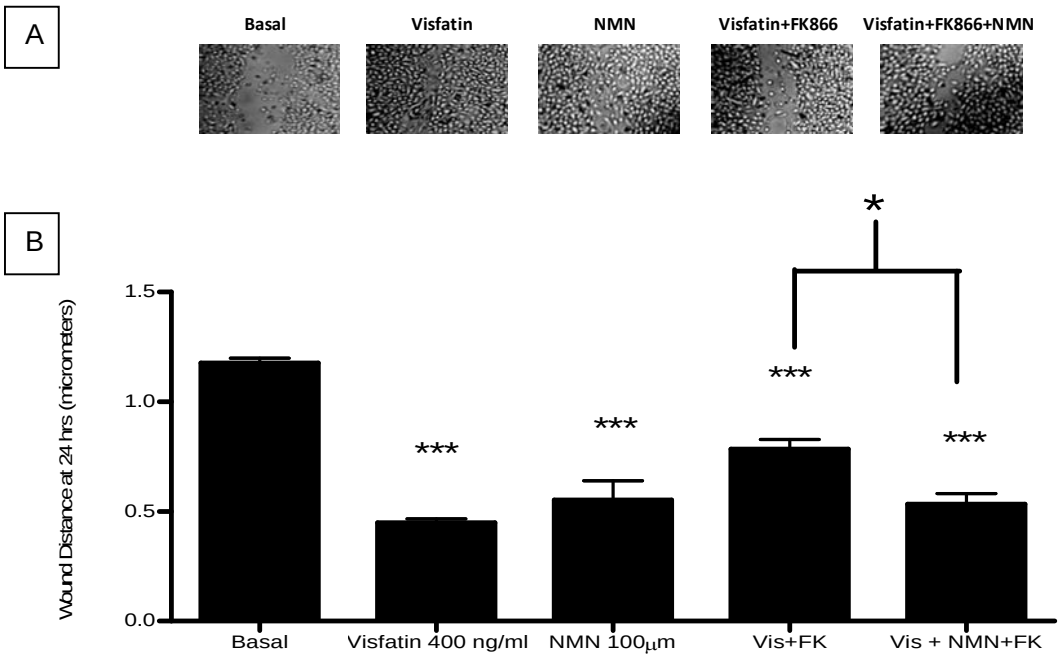

Figure 4.4 Wound Scratch Assay demonstrating the effect of visfatin [400 ng/ml] and NMN [100μM] and in combination with FK866 [10nM] on the PC3 cell migration across an artificial wound. A) representative photographs of each treatment and B) graphical representation of 24-hour data; n=3 for all experiments, *p<0.05, ***p< 0.001

EFFECTS OF VISFATIN ON APOPTOSIS

A DNA fragmentation assay was used to assess for apoptosis. Visfatin treatment did not significantly alter apoptosis. The positive control using H2O2 confirmed that the assay was successful although weakly so ($p < 0.05$). There was a positive result at 400ng/ml of visfatin although not statistically significant. Fig 4.5

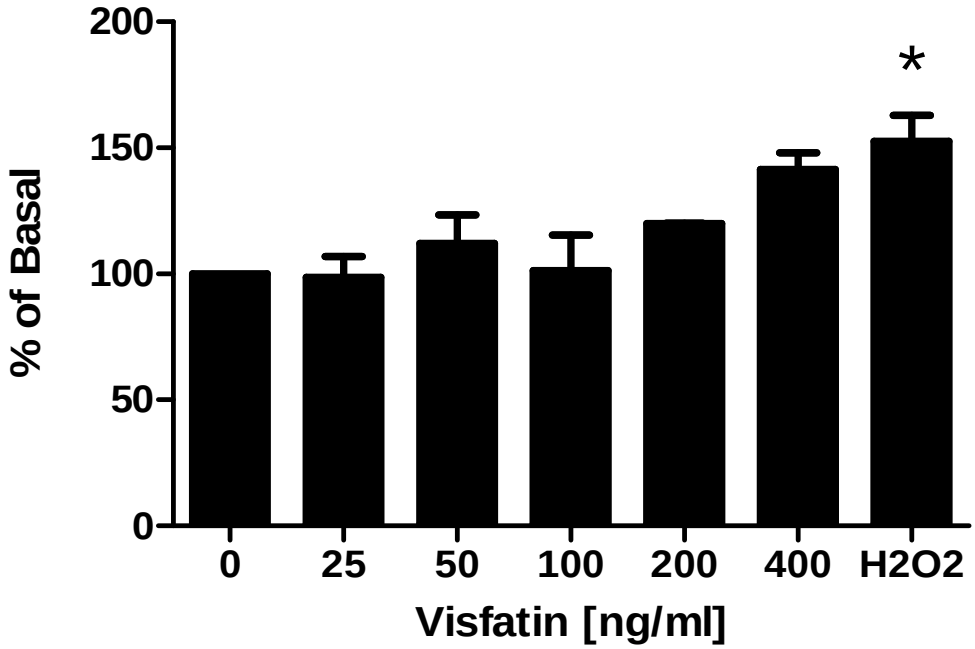

Figure 4.5 Effect of Visfatin [0– 400ng/ml] on PC3 cell apoptosis. Hydrogen Peroxide (H2O2) [10mM] was used as a positive control. 24-hour data are expressed as relative to basal; n = 4 for all experiments, * $p < 0.05$.

DISCUSSION

This is the first study to report a proliferative effect of exogenous visfatin incubation on prostate cancer cells. This study reports clear differences in the proliferative response of two different prostate cancer cell lines to visfatin. Visfatin demonstrated an increase cellular proliferation using the cell proliferation assay at 24 hours but not at the 48 hour time point (Fig 4.1a/b). The assay used is a correlate for cell number and by inference a surrogate for proliferation. The main limitation therefore, is that the assay is not a direct measure of cellular proliferation or apoptosis. At 48 hours the assay suggests the proliferation of cells returned to baseline levels following an initial increase at 24 hours. Examining the result more closely with respect to this assay one could conclude the cell number had actually reduced i.e. undergone rapid apoptosis/necrosis or the rate of proliferation had declined rapidly relative to basal levels. One explanation for this may be that the initial increase in cell number over the first 24 hours allowed the cells to reach full confluence in the treated cells and prevent further increase in cell numbers. Further correlation with DNA quantification assays may increase the validity of these results and help answer the above discrepancy. The SYBR-DNA labelled assay has been shown to be more accurate in quantifying cell number, proliferation and cell cycle arrest as analyzed by DNA content in other cancer models (McGowan, Alling et al. 2011). Visfatin-induced increases in cell proliferation were observed in the androgen-independent PC3 cells, but not the androgen-dependent LNCaP cells. This suggests that visfatin can exert differing effects based on characteristics of the cell exposed to it. One of the key differences in the characteristics of the two cell lines used is the androgen status of these cells. PC3 cells represent an in vitro model for androgen-insensitive prostate cancer, and thus do not express the classical intracellular androgen receptor (AR) as compared to the androgen sensitive LNCaP cells. The

difference in response to visfatin between the two cell lines raises the possibility of androgen or androgen receptor status being of potential significance in the modulation of prostate cancer cell biology by visfatin. It is also a possibility that the absence of additional androgen in the treatment media may have influenced the results seen in the 'androgen sensitive' LNCaP cell line. Future studies to strengthen these findings in this cell line could therefore involve comparing visfatin exposure in the presence and absence of androgen. The possibility of an interaction between AR and visfatin in prostate cancer cells is also an area for future research but fell beyond the remit of this thesis.

The effect on proliferation was demonstrated in a concentration-dependent manner (Fig 4.1a) and perhaps more importantly at levels of visfatin similar to those that have been demonstrated in the obese population (Haider, Schindler et al. 2006). This provides novel evidence that elevated levels of circulating visfatin may influence the growth of prostate cancer tissue. As touched on in the introduction, in addition to being present intracellularly, visfatin has also been detected in the circulation, suggesting that it may function as a cytokine as well as/or an enzyme. However, having said this there is some debate with regards to the function of visfatin as a cytokine-like secreted protein (Rongvaux, Shea et al. 2002; Fukuhara, Matsuda et al. 2005). One of the theories as to why visfatin was thought to not be an extracellular or circulating cytokine was based on the fact that although visfatin cloning and characterization studies have found visfatin mRNA to encode a 52 kDa polypeptide, it was found to lack a typical signal sequence for secretion; despite this theory it has, however, been shown to be secreted from activated lymphocytes and HeLa cells (Samal, Sun et al. 1994). In vivo however as is often the case, multiple factors can contribute to altered homeostasis and thus it was postulated that the high circulating levels of visfatin associated with obesity may be due

to visfatin release during adipocyte necrosis/apoptosis, which occurs in obese states, rather than true secretion by adipocytes (Rongvaux, Shea et al. 2002; Stephens and Vidal-Puig 2006). The presence of visfatin in the circulation cannot be disputed despite the theory, and therefore it is possible that it may exert biological effects via an unknown receptor. In chapter three we have demonstrated that exogenous visfatin down regulates visfatin mRNA expression and the mechanism of action by which exogenous visfatin induces its proliferative effects remains to be elucidated. It may be via the surrogate action of NMN by extracellular production and action by a secondary mechanism. This is a well documented effect in pancreatic islets as demonstrated by Revollo et al (Revollo, Korner et al. 2007) and the theory well described by Garten et al (Garten, Petzold et al. 2009).

Having shown that visfatin up-regulates proliferation I looked to identify a more functional assay to demonstrate the effect of visfatin on PC3 cells. To do this we used a wound scratch assay. This assay demonstrated that an artificially created 'wound' was bridged much faster under the influence of visfatin compared to without (Fig 4.4). Although this assay has been previously cited in the literature, in particular in cancer models (Gkika, Flourakis et al. 2010), it is only a surrogate for migration as the assay does not control for cellular proliferation as a mechanism of bridging the wound. The migratory characteristics of cancer cells is a target of therapeutics and can also serve as an indicator of tumour aggressiveness and malignancy (Gkika, Flourakis et al. 2010). Within the limits of this experiment as described above, this assay gives slightly more insight into functional assessment as it demonstrates the more natural proliferative behaviour of malignant cells to invade and grow into space. It should be commented that the technique is semi-quantitative and not without its flaws and there are more quantitative methods available such as in vitro cell invasion chamber assays

(Wang, Hasan et al. 2011). Due to resource and time constraints I did not proceed to these. We demonstrated that visfatin was more 'proliferative' than NMN. FK-866 did inhibit proliferation; however the effect was partially reversed by visfatin.

FK866 is a long established very selective non-competitive inhibitor of Nampt (Hasmann and Schemainda 2003). The compound interferes with NAD+ production and hence total cell reduction of NAD+ concentration. It was also of interest that FK866 did reduce cell migration however not completely in the presence of visfatin. In this study a dose of 10nM was used in keeping with concentrations used by Hasmann et al in their paper examining apoptotic effects of FK-866 in HepG2 cells. It would be anticipated that FK866 should induce apoptosis of the cells and therefore no migration would be anticipated in its presence and in fact cell death would be observed. Interestingly Hasmann et al found that cell viability based on a tetrazolium salt assay was reduced early after incubation with FK-866 and cell proliferation was still noted up to day 3 and apoptosis followed at a much more delayed time point of around day 4 onwards. This supports the theory that FK-866 induces apoptosis via NAD+ depletion at a delayed state and if NAD+ production/levels are preserved i.e. by the use of exogenous visfatin viability can be preserved. Using the wound scratch assay, it serves as a semi-quantitative assay of cell migration. Accepting these limitations it was noted that visfatin's effect on enhancing cell migration clearly can, in part, be reduced from as early as 24 hours. This effect is enhanced by using NMN as demonstrated in the final wound scratch assay. This corroborates the suggestion that it is indeed the rate limiting step catalysed by visfatin that is of importance. Replenishment of the intermediary product, NMN, can to some extent recover the inhibition of FK-866 lending further support to Garten et al's theory i.e. by replenishing the exogenous NMN its action via an unknown mechanism allows intracellular NAD+ replenishment (Garten,

Petzold et al. 2009). This would explain why a combination of visfatin and NMN incubation closes the wound distance significantly more than visfatin alone in the presence of FK-866. It can be speculated that should a viability study be done in the same population as per Hasmann's findings then it would probably decreased and apoptosis would be noted. This would certainly be a future study.

As part of the functional spectrum of visfatin this part of the study also looked at the effect of visfatin on apoptosis. As discussed neoplasia is a balance between apoptosis and proliferation. The literature suggests that visfatin is a proliferative agent however little is documented as to whether it has a mechanism of action via anti-apoptosis. A study by Kendal-Wright et al suggested that over-expression of visfatin in the amniotic sac cells in response to stretch was anti apoptotic (Kendal-Wright, Hubbard et al. 2008). Jia et al (Jia, Li et al. 2004) demonstrated that in sepsis visfatin secretion by activated neutrophils can also inhibit apoptosis. They suggested that visfatin may inhibit the activity of caspase-8, which suggests the possibility that visfatin acts directly at the cell membrane, possibly via a membrane-associated inhibitor of apoptosis such as FLICE-like inhibitory protein (FLIP) or through regulatory phosphatases in the plasma membrane. Visfatin has also been demonstrated to have anti apoptotic effects in human umbilical vein endothelial cells (HUVECs) (Adya, Tan et al. 2008). I therefore looked to identify whether an anti-apoptotic effect was a potential mechanism for the increased migratory capacity of PC3 cells under the influence of exogenous visfatin. Using the PC3 cell line no significant effect was identified with regards to apoptosis. In this particular experiment it was difficult to obtain good optimization as demonstrated by the use of H2O2 although it was weakly significant suggesting the assay itself worked. The findings have confirmed the absence of a significant effect on apoptosis of visfatin however at the maximum dose there is a suggestion that it had some apoptotic effect

≈40% increase. This was not statistically significant however due to the error involved. One explanation could be the onset of apoptosis due to prolonged starvation of the cells for the assay coupled with high dose visfatin treatment. This is an unusual finding as the proliferation assay performed earlier in the study suggested maximal proliferative effect at this concentration. The apoptosis assay used was a DNA fragmentation ELISA. The Cellular DNA Fragmentation ELISA measures apoptosis, necrosis, or cell mediated cytotoxicity by quantifying the fragmentation and/or release of BrdU-labeled DNA. This is a late stage of apoptosis and often referred to as the hall mark of apoptosis as opposed to necrosis. As a marker of late stage apoptosis it could be inferred that the cells may be in early stage apoptosis and either using an alternative assay to capture this event such as a caspase cascade detection assay or simply performing the study at varying time points may be a better option. As the literature suggests that visfatin was a proliferative agent further functional assays were not undertaken with respect to visfatin and apoptosis.

CHAPTER 5

MECHANISMS OF ACTION OF VISFATIN ON PROSTATE CANCER

CHAPTER 5 MECHANISMS OF ACTION OF VISFATIN ON PROSTATE CANCER

INTRODUCTION

Carcinogenesis is a multi-step process which results in deregulation of cellular homeostasis, allowing premalignant lesions to develop into invasive tumors (Gorgoulis, Vassiliou et al. 2005). Detailed mechanisms pertaining to the cell cycle, apoptosis and angiogenesis have been laid out in chapter 1 pages 31-35. It is therefore imperative that multiple avenues are explored to examine the mechanisms of functional effects of agents suspected of being involved in the carcinogenic process.

Visfatin has been shown to elicit a variety of effects in different cell lines through several second messenger systems. This chapter focuses upon the potential mechanisms through which visfatin may elicit its functional effects on PC3 cells. The specific aim for this part of the study was to identify second messengers and angiogenic mechanisms. Given that adipokines, in particular leptin and adiponectin, have been shown previously to influence oncogenes in prostate cancer models (Mistry, Digby et al. 2008) the study also looked at onco- and tumour suppressor genes that may be regulated by exposure to visfatin.

Given that visfatin has been postulated to have insulin-mimetic effects initially thought to be mediated via the insulin receptor, an evaluation of visfatin's effects on gene expression of IGF-1R was performed.

HYPOTHESIS

Having demonstrated the functional effects of visfatin on PC3 cells it is suggested that visfatin exerts its functional effects on prostate cancer cell models via various mechanisms including MAP-kinases, MMPs and BCL-2, BCL2-L1, MCL-1, BAX, IGF-R, gene over/under expression. Thus this study hypothesizes that visfatin modulates MAPK and also effects MMP-2/-9 activity and may therefore effect CaP evolution. It is also hypothesized that visfatin modulates oncogenes as a mechanism of CaP neoplasia.

AIMS

- To identify if visfatin activates common MAPKs in PC3 cell lines
- To identify if visfatin activates MMPs in PC3 cells
- To identify if visfatin alters the expression of various tumour suppressor and oncogenes

METHODS

Methods are detailed below. Protein data was measured by standard western blotting technique and MMP activity was detailed using gelatin zymography. RT-PCR was undertaken to evaluate gene expression data.

MAPK- ERK 1/2 AND P38 MAPK ACTIVATION, MMP-2/9 EXPRESSION

The effect of visfatin on activation of the MAPK (ERK-1/2 and p38) as well as MMP-2 and MMP-9 protein expression in PC3 cells was studied using Western blotting

analysis as per Patel et al and Adya et al (Adya, Tan et al. 2008; Patel, Mistry et al. 2010). Following treatment with visfatin at times points between 0-60 mins for MAPK activity and at 24-hours for MMP analysis), cells were lysed with RIPA buffer. Samples were centrifuged and quantified and subsequently boiled prior to use.

For MAPK analysis 40 µg of each sample was loaded; for MMP analysis 80 µg was used. Samples were separated by 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE), and electro-blotted onto a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was then blocked by incubating with 5 % bovine serum albumin in TBS-T, pH 7.4 for 1-hour at room temperature. Membranes were then incubated with primary antibody phosphorylated ERK1/2, phosphorylated p38 (rabbit polyclonal 1:2000 diluted in 5% BSA/TBS-T), MMP-2 or MMP-9 (mouse monoclonal 1:400 diluted in 5% BSA/TBS-T) overnight at 4 °C. Membranes were then washed thoroughly for 1 hour at room temperature with TBS-T (0.1%) followed by incubation with a secondary anti-mouse horseradish-peroxidase-conjugated secondary antibody (1:2000 dilution) for 1-hour at room temperature. Antibody complexes were demonstrated using a chemiluminescence detection reagent kit (ECL+). To determine MAPK activity the membranes were stripped by submerging in stripping buffer [10% SDS, 1M Tris-HCl pH 6.8, β-mercaptoethanol and incubated at 50^{o}C for 30 minutes with occasional agitation followed by washing with TBS-T at room temperature (10 minutes – twice) and PVDF membranes were blocked in TBS-T and 5% BSA for one hour at room temperature. The membranes were re-probed with primary rabbit-anti-human antibody for total $ERK_{1/2}$ /p38 MAPK [1:1000 dilution] overnight at 4 °C. The following day, membranes were washed with TBS-T for 1 hour and then incubated with the secondary anti-rabbit horseradish peroxidase-conjugated Ig [1:2500 dilution] for one hour at room temperature.

In addition to quantitative loading of gels, the MMP membranes were also re-probed with β-actin antibody (1:10,000 dilution) to determine equal protein loading. With respect to MMP analysis IGF [10ng/ml] was used as a positive control. Studies have demonstrated a role for IGF in *in vitro* prostate cancer models with respect to MMP-2/9.(Saikali, Setya et al. 2008). Band densities were measured using a scanning densitometer coupled to scanning software Scion Image™.

To calculate potential MAPK activation the band densities (calculated using Scion image) of phosphorylated MAPK in the different treatment groups were normalized by dividing with the band intensity of total MAPK detected with an antibody that detects both phosphorylated and un-phosphorylated forms of the enzyme. This corrected for any differences in protein loading. Fold induction is calculated as normalized band intensity relative to basal. For MMP expression a similar calculation was performed however the band intensity was normalised to β-actin.

GELATIN ZYMOGRAPHY

The gelatinolytic activity of MMP-2 and MMP-9 secreted into culture supernatants following treatment with 0 – 800 ng/ml visfatin was measured by gelatin zymography. 10µl zymography sample buffer was mixed with 10 µl of culture supernatant and resolved in 10% SDS-PAGE containing 1 mg/ml of gelatin (Sigma, St. Louis, USA) under non-reducing conditions. After electrophoresis at 4º C, gels were washed twice for 30 mins with renaturation buffer (2.5% Triton X-100) at room temperature, and then incubated overnight in incubation buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 10 mM $CaCl_2$, 1 µM $ZnCl_2$) at 37º C. MMP activity was determined by inhibition with 10 mM EDTA. Following incubation, gels were stained for 1-hour using a buffer of 0.25 % coomassie brilliant blue R-250 (in 45% methanol and 10% acetic acid), and then destained in the same buffer minus coomassie. Following de-staining, gelatinolytic

activity of secreted MMP was observed as white bands against a blue background, which were quantified by measuring the band intensity (Gel Pro image analysis). IGF [10ng/ml] was used as a positive control.

REAL TIME POLYMERASE CHAIN REACTION (RT-PCR)

For quantitative RT-PCR the BioRad IQ5 I-cycler system with 96 well plates was used. Reaction mixtures contained 10µl PrimerDesign 2X PrecisionTM Mastermix (2x reaction buffer, 0.025 U/>l Taq Polymerase, 5 mM MgCl2, dNTP Mix

(200>M each dNTP), 1µl primer (300nM in a 20µl reaction) and 4 µl RNAse/DNAse free water and 5µl of diluted cDNA (5ng/<l). The final volume in each well was 20µl. Quantitative amounts of the genes of interest were standardized against 2 housekeeping genes as per geNorm™ analysis (see page 75). For each sample, ∆CT (crossing point) values were calculated as the CT of the target gene minus the CT of the β-actin gene. The RNA levels were expressed as a ratio, using “Delta-delta method” for comparing relative expression results between treatments in real-time PCR (Pfaffl *et al*., 2001). and changes in gene expression expressed as x-fold over basal.

Thermal cycling conditions were:

Cycle 1: (1X)

Step 1: (enzyme activation)	95.0 °C	for 10:00.
Cycle 2: (40X)		
Step 1: (denaturation)	95.0 °C	for 00:15.
Step 2: (data collection)	60.0 °C	for 01:00.
Cycle 3: (1X)		

Step 1:	95.0 °C	for 01:00.
Cycle 4: (1X)		
Step 1:	55.0 °C	for 01:00.
Cycle 5: (81X)		
Step 1:	55.0 °C-95.0 °C	for 00:10.

All samples were analysed in triplicate and n=3-5 were used for all treatments. To identify cDNA cross contamination between wells or contamination of one or more reagents 'no template controls' were used where the cDNA was replaced with RNAse/DNAse free water. To identify for genomic DNA contamination 'RT-negative' controls were used with the equivalent concentration of RNA is added minus the reverse transcription step. With respect to MMP analysis IGF [10ng/ml] was also used as a positive control.

All primers were designed using the reference sequence in the NCBI Nucleotide sequence database or pre-validated primers were purchased from PrimerDesign Ltd. Specificity of all primer sets were confirmed using melt-curve analysis and sequencing where appropriate. Figure 2.1 shows the primers used.

RESULTS

THE EFFECT OF VISFATIN ON MAPK ACTIVITY IN PC3 CELLS

Incubation of PC3 cells with visfatin resulted in a significant activation of ERK 1/2 after 10 minutes at a treatment dose of 400ng/ml which corresponded to the maximal proliferative effect of visfatin ($p<0.05$) (Figure 5.1). A significant increase in p38

phosphorylation was also observed after 2 minutes incubation with visfatin [400 ng/ml] ($p<0.05$) with a subsequent rapid return to basal levels after 10 minutes (Figure 5.2).

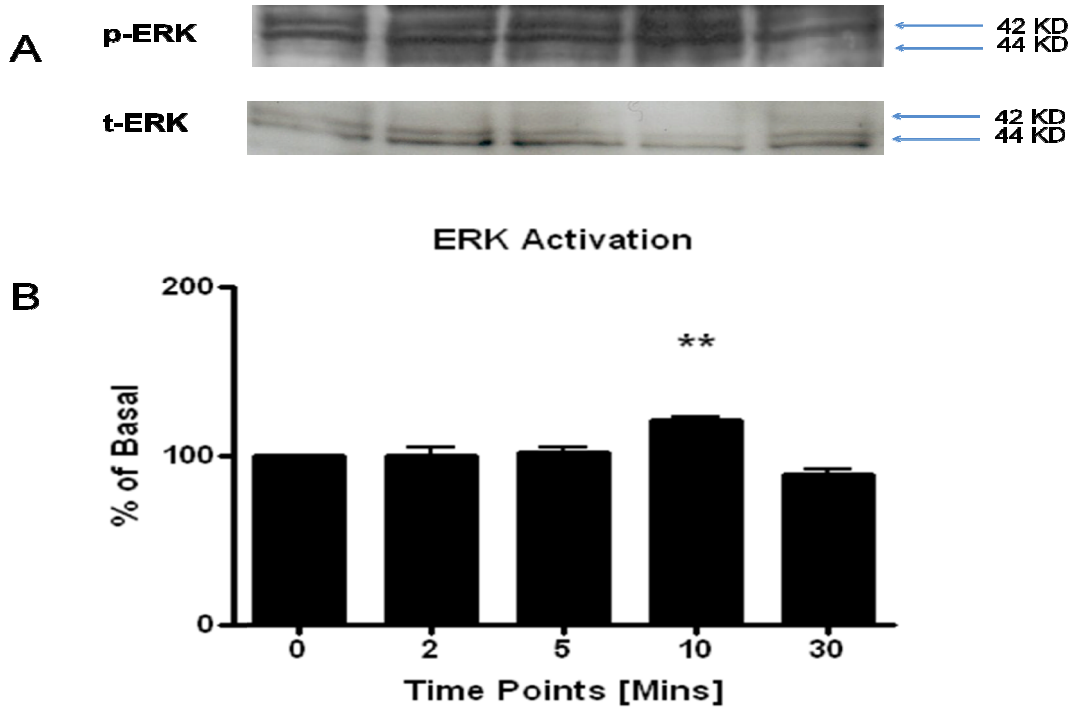

Figure 5.1 The effect of visfatin on MAPK (ERK 1/2) activity in PC3 cells

A: Representative western blot showing changes in p-ERK/ERK expression following treatment with 400 ng/ml visfatin at varying time points of incubation 0-30 mins; lanes correspond to bar chart.

B: Bar chart showing quantification of changes in ERK activation in PC3 cells. Data represent means +/- SEM of 3 experiments; ** $p < 0.05$ compared to basal expression.

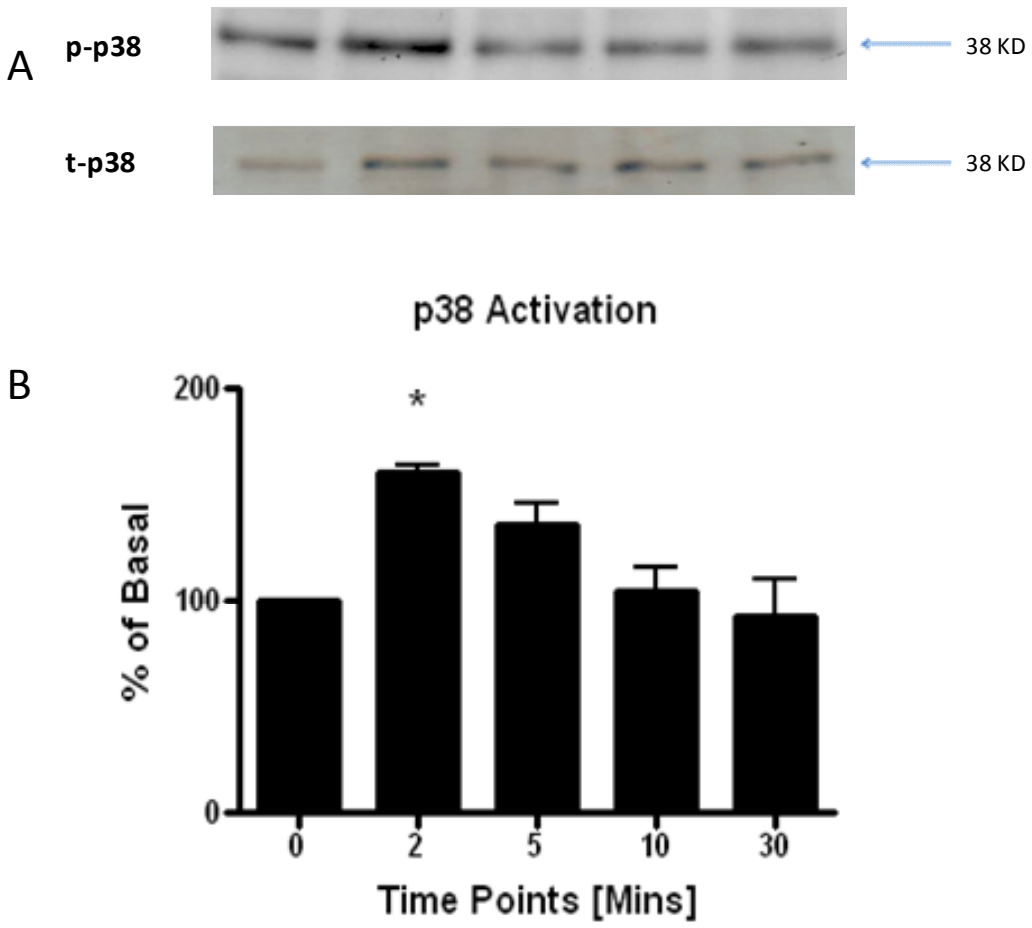

Figure 5.2 The effect of visfatin on MAP-K (p38) activity in PC3 cells

A: Representative western blot showing changes in p-p38/t-p38 expression following treatment with 400 ng/ml visfatin at varying time points of incubation 0-30 mins; lanes correspond to bar chart

B: Bar chart showing quantification of changes in p38 activation in PC3 cells. Data represent means +/- SEM of 3 experiments; * $p < 0.05$ compared to basal expression.

THE EFFECT OF VISFATIN/IGF-1 ON MMP-2 AND MMP-9 MRNA AND PROTEIN EXPRESSION AND SECRETED ACTIVITY IN PC3 CELLS

mRNA expression of both MMP-2 and MMP-9 was significantly increased following treatment with all doses of visfatin studied ($p \leq 0.05$); the greatest increases were seen

in MMP-9 expression. Addition of 10 ng/ml IGF-1 alone also significantly increased both MMP-2 and MMP-9 expression (Figure 5.3).

The effect of visfatin on MMP-2 and MMP-9 protein expression was studied using Western blotting. We found that the changes in mRNA expression of these MMPs were also associated with corresponding significant increases in protein expression ($p \leq 0.05$) (Figure 5.4).

The effect of visfatin +/- IGF-1 on the activity of secreted MMP-2 and MMP-9 by PC3 cells was studied using gelatin zymography. Visfatin induced a concentration-dependent increase in MMP-2 activity that was significant at concentrations over 400 ng/ml ($p \leq 0.05$); 10 ng/ml IGF-1 alone also significantly induced MMP-2 activity ($p < 0.001$) (Figures 5.5). MMP-9 also showed a dose-dependent increase in activity following treatment with visfatin which was significant at concentrations over 400 ng/ml ($p< 0.001$). IGF-1 alone also significantly increased MMP-9 activity ($p < 0.001$) (Figure 5.5).

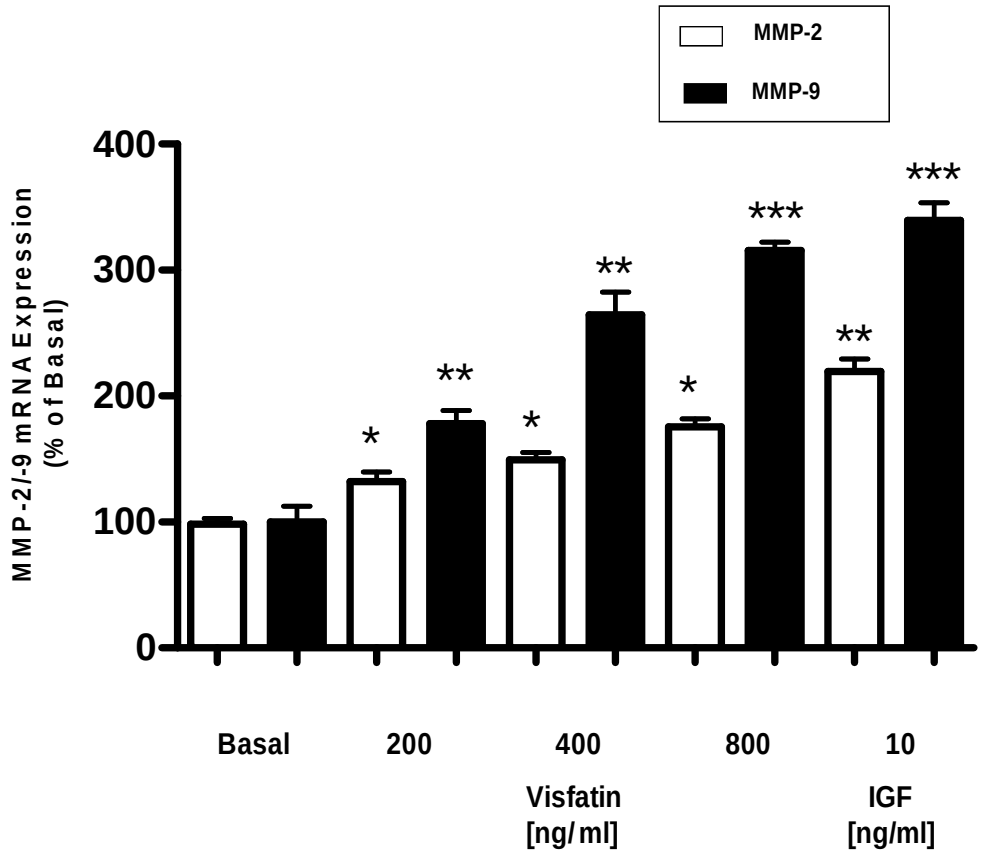

Figure 5.3 The effect of visfatin/IGF-1 on MMP-2 and MMP-9 mRNA expression in PC3 cells. Bar chart showing quantification of changes in MMP-2/-9 mRNA expression in PC3 cells. Data represent means +/- SEM of 3 experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to basal expression.

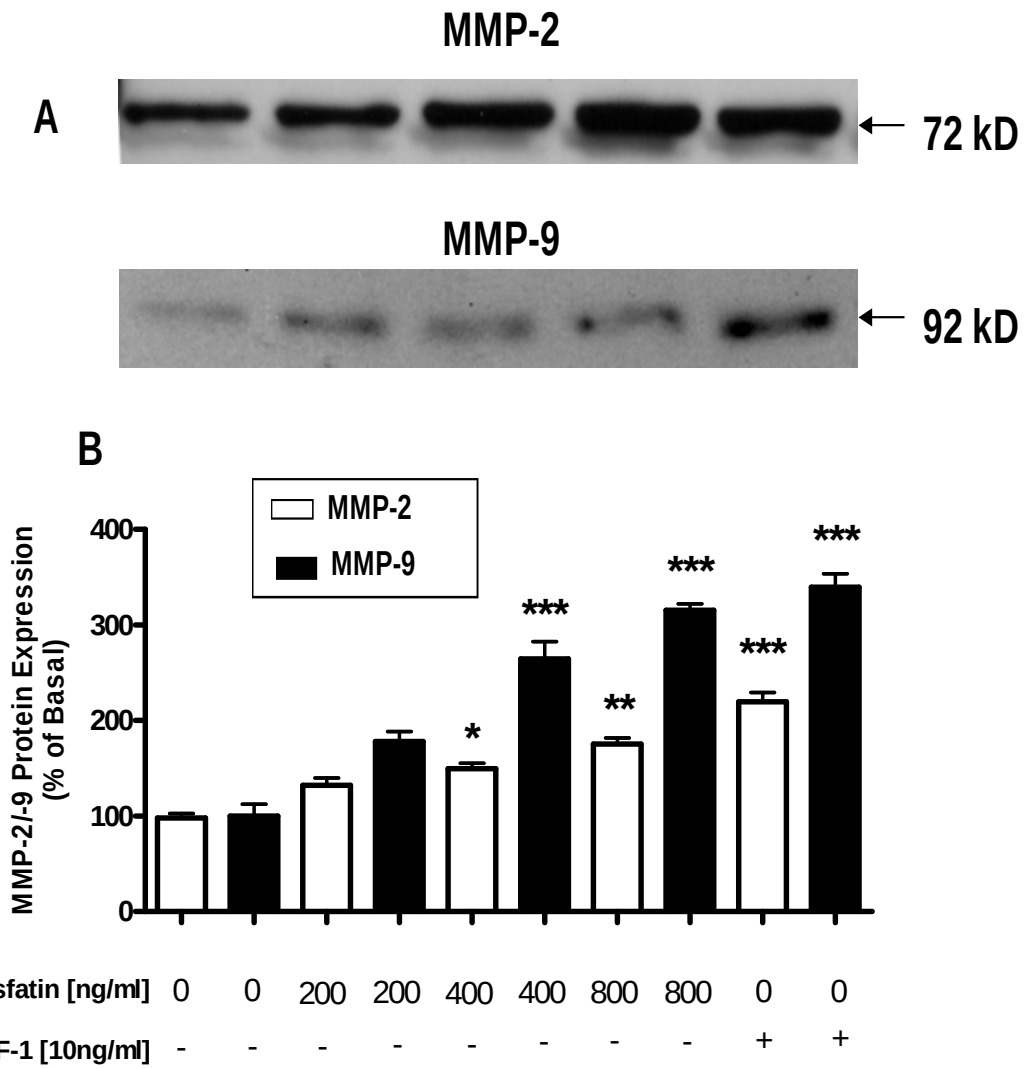

Figure 5.4 The effect of visfatin/IGF-1 on MMP-2/9 protein expression in PC3 cells. A. Representative western blot showing changes in MMP-2/-9 expression following treatment with 0 – 800 ng/ml visfatin or 10 ng/ml IGF-1; lanes correspond to bar chart. B. Bar chart showing quantification of changes in MMP-2/-9 expression in PC3 cells. Data represent means +/- SEM of 3 experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to basal expression.

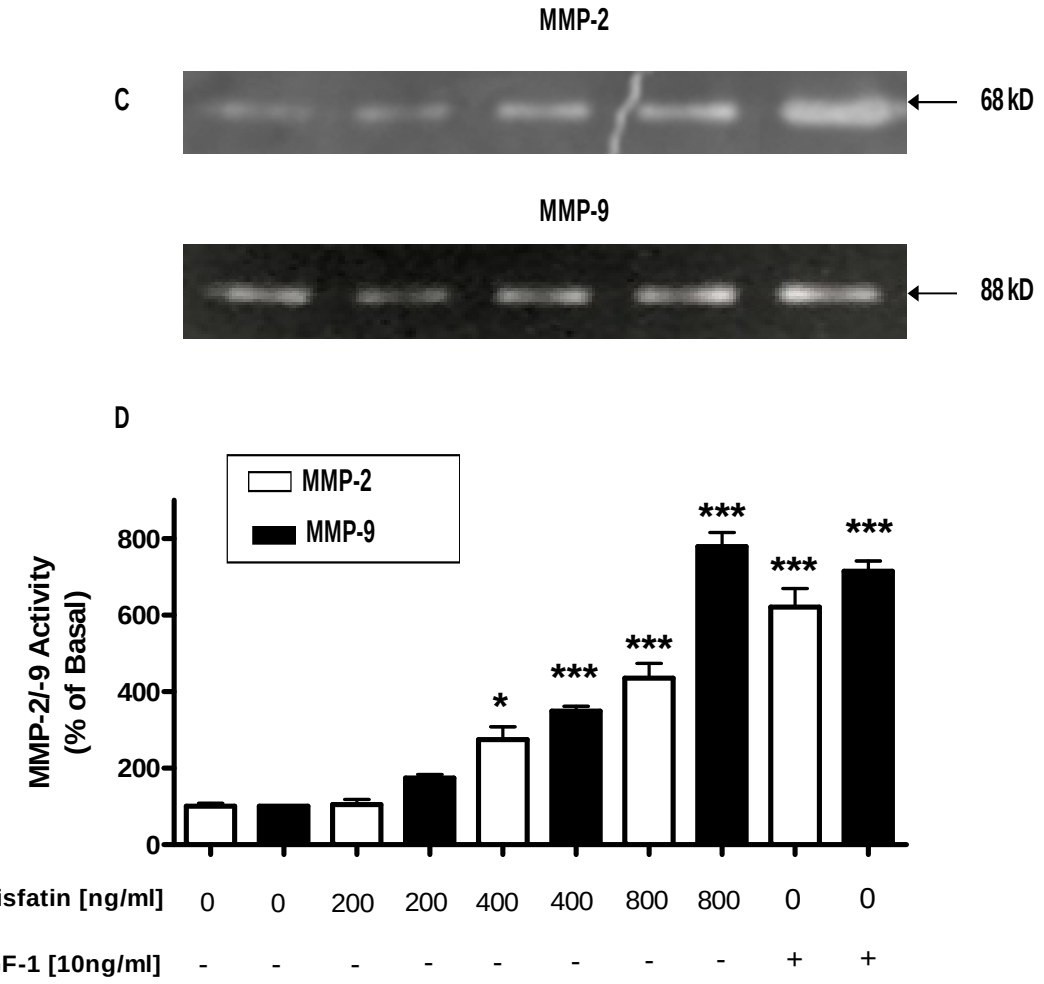

Figure 5.5 The effect of visfatin/IGF-1 on MMP-2 /-9 activity in PC3 cells. C. Representative zymography gel showing changes in MMP-2/-9 activity following treatment with 0 – 800 ng/ml visfatin or 10 ng/ml IGF-1; lanes correspond to bar chart. D. Bar chart showing quantification of changes in MMP-2/-9 activity in PC3 cells. Data represent means +/- SEM of 3 experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to basal expression.

EFFECT OF VISFATIN ON PC3 CELL ONCOGENE EXPRESSION USING REAL TIME PCR

Incubation with visfatin had no significant effect on the level of BCL-2 expression at all concentrations and at both time points used (Figure 5.6).

mRNA expression of BAX was significantly decreased at both 4 and 24 hours following treatment with 50 ng/ml of visfatin studied ($p \leq 0.05$); (Figure 5.7). The trend suggested that there is a consistent fall in BAX expression at all concentrations.

mRNA expression of BCL2-L1 was significantly decreased following treatment with 25 and 50 ng/ml of visfatin ($p \leq 0.05$ and $p < 0.01$ respectively) at 4 hours before returning to basal at higher concentrations. Although statistically not significant the trend appeared consistent at 24 hours (Figure 5.8).

mRNA expression of MCL-1 showed no significant change at 4 hours at any concentration of visfatin. At 24 hours there was a significant decrease in mRNA expression from concentrations of visfatin at 50 ng/ml and upwards ($p < 0.01$) however this was not dose dependent (Figure 5.9).

mRNA expression of IGF-R demonstrates a significant up-regulation from 50 ng/ml visfatin ($p<0.01$) and peaks at a treatment concentration of 100 ng/ml ($p < 0.001$) at 4 hours. The levels then begin to fall towards basal at higher concentrations. There was no significant change at 24 hours although at concentrations ≥100ng/ml there was a trend to increase IGF-R gene expression (Figure 5.10).

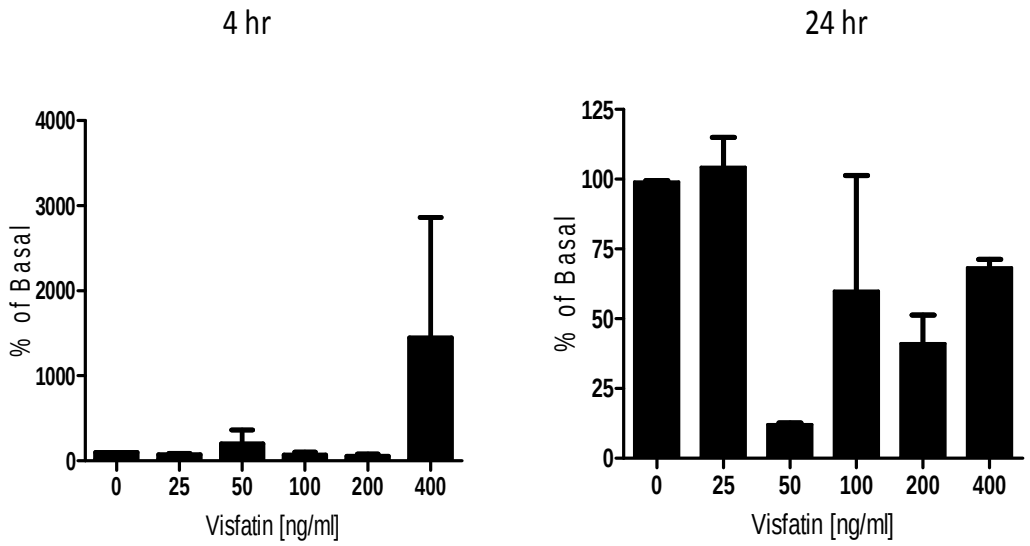

Figure 5.6 Effect of visfatin [0– 400ng/ml] on PC3 cell BCL-2 RNA expression. 4 and 24-hour data are expressed as relative to basal; n = 3.

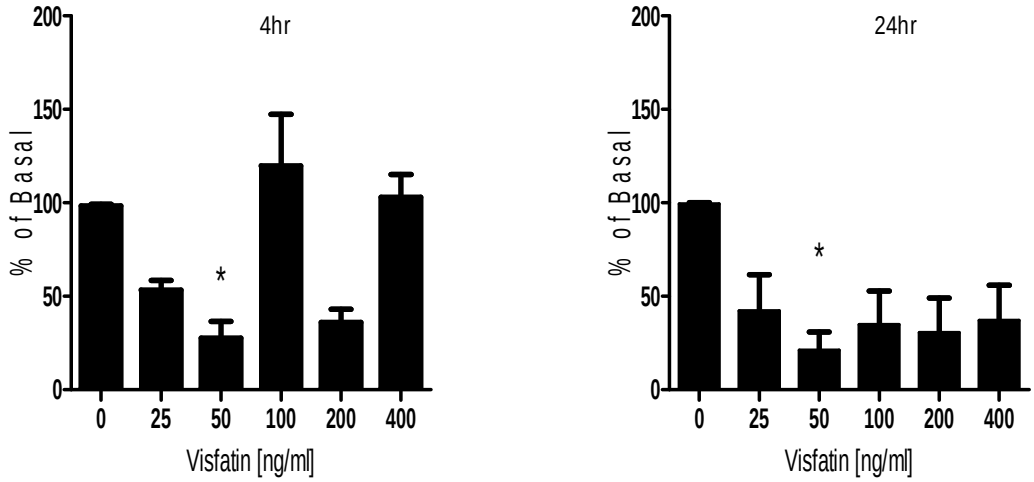

Figure 5.7 Effect of visfatin [0– 400ng/ml] on PC3 cell BAX RNA expression. 4 and 24-hour data are expressed as relative to basal; n = 3 * $p < 0.05$.

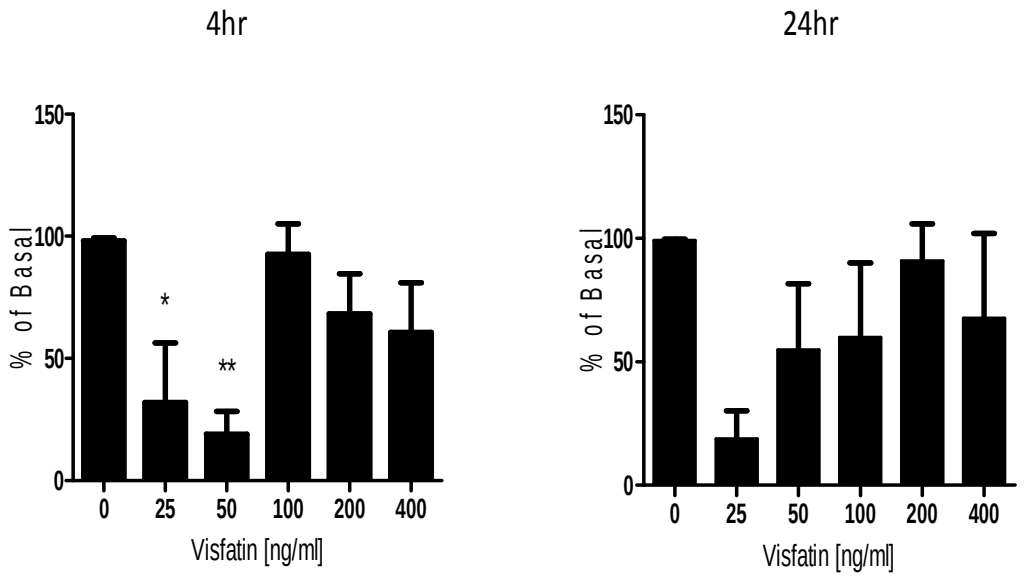

Figure 5.8 Effect of visfatin [0– 400ng/ml] on PC3 cell BCL2-L1 RNA expression. 4 and 24-hour data are expressed as relative to basal; n = 3 * p < 0.05, **p<0.01.

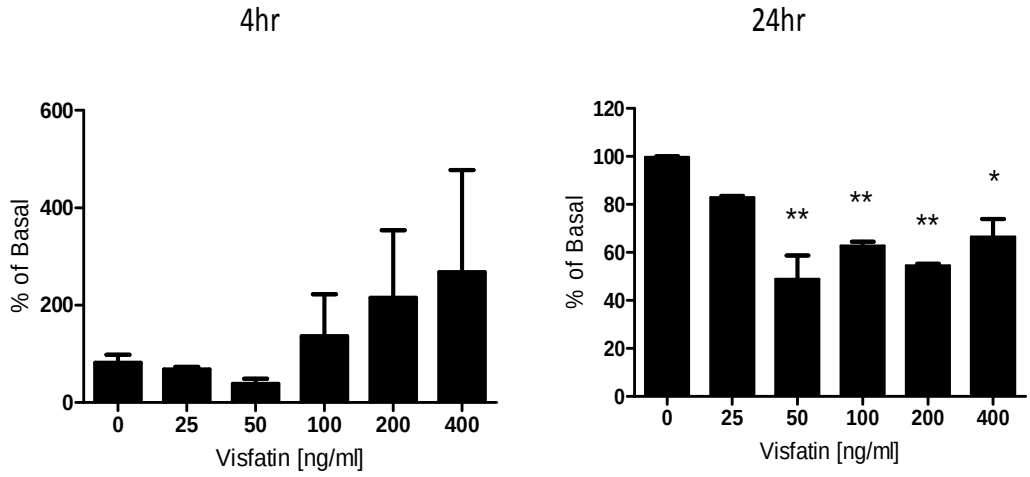

Figure 5.9 Effect of visfatin [0– 400ng/ml] on PC3 cell MCL-1 RNA expression. 4 and 24-hour data are expressed as relative to basal; n = 3, *p<0.05, ** p < 0.01.

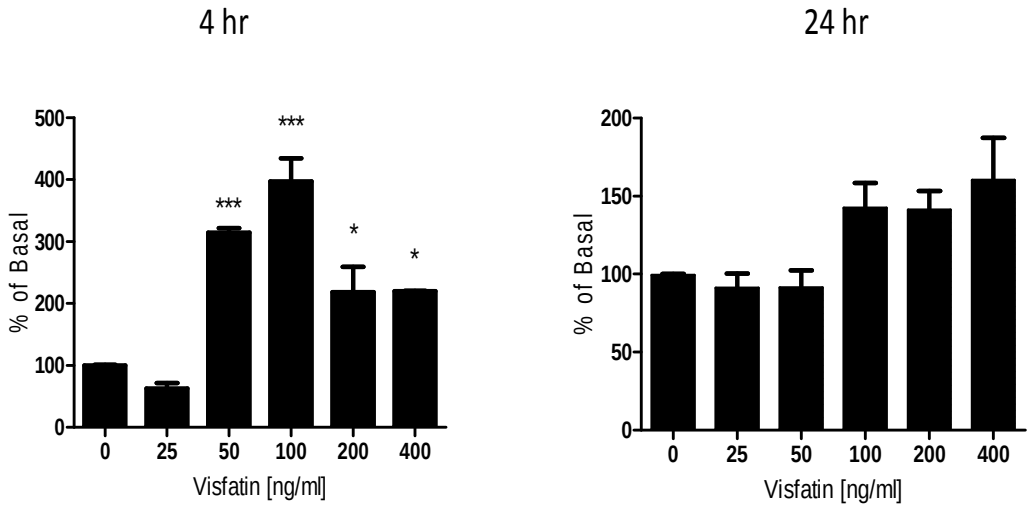

Figure 5.10 Effect of visfatin [0– 400ng/ml] on PC3 cell IGF-R RNA expression. 4 and 24-hour data are expressed as relative to basal; n = 3 * $p < 0.05$, ***$p<0.001$

DISCUSSION

Given that exposure to exogenous visfatin increased cell proliferation, it reasonably followed that MAPKs may be implicated in this observation. ERK1/2 is broadly associated with mitogenesis and therefore inversely related to apoptosis (Cross TG 2002). The results reported here demonstrate a small yet significant increase in ERK1/2 activity after 10 mins using the maximal proliferative concentration of visfatin which was determined with the cell proliferation studies (Fig 5.1). ERK activation has been previously demonstrated in PC3 cells following incubation with leptin, also an adipokine (Deo, Rao et al. 2008). Other groups have also demonstrated visfatin as an activator of ERK1/2 in other tissues including endothelial and smooth muscle cells (Kim, Bae et al. 2007; Wang, Xu et al. 2008). The Ras/Raf/MEK/ERK cascade is known to act as a second messenger pathway from cell surface mitogens via transcription factors ultimately regulating gene expression and this signalling cascade also regulates the activity of many proteins involved in apoptosis (McCubrey, Steelman et al. 2007). Increased expression of this complex Ras/Raf/MEK/ERK pathway is well established in metastatic CaP, androgen independent states and poor outcome (Abreu-Martin, Chari et al. 1999; Gioeli, Mandell et al. 1999; Bakin, Gioeli et al. 2003). This pathway has also been shown to have notable effects on apoptosis regulatory molecules including the BCL2 family members such as MCL-1 (Steelman, Pohnert et al. 2004; McCubrey, Steelman et al. 2007). It was noted in this study that the level of ERK activation was relatively low compared to that seen in the other aforementioned studies in other cell types. This could be attributed to the finding that androgen independent cell line like DU145 and including PC3 tend to express low levels of activated ERK (McCubrey, Steelman et al. 2007). The most widely reported effect of ERK activation in a prostate cancer model is to promote cell proliferation, although some studies have demonstrated

ERK dependent apoptosis in prostate cancer cell lines (Maroni, Koul et al. 2004; Ghosh, Malik et al. 2005). This was the first study to demonstrate ERK phosphorylation in response to visfatin in prostate cancer cell lines. Undoubtedly the conclusion from review of the literature is that ERK activity is implicated in CaP. However, although I present novel data one needs to be cautious in extrapolating these results to be biologically significant as the majority of studies demonstrate a much higher degree of ERK phosphorylation.

Various groups have reported the activation of p38 by agents which include IL-1, TNF-α, EGF, fibroblast growth factor (FGF) IL-6 and vitamin D, all of which have been related to prostate carcinogenesis (Mehta, Robson et al. 2001; Tuohimaa, Lyakhovich et al. 2001; Culig, Bartsch et al. 2002). Ricote et al have also shown that p38 can be induced by provoking a stress response in tissue via UV light and also osmotic changes. They also noted that the activation of p38 pathways can regulate gene expression by modulation of activating transcription factor-2 (ATF-2) and nuclear factor-κB (NF- κB) (Ricote, Garcia-Tunon et al. 2006). p38 has been broadly associated with apoptosis induction (Cross TG 2002). Prostate cancer cells with active p38 have been shown to be highly sensitive to the chemotherapy agent, cisplatin, triggering increased apoptosis (Skjoth and Issinger 2006). Newer data has opposed the above theory proposed by Cross et al and suggested that p38 may indeed have pro-carcinogenic effects in some cell types, conferring protection against apoptosis instead (Luschen, Scherer et al. 2004), an example of this is found in melanoma cells whereby the p38/ATF-2 pathway has been shown to mediate proliferation signals. The precise effects of p38 activation in prostate carcinogenesis are poorly understood at present. Interestingly we have novel data showing significant increased phosphorylation in p38 activation after 2 mins at visfatin concentrations of 400ng/ml (Fig 5.2). Given that the

earlier functional data demonstrated proliferative effects of visfatin as opposed to apoptotic it is likely that in this cell model activation of p38 has a proliferative nature rather than apoptotic. Furthermore p38 has also been shown to play an important role as a mediator in the up regulation of MMP-2 in prostate cancer (Huang, Chen et al. 2005). Our data lends support to the existing data that p38 activation may have proliferative effects dependant on the stimulant applied, in this case visfatin, and also that it may play a role in mediating factors responsible for prostate cancer progression. The literature certainly suggests a significant role for p38 in CaP biology but the exact role is still debated. The data presented here is again in keeping with the literature and serves only to lend support. Ideally the use of a p38 inhibitor such as SB203580 would help demonstrate and verify the true significance of this result and may have a place in future studies.

MMP-2 and -9 are important proteases that are involved in the breakdown of the extracellular matrix under both physiological and pathological conditions (Saarialho-Kere, Chang et al. 1992; Adya, Tan et al. 2008). More specifically, they degrade type IV collagen, the major structural component of basement membranes and are crucial to the process of cell migration, invasion and metastasis. Both MMP -2 and -9 have been shown to play an important role in advanced CaP (Wilson, Gallagher et al. 2004; Incorvaia, Badalamenti et al. 2007). Not only were the effects of visfatin notable in the PC3 prostate cancer cell line, but they also induced significant increases in MMP-2 and -9 expression and gelatinolytic activity. In conjunction with their aforementioned effects on proliferation and apoptosis, it is of great interest that both ERK 1/2 and p38 have been implicated in the regulation of MMP-2 and -9 (Adya, Tan et al. 2008). Visfatin induced ERK 1/2 phosphorylation has been shown to regulate the production and activity of MMPs and play a role in dysregulated angiogenesis (Adya, Tan et al. 2008)

while p38 has also been shown to influence MMP-2 in prostate cancer (Huang, Chen et al. 2005). The data is strong when relating to MMPs to CaP and this novel data supports the current published data. These data allow one to comment on a possible mechanism by which visfatin influences prostate cancer progression i.e. via an increase in MMP production and activity.

The multistep theory of metastatic spread proposed by Liotta et al is helpful in understanding the rational for examining the MMP axis. The three steps are: (1) the adhesion of tumor cells to basement membrane; (2) local proteolysis that leads to the invasion of cancer cells into stroma; and (3) tumor cell proliferation (Liotta 1993). From the above it can be inferred that step 2 is a key factor in prostate cancer progression. This involves the disruption of the basement membrane components such as type IV collagen, laminin, fibronectin, and proteoglycans (Mignatti and Rifkin 1993). MMPs are an important part of this cascade (Liotta 1993) and our novel finding that visfatin upregulates MMP-2/-9 at all levels and most importantly activity is dramatically increased suggests that obese patients with elevated levels of visfatin may in fact have a more aggressive form of the disease capable of metastasis. Studies have shown an association of increased production of MMPs (MMP-2, -3, -7 and -9) with malignant progression of prostate cancer (Nunn, Gibson et al. 1997). The above evidence lends support to metastatic disease although as described in chapter 1 (introduction) the Gleason score is largely thought to represent a key feature in grading CaP into aggressiveness. Stearns *et al* (Stearns and Stearns 1996) have reported that, MMP-2 expression is associated with ≥ Gleason 7 i.e. high grade disease. In keeping with the present study thus far one study has also shown that in normal prostate tissue and low grade disease as per the Gleason score MMP-2/-9 display low levels as compared to high grade tumours (Wood, Fudge et al. 1997).

The findings reported here, particularly the increased gelatinolytic activity seen with visfatin treatment, allows us to tentatively speculate that the elevated circulating visfatin levels associated with obesity may promote prostate cancer progression by enhancing the capability of metastasis via MMP regulation.

Finally, the effect of visfatin on gene expression levels of various oncogenes was measured. As discussed in chapter 1 (p 34-35) adipokines have been shown to regulate oncogenes and tumour suppressor genes but no data exists for gene modulation by visfatin and the BCL family of genes in prostate cancer. It was therefore decided to run a pilot study at this stage to try and establish if this was a novel mechanism by which visfatin may influence CaP pathogenesis. Through-out the PCR analysis it was noted that there was significant variance in the data presented, and to further elucidate this relationship, a formal powered study would be needed with the appropriate number of replicates. Strict post–hoc analysis to compare the data in the presence of large standard error can also influence the likely hood of significance within the data. Given the nature of the results seen graphically in Figs 5.6-5.10 the statistical analysis was reviewed and confirmed. Both the ANOVA and unpaired t-test assume Gaussian or parametric distribution and this is often difficult to confirm in RNA data and a non-parametric test can be an acceptable alternative (Yuan, Reed et al. 2006). These factors have been accounted for in discussing the data.

Although in the past adipokines have been shown to modulate BCL-2 in CaP cell lines (Mistry, Digby et al. 2008) we did not show any similar findings with respect to visfatin. The BCL-2 family have been documented to be expressed in both primary and metastatic CaP (Hughes, Murphy et al. 2005). BCL-2 is anti-apoptotic (Bubendorf, Sauter et al. 1996) and it is therefore unsurprising that given the lack of effect of visfatin on apoptosis that an effect on this key anti-apoptotic gene was not discovered.

We did however note an effect of down regulation of BAX mRNA at both 4 and 24 hr time points following incubation with 50ng/ml visfatin. BAX has the opposite action to BCL-2 and has the effect of permeabilizing the outer mitochondrial membrane which results in apoptosis via caspase release (Wolter, Hsu et al. 1997). From this isolated result it is difficult to discern its significance when applied to the overall picture. The concentration of visfatin exhibiting this effect is close to physiological levels in the obese population and down regulation of BAX at this level of visfatin may therefore be of relevance. The down-regulation of an apoptotic gene provides an isolated mechanism of action of tumour aggressiveness. This result can provide a further mechanism of action of visfatin in increasing tumour aggressiveness as decrease in this pro-apoptotic gene expression would encourage cellular proliferation. A further way of revisiting this finding in the future would have been to examine the ratio of BCL-2:BAX as it has been the balance of the two that has proposed to regulate apoptosis (Kyprianou, King et al. 1997; Johnson, Robinson et al. 1998).

MCL-1 was originally described as an early gene that was induced during differentiation of ML-1 myeloid leukemia cells (Kozopas, Yang et al. 1993). MCL-1's role in the regulation of cell death has been mostly investigated in myeloma cells in which it mediates ‘pro-survival’ i.e. promotion of cell viability via the action of IL-6 (Jourdan, Veyrune et al. 2003) as do BCL2, BCL2-L1, and other anti-apoptotic members of this family (Craig 2002). MCL-1 has been demonstrated to have variable expression from prostate cancer specimens compared to benign tissue (Krajewski, Bodrug et al. 1995; Krajewska, Krajewski et al. 1996) which suggests a role for MCL-1 in prostate cancer although data has been sparse since this discovery. It should be noted however that the increase cell viability has been found to be short lived with the MCL-1 conferring an advantage for only several days longer than controls as found in murine myeloid

progenitor cells (Zhou, Qian et al. 1997). A common thread that should be considered and is expressed in the literature is that control of apoptosis via the BCL-2 family is a complex set of interactions within the family. Given that the adipokine IL-6 is reported as a mediator in BCL-2 family mediated apoptosis it seemed feasible that visfatin would play a similar role. This is novel data and the kinetics of these genes and there expression in response to visfatin has not been elucidated until this study. This is particularly difficult to explain given the knowledge that MCL-1 and BCL2-L1 have been shown to be linked to MAP kinase mediated pathways (either MEK/ERK or p38) (Boucher, Morisset et al. 2000). We have demonstrated that visfatin may regulate both these pathways. The complex interplay is well illustrated by using the Raf/MEK/ERK pathway.

Overall, although I have shown that visfatin treatments at a variety of concentrations and time points appears to modulate these various BCL2 family oncogenes, the data is not in keeping with the literature and the findings presented thus far. These data therefore have to be reviewed with caution. Much of the data is novel yet within the confines of the experiments (in particular low 'n' numbers) and incongruity with published literature significant conclusions cannot necessarily be drawn.

The final result in this chapter showed an increase in IGFR mRNA at 4 hours. This may prove to be highly significant finding. In a paper that was subsequently retracted, Fukuhara et al (Fukuhara, Matsuda et al. 2005) suggested there was a role for visfatin as an insulin-mimetic. Subsequently no further studies have reproduced these results; however there has been some data in support of these interesting findings. Xie *et al.* demonstrated insulin-like effects with visfatin in human osteoblasts (Xie, Tang et al. 2007). They found that visfatin increased glucose uptake, stimulating the expression of osteogenic markers at both gene and protein level. It was also shown to cause an

increase of mineralisation of osteoblasts similar to that of insulin. They also went on to show that when the insulin receptor (IR) was specifically blocked by HNMPA-(AM) (Cell-permeable analogue of HNMPA (Hydroxy(2-naphtyl)methyl]phosphonic acid) that yields the parent compound from cleavage by cytosolic esterases and inhibits tyrosine autophosphorylation of the human insulin receptor). the insulin-like effects were also inhibited. Dahl and associates (Dahl, Yndestad et al. 2007) also published further evidence that visfatin acts via the IR. They showed that in THP cells visfatin mediated secretion of interleukin-8 (IL-8) and tumour necrosis factor-alpha (TNF-α) as well as matrix metalloproteinase-9 (MMP-9) activity was abolished when the IR was inhibited by HNMPA-(AM)

There has been evidence to the contrary and Revollo *et al.* have shown that visfatin does not exert insulin-mimetic effects *in vitro* or *in vivo* but does exhibit NAD biosynthetic activity. It may be that there is a degree of cross talk between visfatin-mediated and insulin signalling pathways in a cell specific manner. An alternative theory is that it is the visfatin mediated NAD synthesis influences insulin signalling. With this evidence it is easy to conclude that visfatin has insulin like effects however further studies are warranted. IGFs are structurally similar to pro-insulin. In prostate epithelial cells, IGFs display significant mitogenic and anti-apoptotic effects, and are hypothesised to be causal in prostate carcinogenesis and progression (Cohen, Peehl et al. 1991; Cohen, Peehl et al. 1994). The IGF ligand family include IGF-I, IGF-II, insulin, as well as several non-classical ligands of which the roles are not fully elucidated (Werner and LeRoith 1996). The IGF receptors display a similar affair and comprise of the insulin receptor (IR), IGF-1R, IGF-IIR, and several atypical receptors, (Nakae, Kido et al. 2001). IR has been shown to have high homology with the to the insulin-like growth factor receptor (IGF-1R) ranging between 45-65% homologous in the ligand

binding site (Ullrich, Gray et al. 1986; Yip, Hsu et al. 1988; Andersen, Wiberg et al. 1995; Mynarcik, Williams et al. 1997). It is therefore unsurprising that cancer cells have an amazing ability to respond to circulating insulin a situation that becomes extremely important in the obese population suggesting further evidence between aggressive CaP and obesity. These findings lend support to other epidemiological data confirming that obesity and a hyperinsulinaemic state are associated with a variety of cancers such as breast, prostate, colon and kidney carcinomas (Bray 2002; Calle and Thun 2004).

Further evidence to the importance of IGF-1R to this system can be found in data from Chan et al who found that IGF-1 serum levels are positively correlated with CaP development risk (Chan, Stampfer et al. 1998). Further studies have also found similar results (Mantzoros, Tzonou et al. 1997; Wolk, Mantzoros et al. 1998). Furthermore it been has shown that prostate cancer cell lines form both androgen sensitive and insensitive lineage show an increase in proliferation in response to IGF-1 (Semjonow, Brandt et al. 1996). All of the above lend support to the significant finding demonstrated in this study that the upregulation of IGF-1R mRNA levels in response to visfatin exposure is a significant finding in potentially explaining a mechanism by which visfatin modulates aggressive disease. There is also evidence in the literature that IGF-1R is involved with CaP directly. Both protein and mRNA for IGF-1R have been found to have altered expression in tissue samples of prostate cancer and benign prostate epithelium (Tennant, Thrasher et al. 1996), and even more significant is the finding by Burfeind et al that antisense RNA to IGF-1R suppresses tumour growth and prevents invasion by rat prostate cancer cells *in vivo* (Burfeind, Chernicky et al. 1996).

With respect to IGF-1R there is a wide evidence base as discussed above as to the role in prostate cancer. The data presented is in keeping with the published literature to date and provides novel mechanisms linking visfatin and prostate cancer.

CONCLUSION

In this study it has been demonstrated with novel data that there are multiple potential mechanisms by which visfatin may modulate CaP further studies inhibiting these pathways would further delineate which second messenger systems are implicated with any significance. The finding that visfatin upregulates MMP levels and activity is highly significant in explaining a mechanism of increased aggressiveness as is the increase in mRNA levels if IGF-1R.

CHAPTER 6

THE INVESTIGATION OF NMN AND FK866 IN PC3 CELLS

CHAPTER 6 THE INVESTIGATION OF NMN AND FK866 IN PC3 CELLS

INTRODUCTION

Visfatin is the rate-limiting step in NAD biosynthesis and therefore becomes a highly important part of normal energy homeostasis, including metabolism and also plays a role in immunity and neoplasia (Galli, Van Gool et al. 2010). The concept of extracellular and intracellular visfatin has already been discussed in chapter 4 (p81-81) and the compartmentalization of visfatin in this fashion becomes of increasing importance when discussing the role of nicotinamide mononucleotide (NMN).

NMN is a by-product of the action of extracellular visfatin in NAD metabolism. This 'by-product' has been suggested to have the capacity to behave in an autocrine and even paracrine fashion on tissue (Garten, Petzold et al. 2009). In addition it has been shown in visfatin knockout mice that the insulin secretion dysfunction is reversible with the addition of extracellular NMN (Revollo, Korner et al. 2007). This finding suggests a role for NMN as a surrogate for extracellular visfatin. Of further interest is that plasma NMN levels have been found to be regulated by extracellular visfatin and that this is independent of intracellular NMN levels and biosynthesis (Revollo, Korner et al. 2007). The role of circulating NMN levels in prostate cancer and neoplasia still remains unexplored and therefore the purpose of this study was to try and elucidate this role in PC3 cancer cells. It would seem logical that cancer cells with high metabolic demand require high levels of both visfatin but also the intermediary products of this metabolic reaction and hence a role for NMN as a neoplastic molecule.

Looking through the literature it becomes apparent that in the past 7 years there has been a renewed interest in the NAD axis as a novel target for an anti-cancer agent. As mentioned above it would be logical that interfering with NAD levels in cells that have a high metabolic requirement, secondary to high proliferative rates may induce tumour cell death following an associated fall in the levels of the pyridine nucleotide. The knock on effect of lowering NAD levels would not only affect the reactions requiring NAD+ as a cofactor but may hamper other cell signaling processes. An example of this scenario can be found in the modulation of sirtuins and mono- and poly-ADP ribosylating enzymes (PARP). NAD is a substrate for the sirtuin subclass of histone deacetylases as well as PARP and the extremely high level of PARP activity of cancer cells should make them more prone to NAD level manipulation (Pollak, Dolle et al. 2007).

Typically human cells have multiple ways of replenishing their NAD levels. Two of these pathways are salvage or recycling pathways and a third involves de novo synthesis from the amino acid tryptophan (Magni, Amici et al. 2004). The pathway that is of particular interest to this study is the pathway that involves visfatin. As we know this co-enzyme controls the rate-limiting step of nicotinamide conversion to NMN, which is then converted further to NAD by NMN adenylyltransferase (NMNAT). Visfatin is the target of the inhibitor molecule FK866 (Hasmann and Schemainda 2003). FK866 induces apoptosis and also lowers NAD levels (Drevs, Loser et al. 2003; Hasmann and Schemainda 2003). The in-vitro results relating to FK866 have been promising and it has indeed reached phase 2 clinical trials (Khan, Forouhar et al. 2007; Holen, Saltz et al. 2008).

FK866 (also known as APO866 or WK175) is a highly specific non competitive-inhibitor of visfatin NAPRT (nicotinamidephosphoribosyl-transferase), which causes a reduction of NAD levels (Wosikowski, Mattern et al. 2002) and thus induces apoptosis

independent of the stage of the cell cycle. Visfatin is known to form a homodimer before it exerts its enzymatic action with FK866, binding at the interface between the two subunits with partial overlap of the NMN binding site (Bae, Kim et al. 2006). FK866 is known to lead to apoptosis via all three phases of the apoptotic pathway as demonstrated in in-vitro studies as follows; It manipulates the induction process via its effect on reducing NAD levels; the effector process via the disruption of the mitochondrial membrane potential; and finally the irreversible degradation process via the manipulation of cellular caspases (Holen, Saltz et al. 2008). In-vitro studies have demonstrated that FK866 has had anti-tumour activity in multiple solid human tumours (Hasmann and Schemainda 2003), leukaemia's and also human tumour xenografts including prostate cancer in mice (Wosikowski, Mattern et al. 2002). We wished to use this compound to assess its action on PC3 cells and identify mechanisms by which FK866 may act. Furthermore we used this compound to try and elucidate the role on NMN in these cell lines.

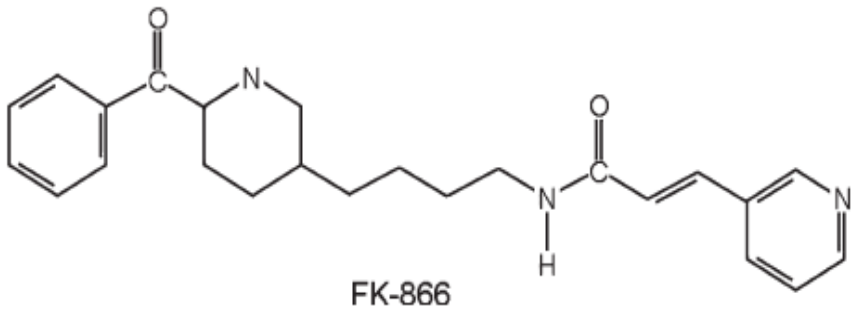

Figure 6.1 Chemical Structure of FK866 (Bae, Kim et al. 2006)

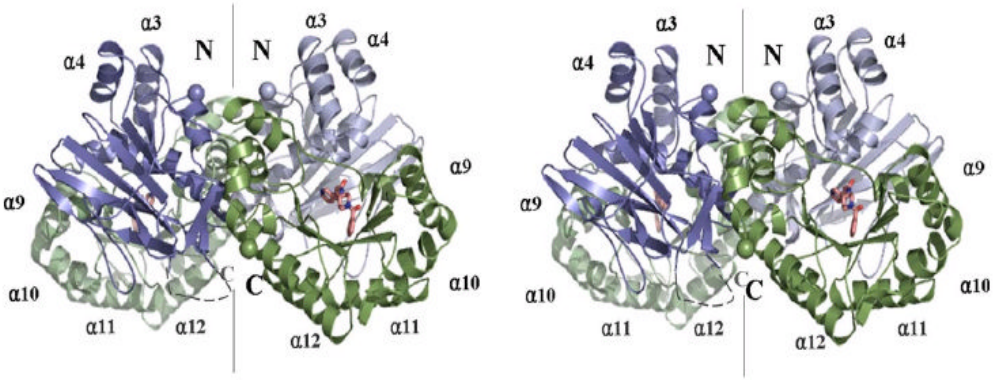

Figure 6.2 figure illustrating the visfatin dimer in complex with FK866; the two monomers are shown in slate and green (subunit A) and pale blue and green (subunit B), respectively. The two FK866 molecules are shown in red. (Bae, Kim et al. 2006)

HYPOTHESIS

NMN is a mechanism by which PC3 cell activity can be modulated as delineated by proliferation and apoptosis. FK866 a potent visfatin inhibitor will act as an anti-tumour agent and its action can be reduced by the use of NMN.

AIMS

- Identify the proliferative and apoptotic effects of both FK866 and NMN in the PC3 cell lines
- To identify if FK866 and NMN alter the expression of various oncogenes in PC3 cell lines

METHODS

Methods were as outlined in the methods chapter and with respect to cell proliferation assay and apoptosis assays see chapter 4 for further details.

Statistical analysis was as described in chapter 4.

RESULTS

FK866 REGULATION OF PROSTATE CANCER CELL PROLIFERATION

Incubation of cells with FK866 alone resulted in a significant decrease in proliferation after 24-hours incubation in PC3 cells compared to control, ($p<0.001$ at 1, 10 and 100 nM) (Figure 6.3). As discussed in chapter 4 (pages 97 and 107) this particular proliferation assay is a surrogate for cellular proliferation based on cellular number derived from metabolic activity and does not take into account cellular proliferation directly or apoptosis. It is also discussed in the literature as cell viability (Hasmann and Schemainda 2003). For the purpose of this chapter in the context of published data we have elected to discuss cell viability/proliferation. The effect appeared to show a dose dependent decrease aside from an aberrant result at 50 nM concentration showing no significant fall in viability.

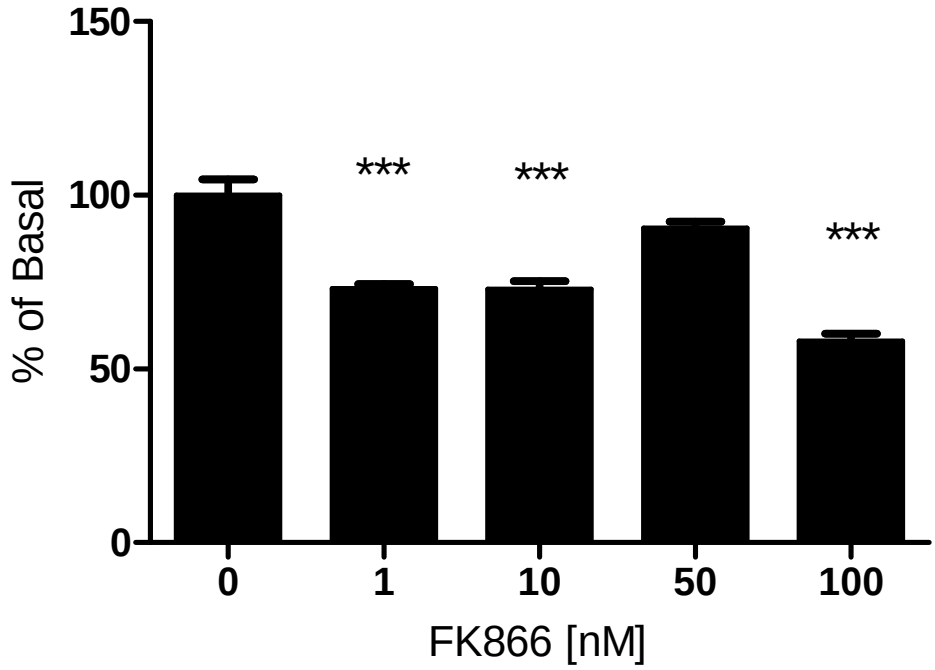

Figure 6.3 The Effect of FK866 [0-100nM] on PC3 cell viability. 24 hour data expressed as relative to basal viability. n = 4 for all experiments, *** $p < 0.001$.

NMN REGULATION OF PROSTATE CANCER CELL PROLIFERATION

Incubation of cells with NMN alone resulted in a significant increase in proliferation after 24-hours incubation in PC3 cells compared to control, ($p<0.01$ at 50 and 100 µM) (Figure 6.4). The effect appeared to show a dose dependent increase before starting to fall to basal at 200µm concentration which still showed a significant increase in viability ($p <0.05$)

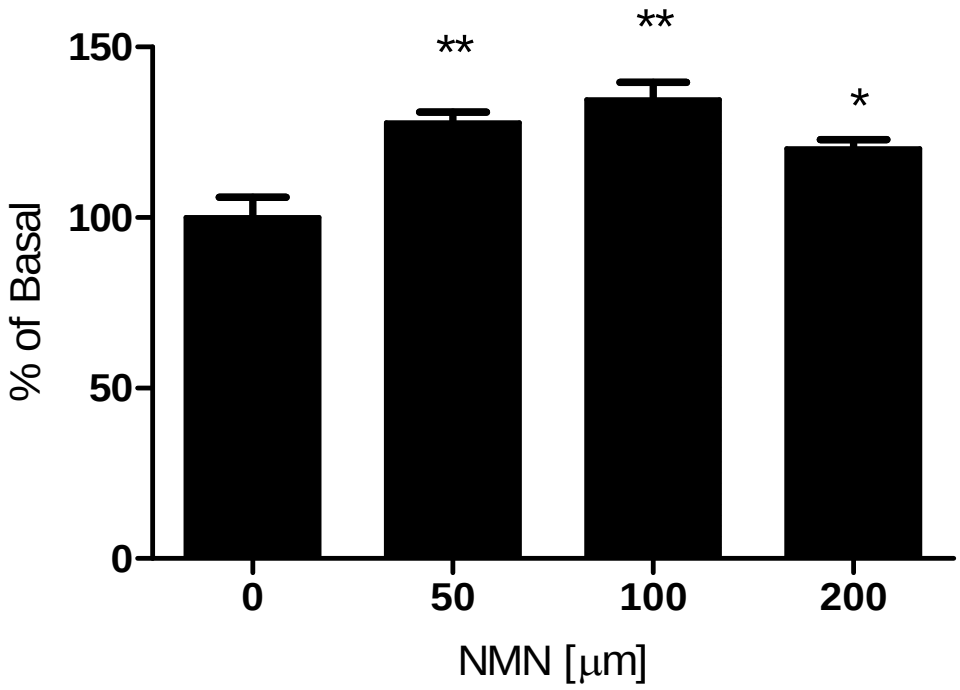

Figure 6.4 The Effect of NMN [0-200μM] on PC3 cell viability. 24 hour data expressed as relative to basal viability. n = 4 for all experiments, *p < 0.05, ** p < 0.01**.**

NMN AND FK866 REGULATION OF PROSTATE CANCER CELL PROLIFERATION

Incubation of cells with FK866 demonstrated a significant fall in cell viability at 10nM concentration ($p<0.01$). This was consistent at 1, 10 and 100nM FK866 and the addition of NMN at the maximum proliferative concentration of 100 μM did not rescue PC3 cell viability. (Figure 6.5)

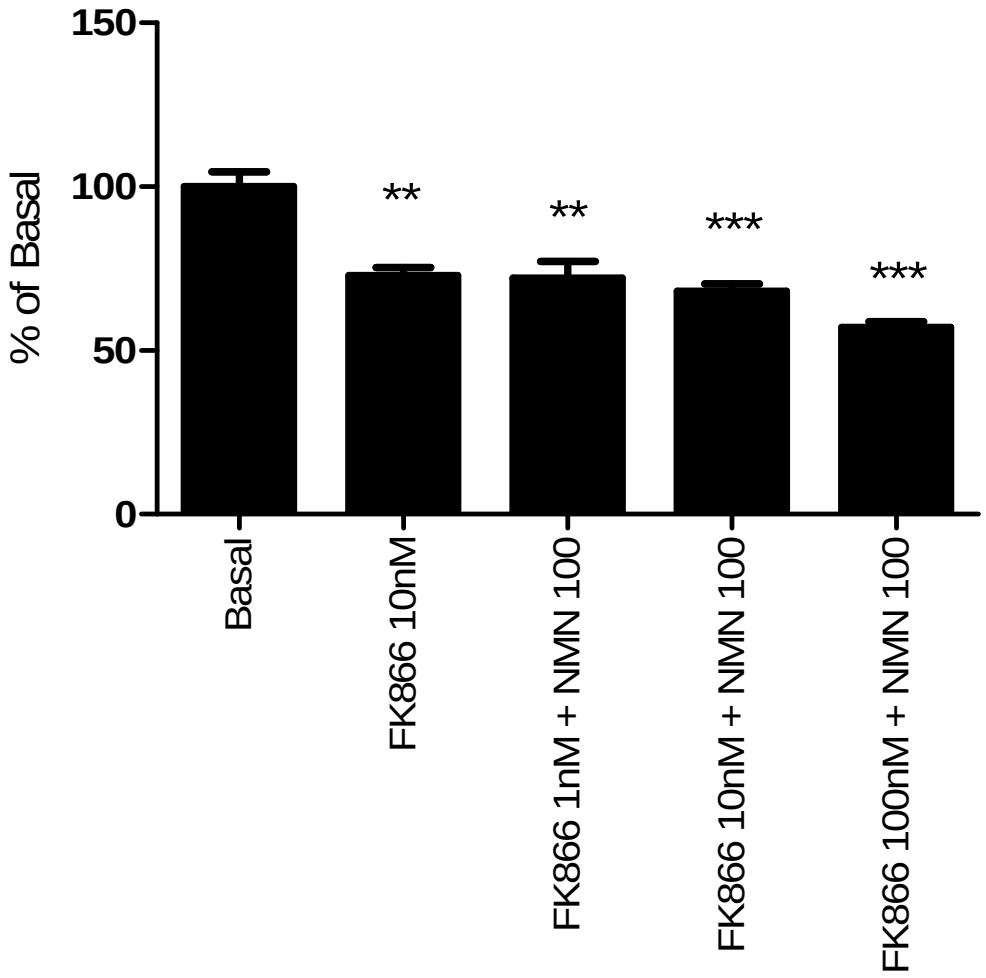

Figure 6.5 The Effect of FK866 alone and in combination with NMN on PC3 cell viability. 24 hour data expressed as relative to basal viability. n = 4 for all experiments, **p < 0.01, *** p < 0.001.

VISFATIN AND FK866 REGULATION OF PROSTATE CANCER CELL PROLIFERATION

Incubation of cells with FK866 demonstrated a significant fall in cell viability at 10nM concentration ($p<0.01$). The addition of visfatin at the maximum proliferative concentration of 400 ng/ml did not rescue PC3 cell viability (Figure 6.6).

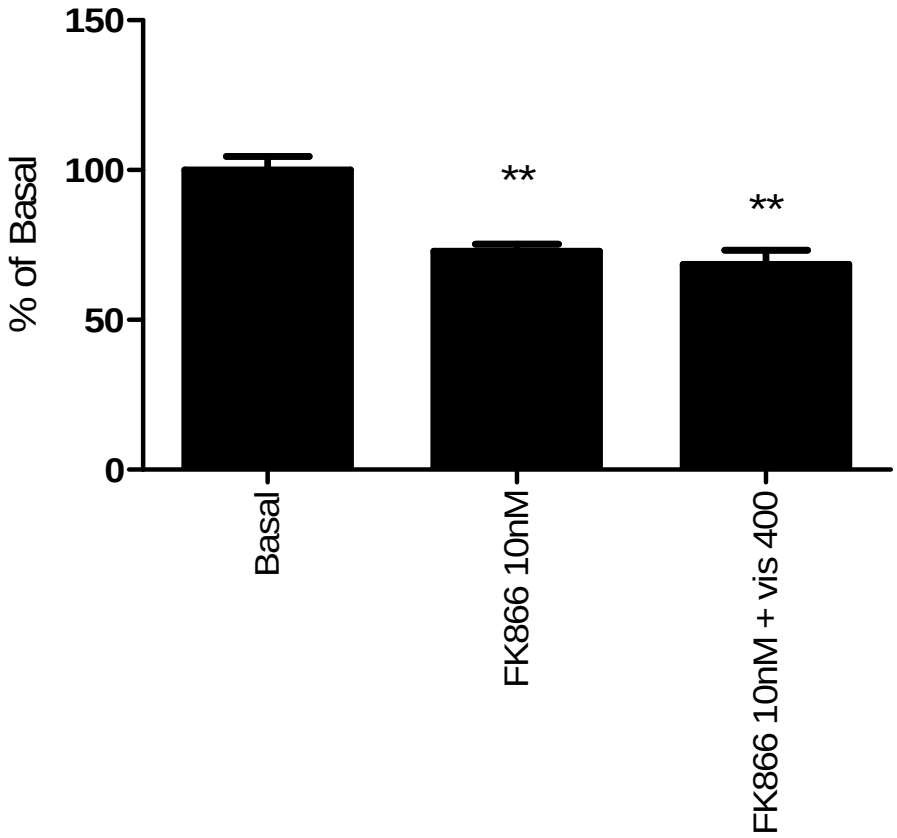

Figure 6.6 The Combination Effect of FK866/Visfatin on PC3 cell Viability. 24 hour data expressed as relative to basal viability. n = 4 for all experiments, ** $p < 0.01$.

FK866 REGULATION OF PROSTATE CANCER CELL APOPTOSIS

Incubation of cells with FK866 alone resulted in a significant increase in apoptosis after 24-hours incubation in PC3 cells compared to control, ($p < 0.01$ at 10 nM) and sustained increase at 50 nM ($p<0.05$) (Figure 6.7) There appears to be a sustained increase in apoptosis although not statistically significant at 100 nm FK866 (Figure 6.8). Camptothesin was used as a positive control at a concentration of 2 µM. This was not only an established positive control within the group, it is also an established agent of apoptosis in androgen insensitive prostate cancer cells (Chatterjee, Schmitz et al.

2001). At 48 hours there was no significant change in apoptosis in PC3 cells following incubation with FK866 at all concentrations.

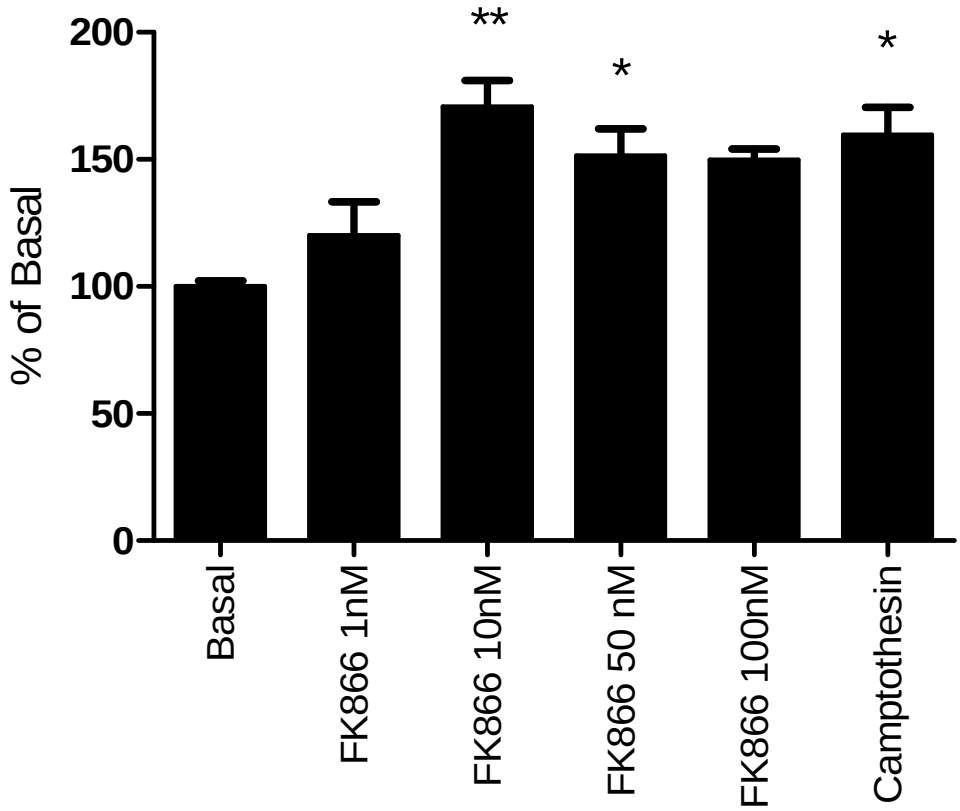

Figure 6.7 The Effect of FK866 on PC3 cell Apoptosis using DNA fragmentation ELISA. 24 hour data expressed as relative to basal apoptosis. n = 4 for all experiments, * $p < 0.05$ ** $p < 0.01$.

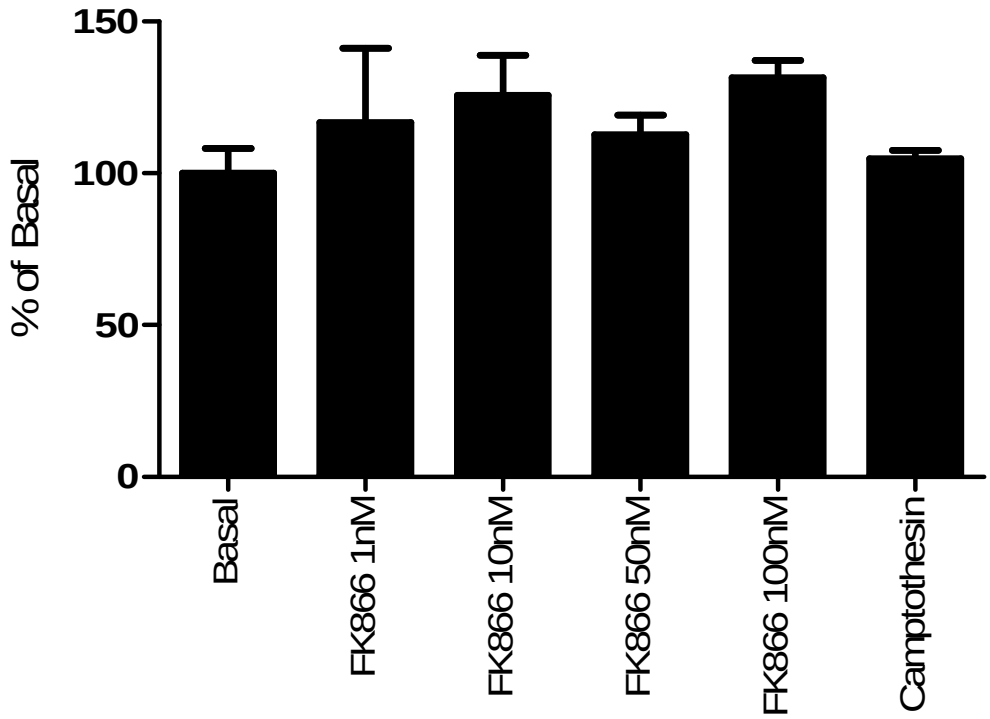

Figure 6.8 The Effect of FK866 on PC3 cell Apoptosis using DNA fragmentation ELISA. 48 hour data expressed as relative to basal apoptosis. n = 4 for all experiments, *** $p < 0.001$.

THE INTERACTION BETWEEN FK866, NMN AND VISFATIN REGULATION OF PROSTATE CANCER CELL APOPTOSIS

Incubation of cells with FK866 10nM alone resulted in a significant increase in apoptosis after 24-hours incubation in PC3 cells compared to control, ($p < 0.001$ at 10 nM). Camptothesin was used as a positive control at a concentration of 2 uM ($p<0.05$). The addition of NMN at 100 µM significantly rescued apoptosis at 24 hours and the addition of visfatin 400ng/ml did not enhance this further. Visfatin alone at 400 ng/ml did not significantly rescue apoptosis, although the result appeared to show a slight reduction in apoptosis from 170%-130% of the control level. Figure 6.9

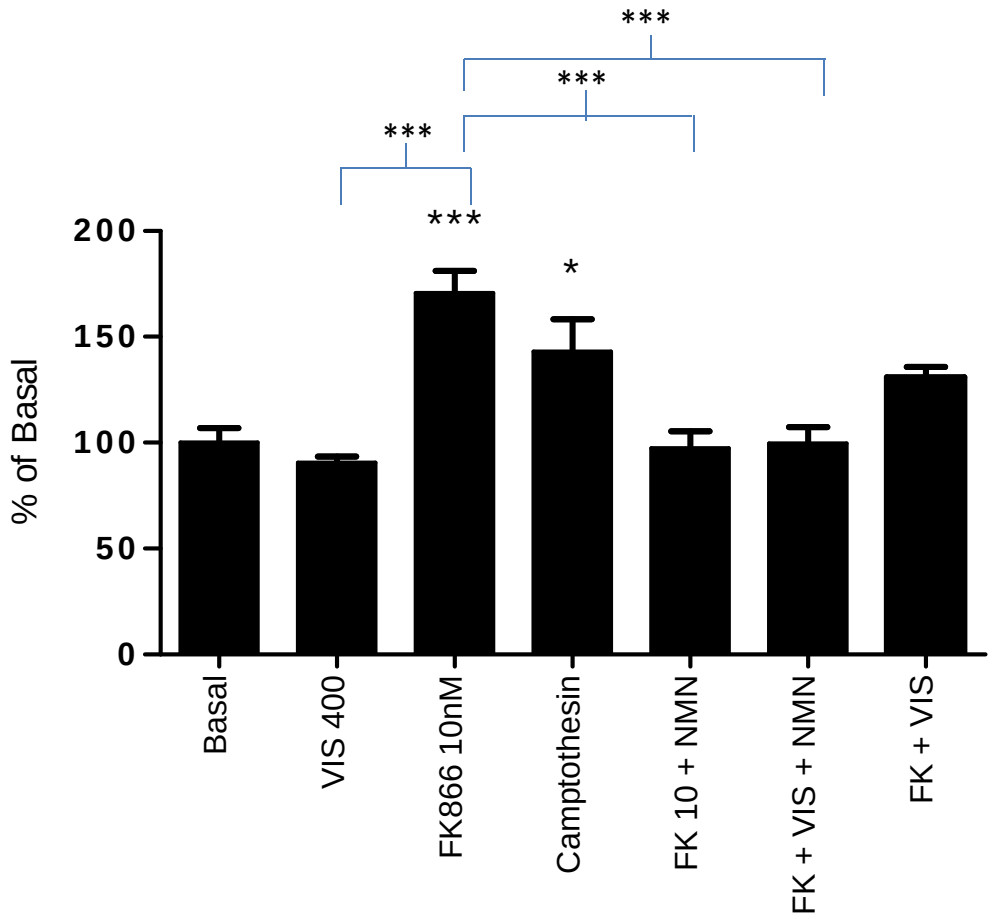

Figure 6.9 The effect of FK866, NMN and visfatin on PC3 cell apoptosis using DNA fragmentation ELISA. 24 hour Data expressed as relative to basal apoptosis. n = 4 for all experiments, *p <0.05, ** p <0.001 *** p < 0.001.

EFFECT OF VISFATIN ON PC3 CELL GENE ACTIVATION USING REAL TIME PCR

NMM 100µM and FK866 10nM both significantly reduce visfatin mRNA expression relative to basal (p<0.001) (Figure 6.10)

mRNA expression of IGF-1R demonstrates a non-significant up-regulation following incubation with 100µm of NMN. No change is demonstrated with FK866 at 4 hours (Figure 6.11).

mRNA expression of MCL-1 showed no significant change at 4 hours after incubation with either NMN or FK866 (Figure 6.12).

NMN 100µM has no significant effect on the level of BCL-2 expression. FK866 10 nM has a dramatic and significant effect in reducing BCL-2 mRNA expression at 4 hours ($p < 0.001$) (Figure 6.13).

mRNA expression of BAX was significantly decreased at 4 hours following treatment with 100µM of NMN ($p \leq 0.05$); (Figure 6.14). At 4 hours however FK866 had no significant effect on BAX mRNA expression.

mRNA expression of BCL2-L1 was not significantly changed following treatment with either FK866 or NMN after 4 hours (Figure 6.15)

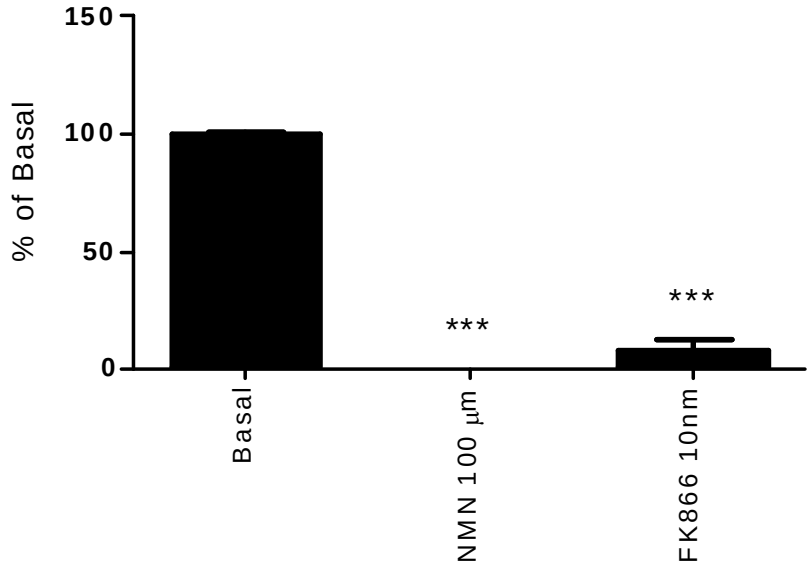

Figure 6.10 Effect of NMN and FK866 on PC3 cell visfatin mRNA expression activation using real time PCR at 4 hours: Effect of NMN [100 µM] and FK866 [10 nM] on PC3 cell visfatin RNA expression. Data are expressed as relative to basal; n = 3 *** $p < 0.001$.

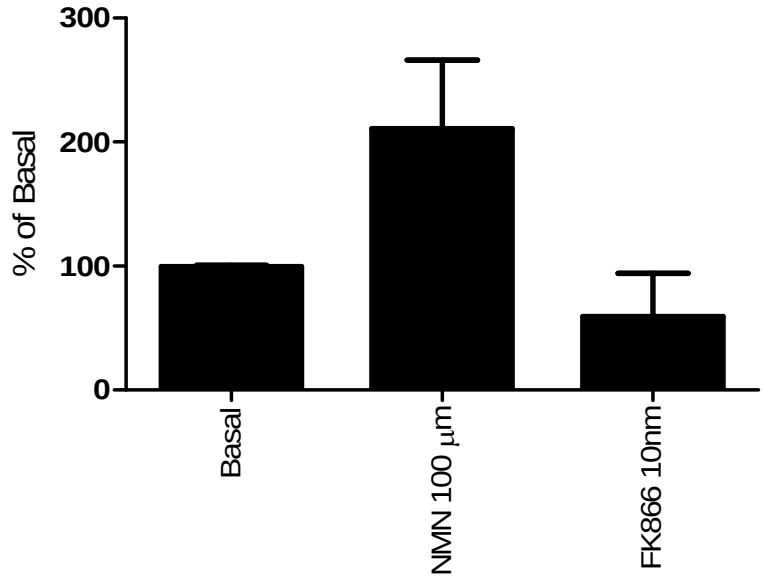

Figure 6.11 Effect of NMN and FK866 on PC3 cell IGF-1R mRNA activation using real time PCR at 4 hours: Effect of NMN [100 µM] and FK866 [10 nM] on PC3 cell IGF-1R RNA expression. Data are expressed as relative to basal; n = 3 *** p < 0.001

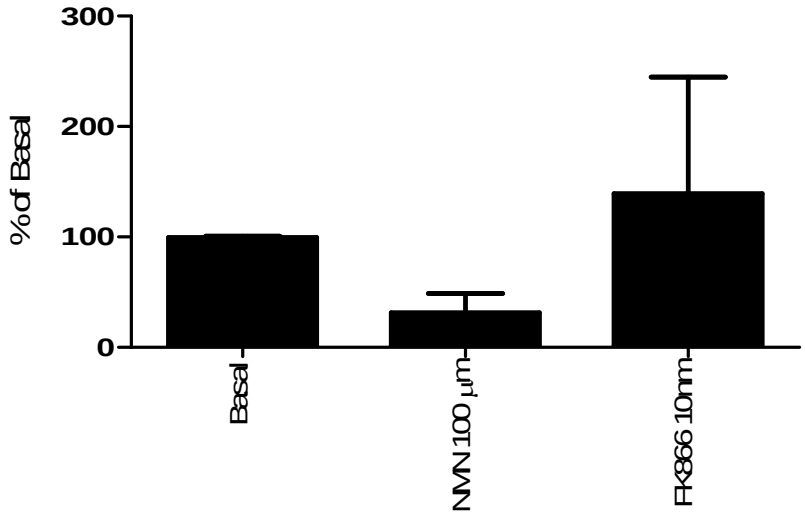

Figure 6.12 Effect Of NMN and FK866 on PC3 cell MCL-1 mRNA expression using real time PCR at 4 hours: Effect of NMN [100 µM] and FK866 [10 nM] on PC3 cell MCL1 RNA expression. Data are expressed as relative to basal; n = 3 *** p < 0.001.

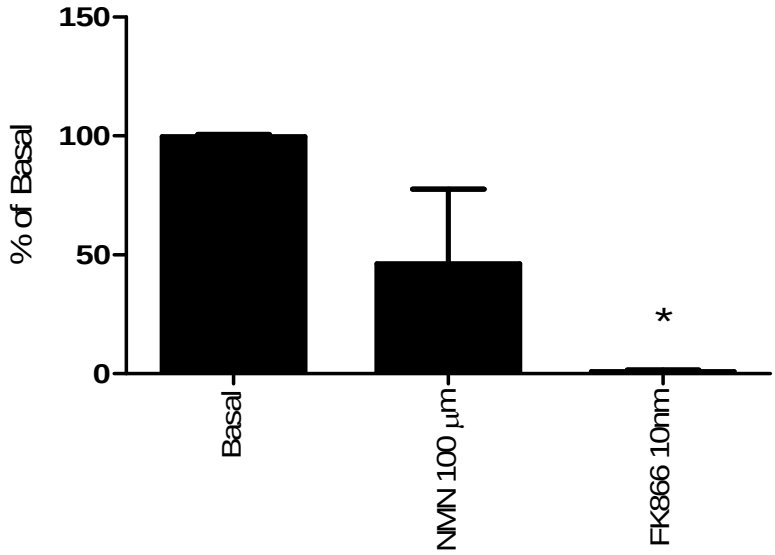

Figure 6.13 Effect of NMN and FK866 on PC3 cell Gene activation using real time PCR at 4 hours: Effect of NMN [100 µM] and FK866 [10 nM] on PC3 cell BCL2 RNA expression. Data are expressed as relative to basal; n = 3 * $p < 0.05$.

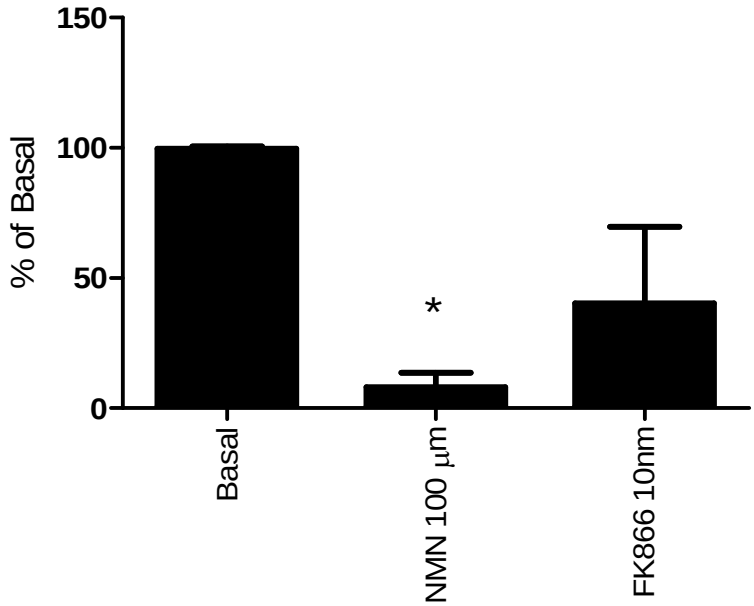

Figure 6.14 Effect of NMN and FK866 on PC3 cell Gene activation using real time PCR at 4 hours: Effect of NMN [100 µM] and FK866 [10 nM] on PC3 cell BAX RNA expression. Data are expressed as relative to basal; n = 3 * $p < 0.05$.

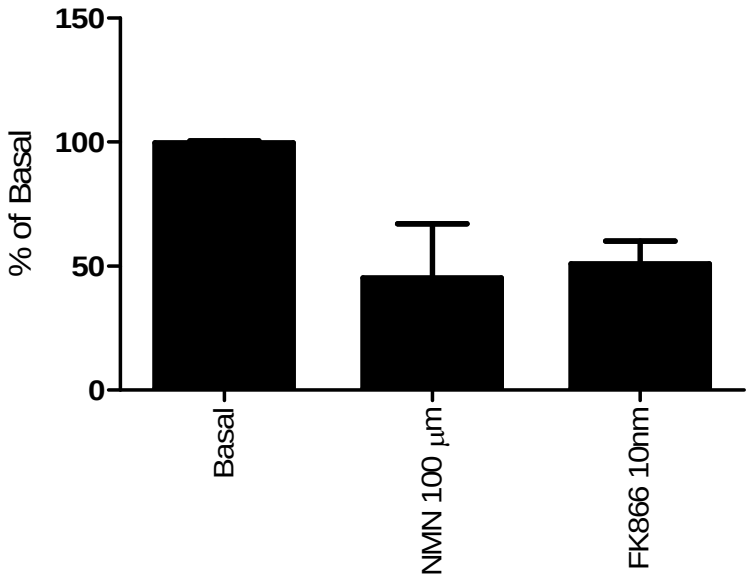

Figure 6.15 Effect of NMN and FK866 on PC3 cell Gene activation using real time PCR at 4 hours: Effect of NMN [100 µM] and FK866 [10 nM] on PC3 cell BCL2L1 RNA expression. Data are expressed as relative to basal; n = 3 * $p < 0.05$.

DISCUSSION

This study elucidated the effect of FK866 on PC3 cells and tried to identify its effect on some of the oncogenes already discussed. Unsurprisingly, FK866 reduced the cell viability at 24 hours of PC3 cells as demonstrated using a MTS cell proliferation/viability assay by 42% at the maximum dose of 100 nM and 27% at 10 nM ($p<0.001$). This is in keeping with other in-vitro studies in other human cell types that have also shown that cell viability reduced at 24 hours. It was noted in a study by Hasman et al that after 24 hours of incubation with FK866 HepG2 cells were noted to have reduced cell viability using a similar assay to the one used in this study (Hasmann and Schemainda 2003). They noted a decreased WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) reaction (another form of tetrazolium salt reaction measuring metabolic activity as a correlate for viability (Hasmann and Schemainda 2003). During this study it was also noted that FK866 induced apoptosis at all concentrations at 24 hours (70% increase in apoptosis at 24 hours with 10nM FK866 $p<0.01$, using a DNA fragmentation assay). However, no significant increase was seen at 48 hours. Again this is in keeping with published results e.g. in a study by Wosikowski et al using THP-1 cells, they have demonstrated apoptosis using a flow cytometric assay following 28 hours of incubation with FK866. Of interest in this study was the relatively rapid decrease in NAD concentration, with levels falling as early as 2 hours after WK175 (FK866) was added to the cell culture media and had fallen undetectable levels by 24 hours. These findings suggest that there is a lag period between the primary apoptotic trigger and the initiation of programmed cell death. The observation of a lag period was noted in the Human acute monocytic leukemia cell line (THP-1) cells but also human hepatocellular carcinoma (HepG2) cells and chronic myelogenous leukemia (K562)

cells (Wosikowski, Mattern et al. 2002). From these data it is concluded that the NAD levels need to reach a critical level prior to acting as a trigger for apoptosis.

As it was not within the confines of this project to measure NAD levels we looked to identify alternative mechanisms of action in the PC3 cell line. The study then looked to examine if FK866 had any effect on various mRNA gene expression. It was found during this study that FK866 significantly reduced visfatin gene expression ($p<0.001$) and also significantly reduced BCL-2 expression ($p<0.001$). The above theory of NAD depletion is now well established in the literature (Wosikowski, Mattern et al. 2002; Hasmann and Schemainda 2003) and it has been suggested that FK866 has no DNA-damaging effect and has been shown not to alter p53 or p21 expression (Wosikowski, Mattern et al. 2002). It was therefore surprising that FK866 had any gene expression effects, in this case BCL-2 expression.

Intuitively one would anticipate that a non-competitive inhibitor of visfatin, which acts by complexing itself with visfatin, would in turn reduce the amount of active visfatin and thus trigger a stimulus for de-novo visfatin production via a putative positive feedback loop. In this study it was discovered that visfatin gene expression is significantly reduced at 4 hours, which is the opposite effect to that was expected. It is feasible that the effect of visfatin up-regulation is of delayed onset and therefore longer time points than 4 hours would be necessary to examine in future studies to answer this question further. It is also possible to hypothesise that this represents another mechanism of action of not yet reported.

With respect to BCL-2 this is a novel finding in keeping with the expected results. As discussed in chapter five BCL-2 is not expressed in the normal prostate and the family of genes are commonly expressed in both primary and metastatic CaP (Hughes,

Murphy et al. 2005). Various evidence in the literature, as discussed in chapter 5 (p 129), has demonstrated that adipokines influence BCL-2. It has been established that BCL-2 exerts its anti-apoptotic effect via modulation of the mitochondrial membrane potential and cytochrome c release (Kluck, Bossy-Wetzel et al. 1997). The down-regulation of BCL-2 gene expression by FK866 would lead to an increase in apoptosis and thus this study has demonstrated a further novel mechanism of apoptosis by FK866 which is unreported.

Further mRNA studies exploring the influence of FK866 on PC3 cell gene expression revealed no effect on MCL-1, BCL2-L1, BAX or the IGFR genes.

The effects on NMN were then elaborated in this cell model. NMN has been shown to increase cell viability at all concentrations in a dose dependent fashion till 100 μM and then begins to decrease in effect at higher doses Fig 6.4. The effect was demonstrated at 24 hours ($p<0.01$). This effect is anticipated based on the theory proposed by Garten et al (Garten, Petzold et al. 2009). One anticipated that bypassing the rate-limiting step modulated via visfatin and with the addition of NMN in the culture media cell viability will inherently be improved. This theory is in keeping with work described by Revollo et al (Revollo, Korner et al. 2007) and the notion that extracellular visfatin acts as an NAD biosynthetic enzyme. It is however distinctly possible that NMN modulates via alternative mechanisms in addition to this and to that effect the study looked at the modulation of tumour genes by NMN.

NMN was found to reduce visfatin mRNA gene expression at similar levels to FK866 Fig 6.10. This was highly significant and is a logical outcome. With excessive levels of NMN one would anticipate that both gene and protein expression of visfatin would reduce as high levels of visfatin would be unnecessary in this scenario. Slightly

unexpected however was the effect of FK866 on visfatin gene expression. One would anticipate that an inhibitor of visfatin would reduce the bioavailable concentration and prompt further gene expression. This is a novel finding and would merit further investigation as no explanation can be offered at this juncture. Furthermore I showed that NMN significantly reduced the mRNA expression of BAX ($p<0.05$) at 4 hours. BAX is a pro-apoptotic protein that is part of the BCL-2 family. By switching off the gene for BAX it provides a mechanism by which NMN can increase cell viability and proliferative response. It has been demonstrated that control of membrane channels BAX can control levels of apoptosis via cytochrome c levels (Shimizu, Narita et al. 1999). Further mRNA studies exploring the influence of NMN on PC3 cell gene expression revealed no significant effect on MCL-1, BCL2-L1, BCL-2 or the IGFR genes.

In chapter four the study touched on the functional interaction of FK866 and NMN using the wound scratch assay. During this chapter the study looked to evaluate if NMN could salvage the effect of FK866 with respect to cell viability and apoptosis. In this experiment it was shown that FK866 reduced the cell viability of PC3 cells. This effect was not negated or reduced by the addition of NMN. This is another surprising outcome as one would expect NMN to rescue visfatin inhibition by bypassing the visfatin driven step. This would be via the same theory described earlier on page 157. However, when the effect of NMN and FK866 in combination on apoptosis was evaluated the results were far more rewarding. As expected FK866 induces significant apoptosis at 24 hours and this effect was significantly reduced by the addition of extracellular NMN (10µM $p<0.001$). Interestingly visfatin did not enhance this effect further nor did it have the same effect in isolation. These findings are in keeping with Wosikowski and Hasmann who suggested that NMN is required to reach a critical level prior to the induction of the apoptotic process. By replacing NMN, the primary mechanism of

action of FK866 is negated and apoptosis averted. The apoptotic effect demonstrated in Fig 6.7 was highly significant and was approximately 70% greater relative to basal. This in itself in not surprising, however in Fig 6.5 we noted that viability/proliferation only reduced by 25% at the same concentrations. This discrepancy can be accounted for when considering the assays involved. The proliferation assay monitors metabolism and correlates with cell number. It does not account for necrosis or apoptosis. The combination of the two assays and their respective results suggest that FK866 mechanism of action is primarily apoptotic and not anti-proliferative.

CONCLUSION

This study has shown novel mechanisms for the action of FK866 and NMN in PC3 cell lines via the modulation of BCL-2 and BAX. The study also supports the findings in the literature with respect to NMN and FK866 with respect to apoptosis. This study also supports the theory of critical NMN levels precipitating apoptosis. By elucidating these effects these novel data with respect to the prostate cell model could assist with the therapeutic potential of FK866.

CHAPTER 7

HUMAN PLASMA VISFATIN LEVELS IN PROSTATE CANCER

CHAPTER 7 HUMAN PLASMA VISFATIN LEVELS IN PROSTATE CANCER

INTRODUCTION

Visfatin (also known as Pre-B cell colony enhancing factor/PBEF and nicotinamide phosphoribosyltransferase - Nampt) is a 52kDa protein highly expressed in visceral adipose tissue, and in other tissues such as liver, muscle and bone marrow cells. Circulating visfatin levels have been shown to be increased proportionally with visceral fat accumulation (Berndt, Kloting et al. 2005; Sandeep, Velmurugan et al. 2007) and pro-inflammatory states (Moschen, Kaser et al. 2007). The results of epidemiological studies have shown a correlation between obesity and solid cancers such as prostate, breast and colorectal (Nishii, Kono et al. 2001; Wolk, Gridley et al. 2001; Calle and Thun 2004). A topic of interest in the literature over the past decade has been the investigation of adipokines as biomarkers for a variety of pathological states such as endothelial dysfunction and chronic kidney disease (Bessa, Hamdy et al. 2010), and including various cancers and logically these have been those cancers for which obesity has been positively correlated as detailed above. Nakajima et al have demonstrated using a case control study that visfatin may be a good marker of colorectal malignancy independent of BMI and also stage progression of the disease (Nakajima, Yamada et al. 2009). The same group had also earlier demonstrated a similar finding in gastric cancer (Nakajima, Yamada et al. 2009). During this study it has been demonstrated that visfatin is expressed in prostate cancer tissue and cell lines. It has also been demonstrated that it promotes cell viability and cell migration in the PC3

cell line. It has also been shown to activate the MAPK pathways specifically p38 and ERK 1/2 and also cause dramatic up-regulation of MMP-2/-9 expression and more importantly their activity. These novel findings have since been corroborated by a recent study by Wang et al who have shown that visfatin promotes prostate cancer cell growth, survival via regulation of SIRT1 functions (Wang, Hasan et al. 2011). The same group also demonstrated that visfatin expression was up-regulated in multiple solid cancers including prostate, colon, brain and lung as well as lymphoma when compared with their benign equivalents. Given the above evidence from other solid tumours and the in-vitro functional as well as tissue findings in prostate cancer the final part of this thesis looked to evaluate if human plasma visfatin levels correlated with prostate cancer and therefore whether there is a role for visfatin as a biomarker in prostate cancer.

As stated above and in chapter 1 (pages 41-49) several studies have correlated obesity with prostate cancer and in particular the incidence of high grade or aggressive disease. On review of the evidence this is conclusive. Furthermore adipokines have been associated with prostate cancer as described and the body of evidence is growing in particular in relation to visfatin and prostate cancer (Wang, Hasan et al. 2011). The literature remains unclear when linking conclusively a single mechanism or cytokine as the mechanism by which obesity is linked with prostate cancer. Levels of visfatin have been documented to be elevated in obesity as have other adipokines. This may be one mechanism by which obesity is linked with prostate cancer, but, I cannot stress enough that it is highly unlikely to be the only mechanism.

HYPOTHESIS

Plasma visfatin levels are correlated with prostate cancer and can potentially be used as a novel biomarker in prostate cancer.

AIMS

- To determine if levels of human plasma visfatin levels are positively correlated with CaP compared to benign prostate disease.
- To identify if human plasma visfatin levels correlate with gleason grade of prostate cancer

METHODS

Methods were as outlined in the methods chapter. This part of the study was performed in collaboration with Dr Andrew Blann (University Hospital Birmingham and City Hospital Birmingham). For human plasma samples, following ethical approval, patients were identified from 3 groups; healthy controls, histologically proven benign prostatic hypertrophy (BPH) or histologically proven prostate adenocarcinoma (CaP). Venous blood was obtained by uncomplicated venepuncture into sodium citrate and was immediately placed on ice before centrifugation at 3000 r.p.m. (1000 g) at 4 °C for 20 min. Citrated plasma samples were then aliquotted into clean tubes and stored at −70 °C until the time of analysis. Serum from the patients was passed to the routine hospital Biochemistry Department for measurement of prostate-specific antigen.

A visfatin ELISA was used to determine plasma visfatin concentrations. This was based on published data (Tan, Chen et al. 2006; Reddy, Umesh et al. 2008) and the ability to process significant sample numbers efficiently. The details are below.

In addition to univariate parametric analysis, results were evaluated between groups by using ANOVA, with significance determined at the level of $P < 0.05$. Spearman rank correlation (non-parametric) was used for calculation of associations between variables (e.g. BMI, visfatin and cancer status) using SPSS version 18.0 (SPSS, Chicago, USA) software and multivariate regression analysis was performed where appropriate.

VISFATIN ELISA

Human plasma samples were collected as described above. A human visfatin ELISA kit was purchased from Phoenix Secretomics and used as per manufacturer's protocol. The assay employs a quantitative sandwich enzyme immunoassay technique.

Visfatin standards were prepared in advance by reconstituting visfatin in 1000µl of reagent diluents. This gives a stock solution of 1000ng/ml. 7 Serial dilutions were then made giving concentrations ranging from 0.78ng/ml-1000ng/ml. 100µl of reagent diluents was used as blank. 100µl of standards were added to each well as were the samples and supplied positive control. All samples were added in duplicate. The plate was sealed and then incubated for 2 hours.

Aspiration and wash of the plate was done 5 times using 250µl per well of assay buffer. 100µl of detection antibody was added to each well the plate sealed and incubated for 2 hours. The aspiration and wash step was then carried prior to adding 100µl per well of secondary antibody horse raddish peroxidase conjugate solution. The plate was again resealed and incubated for 1 hour. Following plate washing 100µl of substrate solution was added to each well for 30 mins avoiding exposure to light. 100µl of stop

solution was then added directly and mixed thoroughly using a plate rocker. The absorbance O.D. was then measured at 450nm.

To calculate the results graphpad software was used to generate a log-log curve fit of the standards. To determine the visfatin concentration data was extrapolated from the standard curve generated using a cubic spline regression. The minimal detectable concentration is reported at 0.78 ng/ml. The manufacturer states an intra-assay precision of 4% and an inter-assay precision of 10%.

RESULTS

N=78	n		Age (Yrs)	Visfatin (ng/ml)	BMI	PSA (ng/ml)	Gleason Grade
CONTROLS	25	Median	68	2.38	27.7	1.1	n/a
		Mean	67.76	2.60	27.08	1.82	n/a
		Sdev	6.76	1.65	3.84	2.27	n/a
BPH	23	Median	69	2.75	25.5	3.8	n/a
		Mean	69.35	3.14	27.17	5.07	n/a
		Sdev	5.97	1.81	5.15	6.51	n/a
PROSTATE CANCER	30	Median	71.5	2.53	26.3	5.7	7
		Mean	69.27	2.85	27.25	50.06	7.14
		Sdev	8.46	1.84	4.92	149.57	1.17

Figure 7.1 A table summarising the results with respect to plasma visfatin levels (ng/ml), prostate specific antigen (PSA/ng/ml), BMI, gleason grade and age.

With respect to age and BMI all three groups were well matched with no significance between them. Figs 7.2a/b

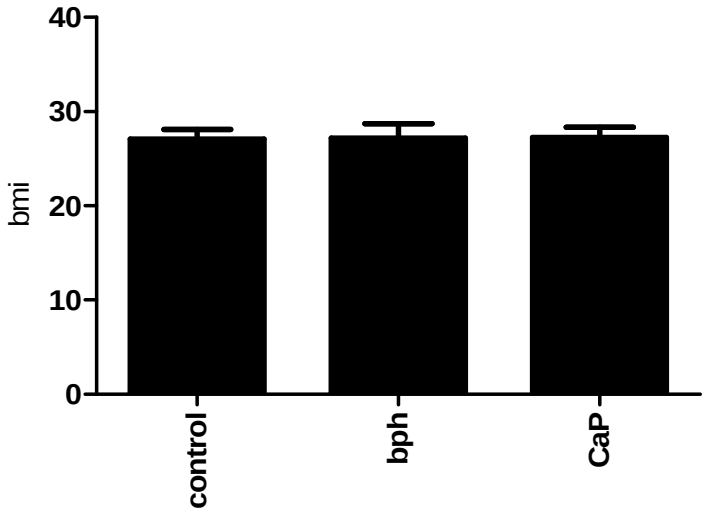

Figure 7.2a Graphical representation of BMI variation between the three groups of patients. (BMI calculated as mass (kg)/ (height (m))^2)

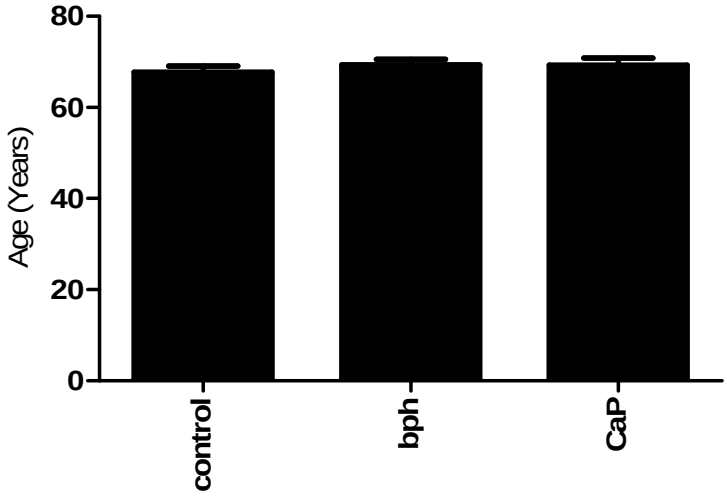

Figure 7.2b Graphical representation of age (years) variation between the three groups of patients.

Using a human visfatin ELISA (Phoenix secretomics) the group of patients as a whole did not demonstrate a significant correlation with BMI and visfatin (Figure 7.3). Using univariate analysis, in the overweight category (BMI 25-29.9) there appears to a slight increase in visfatin levels compared to basal with a mean level of 3.31 ng/ml as compared to 2.94 ng/ml in the normal BMI population. With respect to those that were obese based on BMI alone, there was no statistical difference and the trend appeared to show a decrease in visfatin levels.

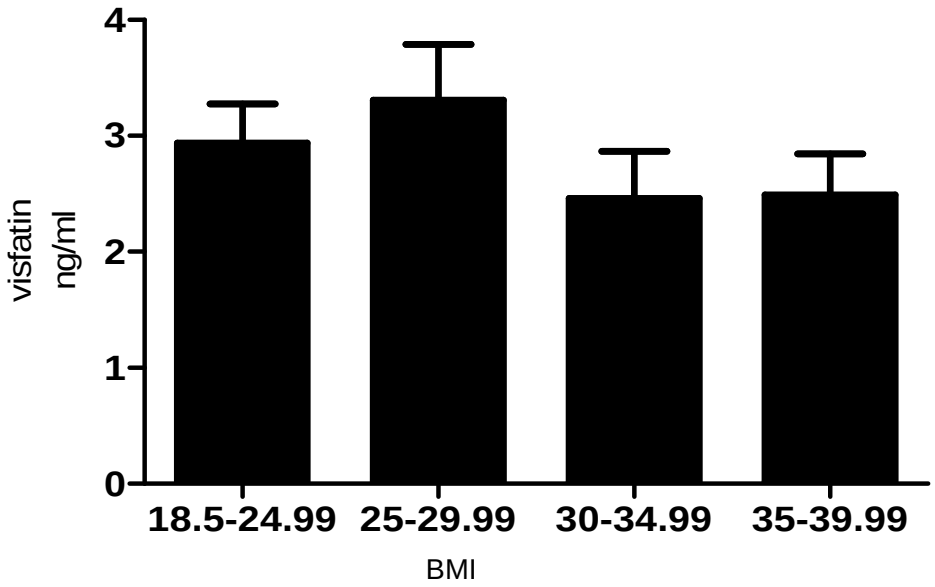

Figure 7.3 Graphical representation of visfatin levels (ng/ml) relative to BMI levels categorised as per WHO guidelines on obesity. 18.5-24.99 normal (n=30); 25-29.99 overweight (n=35); 30-34.99 obese I (n=7); 35-39.99 obese II (n=6); ≥40 obese III (WHO 2004).

Using the spearman rank correlation it was noted that within the control group, there was a significant positive bi-variate correlation between plasma visfatin levels and BMI (r=0.416, P=0.043). No other significant bi-variate correlations with plasma visfatin

levels were noted. In the other groups, no significant bi-variate correlations were noted between plasma visfatin levels and variables. Multiple regression analyses with plasma visfatin as the dependent variable against the other variables was also performed and this did not yield any significant results.

Plasma visfatin levels were analysed with respect to disease state irrespective of other variables. There was no significant difference between the three groups compared. It was noted however the BPH group appeared to have a higher mean level of visfatin 3.14 ng/ml as compared to the control 2.60 ng/ml and the CaP group 2.85 ng/ml

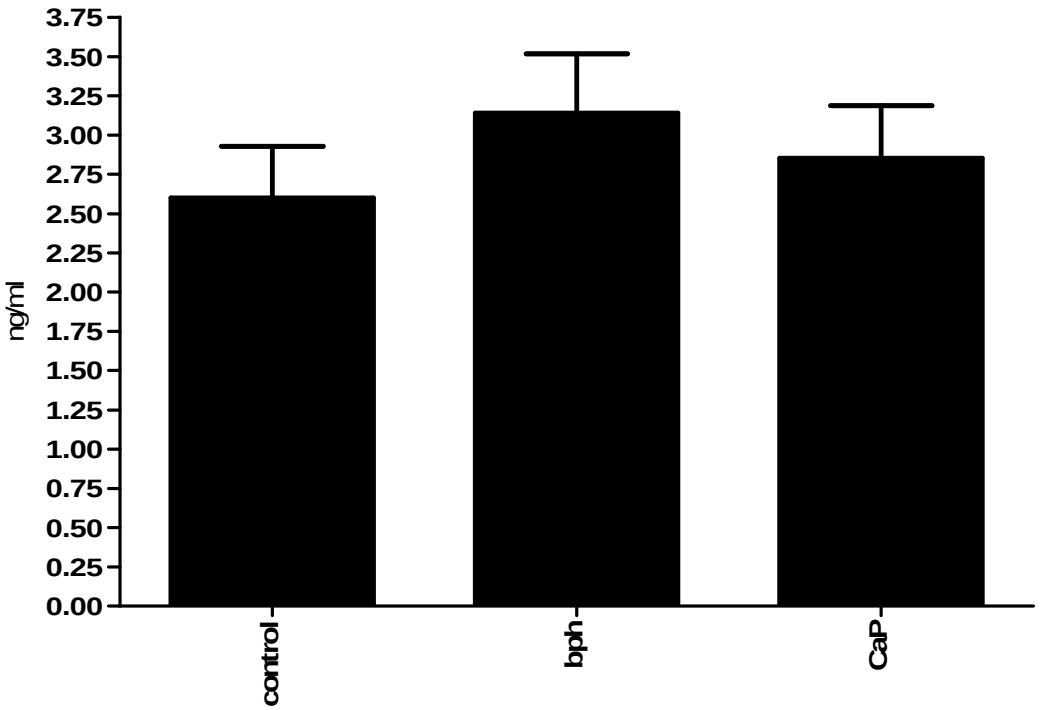

Figure 7.4 Graphical representation of visfatin levels (ng/ml) in disease free control groups, benign prostatic enlargement (BPH) and CaP.

A further subset analysis of patients that were overweight was conducted based on disease state. Although there was statistically significant difference between the three groups interestingly the data suggests that when accounting for BMI >25 i.e. overweight and obese visfatin levels are lower in the BPH group and marginally elevated in the CaP group when compared to the normal weight category (Figure 7.5).

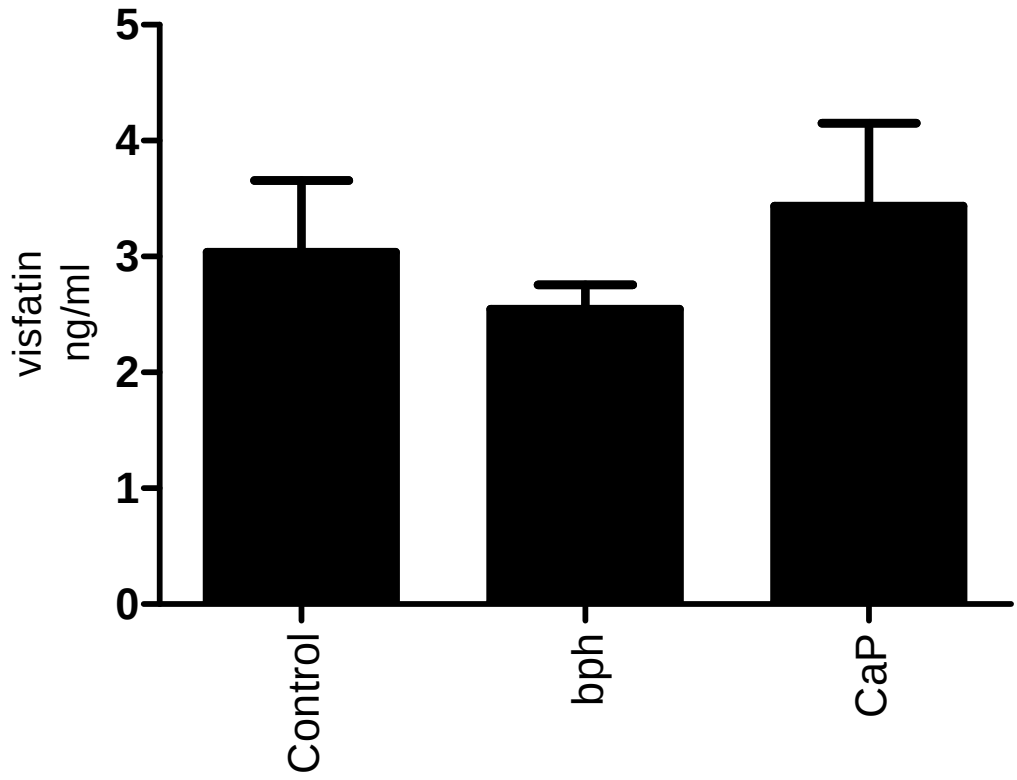

Figure 7.5 Graphical representation of visfatin levels (ng/ml) in disease free control groups, benign prostatic enlargement (BPH) and CaP in the cohort of overweight and obese patients.

Data pertaining to gleason score was available from case note review in 14/30 patients. The median gleason grade was 7 with standard deviation of 1.17. Although there was no statistically significant difference between the varying grades it appears that visfatin levels may show a trend to increase with increasing tumour grade.

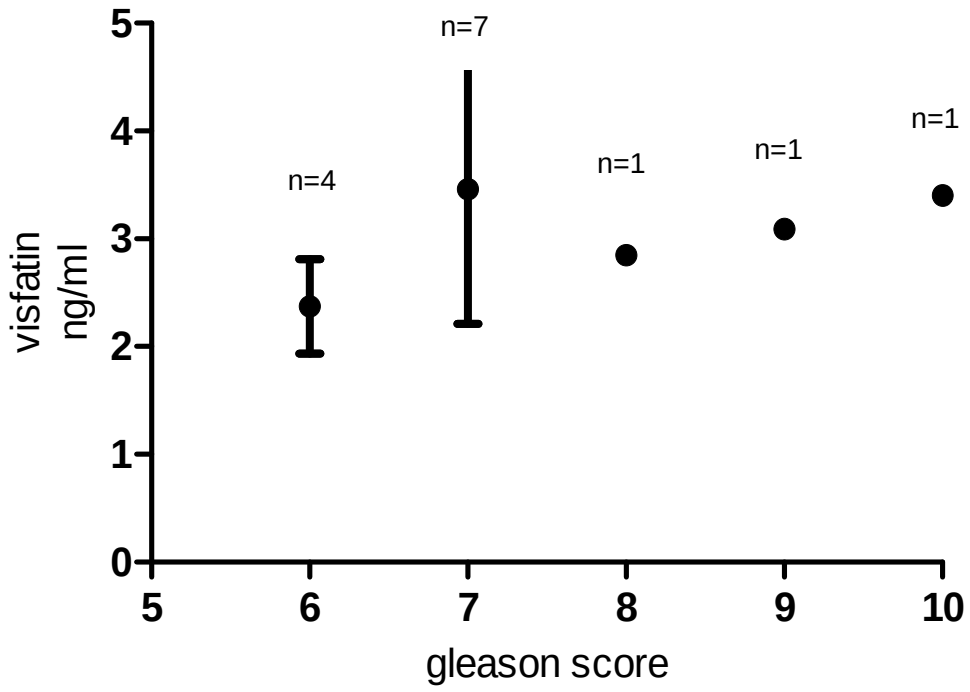

Figure 7.6 Graphical representation of gleason grade vs. visfatin concentration (ng/ml)

DISCUSSION

Based on the previous discovery of visfatin as a novel biomarker in various cancers to date such as glioblastoma, colon cancer and gastric carcinoma (Reddy, Umesh et al. 2008; Nakajima, Yamada et al. 2009) this study tried to elucidate a relationship between plasma visfatin and CaP. Thus far it has been demonstrated that visfatin has a role in prostate cancer and it the effect appeared to be mediated by exogenously applied visfatin. It was logical therefore to explore the levels of circulating plasma visfatin to identify if indeed patients with CaP have higher levels of visfatin than their non-affected counterparts and then to evaluate its potential as a potential biomarker.

This study used age and BMI matched cohorts and looked to primarily evaluate plasma visfatin levels using ELISA methods. The study showed that there was a significant difference in plasma visfatin levels with varying BMI levels within the control group only. Overall in the population group studied no significant correlation was observed between visfatin plasma concentrations and obesity. At face value this would be congruent with some of the studies listed below and perhaps indicate that it is not elevated levels of visfatin that mediate obesity related CaP risk. It should be noted that this study was not matched for obese vs non obese and sample numbers particularly of those in the obese category were small. These data therefore have to be observed with care. Overall the literature reports conflicting results with respect to visfatin levels in obesity. Several studies have concluded that visfatin levels are elevated with respect to obesity (Berndt, Kloting et al. 2005; Filippatos, Derdemezis et al. 2007; Sandeep, Velmurugan et al. 2007). However other studies have been to the contrary (Axelsson, Witasp et al. 2007) and some have in fact reported lower levels of visfatin in relation to obesity (Pagano, Pilon et al. 2006).

Furthermore no significant difference was noted between the control group, BPH and CaP with respect to visfatin. The trend suggested that visfatin levels were elevated in the control group with respect to the CaP group. Interestingly this was reversed when looking at the overweight and obese population, where there was a suggestion that the visfatin levels were highest in the CaP group. It is of note that our population group demonstrated a comparable serum visfatin level compared to that published in the literature (Haider, Schindler et al. 2006).

In a breakdown of Gleason grade the trend suggests that there may be a relationship between visfatin levels and Gleason score. The numbers in the study however are low and they do therefore do not reach any statistical significance. Further future studies would involve a higher sample size in an effort to identify statistically significant results.

The differences in outcomes of the various clinical studies may be in part due to the postulated multifactorial regulation of visfatin (Prieto-Hontoria, Perez-Matute et al. 2011) as well as discrepancies between the obesity measurements and also lack of concordance between commercially available visfatin assays.

Visfatin can be measured by three readily commercially available techniques. These immunoassays include Enzyme immunoassay (EIA), Enzyme linked immunoassay (ELISA) and Radio immunoassay (RIA). A study by Korner et al involving 30 patients forming 3 groups (visceral obese, subcutaneous obese and control group) evaluated the three different assays and found absolutely no correlation between them and showed the ELISA was the most specific assay (Korner, Garten et al. 2007). At the time of the Korner study the EIA was the most commonly employed assay and they suggested that a reason for the lack of sensitivity of the assay may be due to the interference by proteins other than the visfatin monomer or dimer in human serum

(Korner, Garten et al. 2007). A significant difference between the EIA and ELISA method is that the EIA detects the C-terminal visfatin compared to ELISA which detects the full length visfatin (Mahmood, Jamal et al. 2010). Pitfalls of the ELISA techniques are that despite a wide overall range there is a narrow linear detection range (1.85-19.5ng/ml) on the standard fit curve in which results are most accurate. Other possible pitfalls with ELISA techniques and the discrepancies of varying studies include different sample additives, storing conditions and times, and other pre-analytical conditions (Nusken, Nusken et al. 2007). In our study venous blood was obtained and placed directly into blood tubes supplemented with sodium citrate (for plasma). Plasma was obtained by immediate placement on ice prior to centrifugation at 3000 rpm (1000g) at 4 °C for 20 min, before being aliquotted into clean tubes and stored at -70 °C until the time of analysis (Blann, Li et al. 2001). It has been found that the use of citrate additives can influence the protein concentrations as well generate highly variable results (Nusken, Nusken et al. 2007). For future studies EDTA appears to be a more stable additive with respect to visfatin. The trend demonstrated a possible correlation of visfatin with gleason grade of prostate cancer. This is only a trend and to draw any significant conclusions increasing the sample size may yield more robust and statistically significant results.

This study was a longitudinal observation study and to that effect it was not necessary to perform a formal power calculation. To confirm the findings a formal powered study with the appropriate sample size would be needed to formally confirm or refute and correlations observed here.

ELISA assay methods typically exhibit experimental variability at intra- and inter-assay levels. Increased variability can increase the sample size necessary or generate inaccurate conclusions secondary to significant undetected bias. Therefore, variability

and bias effects impact the system. Although the identification of potential sources of error may be possible eradication of these are difficult because they arise from numerous sources and impact the bioassay process at many levels.

There is conflicting published data with respect to serum visfatin levels in particular relating to obesity. The data presented in this chapter relates to a small sample size and various pitfalls have been discussed when reviewing both the literature and study design. Overall the data suggests trends only with no definitive correlations identified..

CONCLUSION

The results of this study suggest that there is no statistically significant correlation between plasma visfatin levels and CaP. Furthermore this study did find a correlation between obesity and plasma visfatin levels within the population studied.

CHAPTER 8

GENERAL DISCUSSION

CHAPTER 8 GENERAL DISCUSSION

The literature is inconsistent with respect to obesity and its link to prostate cancer incidence and is largely based on epidemiological studies. The literature does however suggest that obesity is positively correlated with prostate cancer mortality as opposed to incidence itself (Andersson, Wolk et al. 1997; Calle, Rodriguez et al. 2003; Wright, Chang et al. 2007). The inconsistency in the epidemiological data could be attributed to multiple factors such as the interaction between obesity and age, or the differing effects of obesity on varying histological grades of CaP (Freedland, Giovannucci et al. 2006). Obesity and prostate cancer prevalence appear to be on the increase. Both also are a significant contributor to morbidity and mortality (Adams, Schatzkin et al. 2006). Coupled with this growing health concern is the knowledge that advanced prostate cancer has considerable therapeutic limitations and therefore a greater understanding of any mechanisms responsible for high risk CaP would indeed be of value in developing treatment modalities.

The concept of adipose tissue as a endocrine organ along with a wide body of evidence linking obesity related cytokines or 'adipokines' to CaP models via a variety of mechanisms lends support to the hypothesis that obesity is linked to CaP. The varying circulating levels of some of these in states of obesity also lend further support to their role in mediating prostate cancer.

This study has explored the mechanisms involved with a single adipokine, visfatin, and its by- product NMM using in-vitro studies. It is duly noted that these studies will inherently have limitations, most notably, the isolated pathways and interactions do not necessarily translate to the circulating in-vivo environment and multiple factors can

influence the final outcomes. For the purpose of this study LNCaP and PC3 prostate cancer cell lines were used as surrogates for CaP rather than in-vitro cultures of human prostatic cells. The cell lines are immortalised cell cultures derived from metastases of advanced CaP with hormone refractory disease representing both androgen sensitive and androgen insensitive disease respectively. One possible pitfall of using immortalised cell lines is that repeat passage and culture of these cells may affect their biological properties. Where possible, passage of the cells was kept to a minimum and fresh batches used whenever possible. Despite these efforts however it should be noted that these cell lines do not address the wide range of CaP phenotypes nor are they representative of primary adenocarcinoma of the prostate (Peehl 2005). Despite these limitations that are widely accepted these cell lines provide valuable in-vitro models upon which to base preliminary studies and have been heavily validated by extensive use in the literature (Kaighn, Narayan et al. 1979; Horoszewicz, Leong et al. 1983). Technical advances have provided the use of primary prostatic epithelial cells to be used but malignant cell models of this nature are still limited (Peehl 2004).

One of the key biological differences between the two cell lines is the androgen receptor (AR) status. LNCaP cells are also known to have oestrogen receptors and are PSA producing cells in contrast to the PC3 cells. (Kaighn, Narayan et al. 1979; Horoszewicz, Leong et al. 1983).This significant biological difference may be responsible for the absence of the effect of visfatin in LNCaP cells compared to the PC3 cells. There is some data showing that adipokines do indeed interact with steroids, for example testosterone has been shown to reduce adiponectin secretion from adipocytes however there are no studies looking at the links between visfatin and androgen or androgen receptors.

Since visfatin was first described by Samal et al with respect to B-cell differentiation (Samal, Sun et al. 1994) the function of visfatin has been the interest of many studies. Intracellularly, visfatin has been described as modulating inflammatory responses and innate immunity (Moschen, Kaser et al. 2007; Luk, Malam et al. 2008) in different cell types. It also functions intracellularly with a key role in the biosynthesis of NAD (see chapter 1 and 4). This role with respect to NAD production not only serves great importance with respect to energy homeostasis but also is highly significant in NAD-dependent enzymes such as sirtuins (Wang, Zhang et al. 2006). Following on from the controversy of Fukuhara et al whom first described visfatin as an adipokine with insulin-mimetic properties further studies have since supported this notion (Xie, Tang et al. 2007), but none have reproduced the findings. Revollo et al and also suggested a key role in insulin secretion via NAD biosynthesis (Revollo, Korner et al. 2007). Visfatin has also been shown to have significant roles in cancer (Garten, Petzold et al. 2009).

Extracellularly visfatin has been implicated in multiple inflammatory processes such as sepsis, arthritis and atherosclerosis (Jia, Li et al. 2004; Moschen, Kaser et al. 2007). The anti-apoptotic action of visfatin has also been described in relation to neutrophils and endothelial cells (Jia, Li et al. 2004; Adya, Tan et al. 2008)

During this study several aspects of visfatin with respect to prostate cancer were investigated. The first step was to identify visfatin in the prostatic cell lines as well as human prostate cancer tissue as well as benign prostatic tissue. The novel discovery of visfatin gene expression as well as protein in both the prostate cancer cell lines studied, as well as in benign and malignant prostate cancer tissue, generated a starting point for the study as its presence suggested a role for visfatin in both normal and pathological prostate physiology (Chapter 3 Pages 85-88). The next logical step would have been to identify the receptor however to date a visfatin receptor has not been discovered and

the mode of action of visfatin is the topic of much debate and ongoing research. Interestingly it was also shown that exogenous visfatin reduces visfatin gene expression as early as 4 hours after exposure and levels remain low up to 24 hours (Chapter 3 Page 88). This suggests a feedback loop for the regulation of visfatin as well as the implication that prostate cancer cells lines respond to extracellular visfatin levels.

Functionally, it was clearly demonstrated that visfatin has differential effects on differing prostate cancer cell lines e.g. had no effect on the LNCaP cell line as compared to the PC3 cell lines, which clearly responded to exogenous visfatin in a proliferative fashion. The differential effect may be due to the different androgen sensitivities of the two cell lines and a hypothetical link between steroids and visfatin and investigating this was beyond the scope of the thesis. It was also noted that visfatin was neither pro- nor anti-apoptotic in the PC3 cell line. It is of great interest with particular reference to cancer that visfatin when exogenously applied to PC3 cells causes a significant migration of cells across a wound relative to basal and this action is opposed with the addition of FK866. It was also noted that NMN was able to reverse this effect. Given there were no significant effects in the LNCaP cell line with respect to functional studies further experiments were not undertaken in this cell model as it was concluded that without functional outcome dissecting signalling pathways and gene regulation would serve no further purpose. (Chapter 4 pages 102-106)

The third facet to the study (Chapter 5 pages 118-127) looked to identify whether visfatin modulated the phosphorylation of some key signalling molecules known to be involved in the pathology of prostate cancer, namely p38 and ERK 1/2. This study showed that visfatin significantly activated the p38 and ERK MAPKs at levels consistent with those found to be proliferative in the cell lines. Further studies using specific

inhibitors of these MAPKs, especially in relation to the functional effects of visfatin, would help elucidate with much more certainty the relationship between visfatin and MAPK related cell proliferation. Immunohistochemistry has been used to demonstrate the distribution of ERK1/2 in prostate tissue (Royuela, Arenas et al. 2002). They have shown that ERK 1/2 is predominantly localised to the cytoplasm of prostatic cells and is not particularly active in the epithelial layer of normal prostatic tissue where the vast majority of prostate adenocarcinomas originate, whereas 80% of stromal and basal cells are positive for pERK 1/2 staining. Gioelli et al demonstrated that the identification of activated ERK 1/2 in prostate tissue is directly related to poor histological and prognostic factors as well as demonstrating that pERK 1/2 staining has been found in normal tissue directly adjacent to CaP tissue (Gioeli, Mandell et al. 1999). With this evidence from the literature and the novel findings presented in this study thus far; visfatin having been demonstrated in prostate cancer tissue and exogenous visfatin leading to ERK phosphorylation *in-vitro*, it lends further weight to the hypothesis that visfatin is implicated in CaP progression.

The effects of visfatin on ERK activity in prostate cancer cells have not previously been reported. In this study, visfatin increased ERK 1/2 phosphorylation in the PC3 prostate cancer cell line. This is a significant finding as it has been suggested that ERK signalling mediates some key mitogenic molecules such as endothelial derived growth factor (EGF), transforming growth factor-α (TGF-α) and IGF (Maroni, Koul et al. 2004). Further importance can be ascribed to this finding with the knowledge that cellular proliferation induced by EGF and other EGFR ligands can be prevented by inhibition of the ERK signaling pathway (Guo, Luttrell et al. 2000). ERK has also been put forward as a mediator of IL-6 AR activation and the subsequent development of hormone refractory prostate cancer (Culig, Bartsch et al. 2002; Heinrich, Behrmann et al. 2003).

The role of ERK signaling in CaP has not been definitively identified and its role is still debated. Most commonly ERK 1/2 is reported to lead to cell proliferation or an increase in cell viability, however there are also studies that have shown ERK to be implicated in an apoptotic role (Maroni, Koul et al. 2004; Ghosh, Malik et al. 2005). It was also demonstrated that the levels of ERK 1/2 and related kinases showed low expression in the same cell types as used in this study i.e. LNCaP and PC3 during normal cell culture conditions, however MAPK signalling was increased in response to cellular stress and in particular when exposed to chemotherapy agents (Lee, Steelman et al. 2005). A specific example of this was demonstrated with the use of the chemotherapy agent Docetaxel, which demonstrated increased ERK expression in androgen insensitive cells following treatment (Zelivianski, Spellman et al. 2003). These findings suggest that ERK activation by visfatin or other agents may be a stress response, which would be in keeping with the cell viability studies. This would be in keeping with studies by Koul et al who demonstrated that the ERK 1/2 signaling pathway has only a small part to play in PC3 cell proliferation but was highly integral to cell migration and invasion (Koul, Huang et al. 2004). Further studies that would help delineate this theory would be demonstrable using the wound scratch assay or an invasion assay in PC3 cells following treatment with visfatin and in the presence of an ERK inhibitor. It would therefore be of interest in future studies to try and elucidate the true role of ERK pathways in prostate cancer cell models using ERK inhibitors and cell invasion studies to try and identify if ERK activation by visfatin is a more important pathway in prostate cancer invasion than simply cell proliferation. It is also of interest that in pancreatic cancer cells it has been shown that ERK 1/2 inhibition causes cell cycle arrest and down-regulation of the expression of the anti-apoptotic genes BCL-2, MCL-1, and BCL-XL suggesting that activation of the ERK pathway functions to protect pancreatic cancer from apoptosis as well allowing cell cycle advancement (Boucher, Morisset et

al. 2000). During this study it was found that BAX, BCL2-L1 and MCL1 genes were all down regulated in response to visfatin exposure. This would suggest that the visfatin induced ERK activation is unlikely to be related to gene manipulation in this model.

The study also looked at p38 phosphorylation by visfatin in PC3 cells (page 124). A significant increase in p38 phosphorylation was seen after 2 minutes and then a gradual reduction. p38 has been shown to be phosphorylated by a number of different triggers including IL-1, TNF-α, EGF, fibroblast growth factor (FGF), IL-6 and vitamin D, all of which have been shown to play a role in CaP (Mehta, Robson et al. 2001; Tuohimaa, Lyakhovich et al. 2001; Culig, Bartsch et al. 2002). As well as direct ligand activation, p38 is also activated by physical stressors such as ultraviolet light and osmotic stress (Ricote, Garcia-Tunon et al. 2006). p38 has been largely thought to be a mediator in cell apoptosis and in prostate cancer p38 activity has been shown to sensitize cells to cisplatin based chemotherapeutics (Skjoth and Issinger 2006). However there is now evidence to suggest that p38 activity may indeed be carcinogenic and in fact protect against apoptosis and this has been shown in murine fibroblasts (Luschen, Scherer et al. 2004) and melanoma cells.

Overall the role of MAPK activation in prostate cancer is debated with evidence suggesting both pro- and anti-apoptotic mechanisms. Given the data presented in this study it is tempting to speculate that visfatin activation of these two MAPKs may be implicated in proliferative effects on PC3 cells or in the manipulations of anti-apoptotic genes. Future studies with the use of specific inhibitors would be needed to elucidate these speculative links further.

This study clearly showed that visfatin modulated MMP with a significant up-regulation at both RNA and protein level and also caused a highly significant up-regulation of

zymographic activity in a dose dependent fashion. Neovascularisation, which is a form of dys- or unregulated angiogenesis, is a highly important part of solid tumour progression and as discussed in chapter five MMP-2/-9 have been heavily implicated in prostate cancer. The findings of this study are in keeping with that in the literature. This novel data is of great interest in the quest to find a reliable marker for prostate cancer and more importantly a marker of aggressive disease. A study by Di Carlo et al has suggested promising data suggesting the potential use of MMP zymography in concentrated urine samples to identify CaP patients (Di Carlo, Mariano et al. 2010). The findings during this study are in keeping with the published literature and also suggest a strong mechanism by which visfatin may be linked to prostate cancer. I have discussed in chapter 7 the data obtained with respect to human plasma concentrations of visfatin. These data imply that it is not elevated levels of visfatin that are responsible for obesity related CaP. This would go against the published literature. Furthermore it would go against a hypothesis detailing elevated visfatin levels in obesity as the mechanism linking obesity and CaP. It was discussed however, that the strength of these findings must to be viewed with caution as sample size was low and the study was not powered to identify this relationship. One therefore needs to address the study based on the original findings outlined in the literature to date.

Interestingly visfatin has been heavily implicated as a mediator of inflammation (Busso, Karababa et al. 2008) as well as an angiogenic factor and promotes cell survival (Jia, Li et al. 2004; Moschen, Kaser et al. 2007; Adya, Tan et al. 2008). It is also well reported that elevated levels of visfatin have been found in chronic inflammatory states such as arthritis (Nowell, Richards et al. 2006; Otero, Lago et al. 2006). This is of great interest when attempting to identify links between obesity and prostate cancer risk in particular the role of visfatin. The chronic inflammatory effect that obesity has may be in part due

to elevated visfatin levels and it is of great interest that chronic inflammation is a known risk factor for CaP with population studies, epidemiological genetics and molecular pathologies all lending evidence to this hypothesis (Palapattu, Sutcliffe et al. 2005). This may well be a further mechanism by which visfatin is implicated in CaP.

As well as some of the signalling pathways seen during this study other possible mechanisms by which visfatin may modulate CaP and provide an insight in to the role of obesity in CaP may be found in the link between obesity and insulin resistance and the ensuing compensatory chronic hyperinsulinaemia. This hyperinsulinaemia state leads to reduced IGF binding protein (IGFBP) and a theoretical increase in the bioavailability of free IGF (Renehan, Frystyk et al. 2006). There is also a reported lower level of IGF-1 production by the liver. Overall it is suggested that the alteration in IGF biology is enough to influence cancer risk (Calle, Rodriguez et al. 2003; Renehan, Frystyk et al. 2006). This study discovered that visfatin leads to a highly significant up regulation of IGFR at four hours with exogenous visfatin treatments and this appeared to be sustained at 24 hours although not statistically found to be significant. Given that visfatin levels are postulated to be elevated in obesity this is further novel data that lends support to the link between obesity and CaP via the IGF axis. Insulin has also been shown to up-regulate fatty acid synthase (FAS) expression in hepatocytes (Claycombe, Jones et al. 1998). FAS is a key enzyme involved in mammalian energy homeostasis by converting surplus dietary carbohydrates to fat. FAS is also over expressed in prostate carcinomas and is associated with an aggressive phenotype (Baron, Migita et al. 2004), but whether hyperinsulinaemia stimulates an increase in FAS expression in CaP cells or is linked with visfatin is as yet undocumented. Nonetheless, this could be another feasible mechanism to link obesity/metabolic syndrome and CaP.

With regards to growing obesity levels worldwide it has been long publicized in the media that increasing physical activity and dietary modifications are highly important and can lead to dramatic improvement in obesity related morbidity and mortality with emphasis being played on cardiovascular disease and diabetes (WHO 2000). It is however also highly likely to reduce the risk of a variety of cancers by modulating the many mechanisms discussed in this study including CaP. A study has shown that physical exertion has a reduced risk of advanced prostate cancer and cancer death (Nilsen, Romundstad et al. 2006). The evidence with respect to physical activity and prostate cancer is very difficult to confidently discern as one cannot conclusively say that the beneficial effects are not due to simple weight loss and to date there are no studies showing weight loss effects on prostate cancer risk and progression.

CONCLUSIONS

In conclusion, this study has shown that visfatin may well have a role in prostate cancer and supports the hypothesis linking obesity and prostate cancer and this has been demonstrated by *in-vitro* in PC3 cells. Thus this study has provided evidence that visfatin is proliferative in androgen insensitive cells models as well as providing potential mechanism by which it may modulate aggressive disease by virtue of its action on MAPKs, MMPs, and oncogenes. It has also provided some supporting evidence that the effects of visfatin may be primarily mediated via its relevance to NMN production. Finally it has been concluded that although this study has not confirmed a link between obesity and circulating levels and visfatin nor increased levels in prostate

cancer it has suggested preliminary evidence that levels may be elevated in relation to grade of disease.

These data presented here are by in large preliminary studies. However that being said, study has shown significant novel data and provides a basis for further investigation into prostate carcinogenesis. It would also not be unfair to suggest that these data have predominantly looked at prostate cancer cells lines and investigation into visfatin and normal prostate physiology would yield helpful information into the role visfatin may play in the development and progression of prostate cancer.

When evaluating the effects of visfatin in cell models it has been done for the most part in isolation using an in-vitro model and needless to say, this is a far cry from the in-vivo environment in which pathology normally ensues. Investigation using in-vivo models as would yield more accurate data in the future. A greater insight into the mechanisms linking obesity to cacner is important as it would allow the further development of new therapeutic markers and prognostic indicators. It may also lend greater emphasis to lifestyle modification and weight loss as adjuvant therapies for prostate cancer prevention and treatment.

FUTURE STUDIES

- Objective quantification of visfatin levels in prostate cancer tissue compared to benign prostatic tissue.

- Use of specific MAPK inhibitors to identify if visfatin mediates its proliferative effects via MAPK activity and also its MMP activity.
- Further studies looking at visfatin plasma and or serum levels to identify if there is significant difference in the levels between prostate cancer patients and benign controls and further correlations with MMP as potential biomarkers for aggressive disease.
- To ascertain whether visfatin modulates androgen receptor status in prostate cancer cells or vice versa to indentify if androgen status is a defining factor in visfatin mediated effects.

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APPENDIX 1: PUBLICATIONS

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A novel role for the adipokine visfatin/pre-B cell colony-enhancing factor 1 in prostate carcinogenesis☆

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ABSTRACT

Adipose tissue is now well established as an endocrine organ and multiple hormones termed 'adipokines' are released from it. With the rapidly increasing obese population and the increased risk mortality from prostate cancer within the obese population we looked to investigate the role of the adipokine visfatin in LNCaP and PC3 prostate cancer cell lines. Using immunohistochemistry and immunocytochemistry we demonstrate visfatin expression in LNCaP (androgen-sensitive) and PC3 (androgen-insensitive) human prostate cancer cell lines as well as human prostate cancer tissue. Additionally, we show that visfatin increases PC3 cell proliferation and demonstrate the activation of the MAPKs ERK-1/2 and p38. Moreover we also demonstrate that visfatin promotes the expression and activity of MMP-2/9 which are important proteases involved in the breakdown of the extracellular matrix, suggesting a possible role for visfatin in prostate cancer metastases. These data suggest a contributory and multifunctional role for visfatin in prostate cancer progression, with particular relevance and emphasis in an obese population.

1. Introduction

Prostate cancer is the third most common male cancer worldwide [21] and treatment modalities for advanced disease remain limited. Early prostate cancer growth is dependent on the presence of androgens, with androgen ablation therapy the mainstay of treatment; however, many men fail this therapy and develop androgen-independent disease [8]. Progression of established prostate cancer has been associated with obesity and its metabolic sequelae. Both tumor aggressiveness and mortality from prostate cancer are correlated positively with body mass index (BMI) [5]. Obesity is becoming one of the most significant and growing public health concerns and has reached epidemic proportions with a prevalence of 60% or more in some Western societies [26]. Obesity is well established as a risk factor for type 2 diabetes, cardiovascular disease and cancer. Specifically, visceral obesity is associated with insulin resistance, hyperinsulinaemia and prothrombotic/proinflammatory states [16], has also been identified as a risk factor for prostate cancer [27]. Evidence also suggests that obesity increases the aggressiveness of the disease [10].

Recent studies have investigated the role of adipokines as potential mediators of molecular association between these two disease states [20].

Pre-B cell enhancing factor (PBEF), was originally isolated from peripheral blood lymphocytes, and described as a secreted growth factor for early B cell proliferation [25]. More recently, however, PBEF has also been characterized as an adipokine, being highly expressed in and secreted by visceral adipose tissue, and has subsequently been termed visfatin [30].

Circulating visfatin plasma concentrations as well as visceral adipose tissue visfatin mRNA expression correlated with measures of obesity and visceral fat accumulation [3]. Visfatin plasma concentrations have also been shown to be markedly elevated in obese subjects (0.037 ± 0.008 μg/ml), compared with controls (0.001 ± 0.000 μg/ml) [11].

Intracellular visfatin expression has been demonstrated in normal, inflamed and tumor tissues [2], and has been implicated in chemo-resistance in breast cancer patients [9]. In human endothelial cells, incubation with exogenous visfatin has been shown to stimulate cell proliferation as well as inducing matrix metalloproteinases 2-&-9 (MMP-2/9) expression and gelatinase activity via the PI3K/Akt and mitogen-activated protein kinase (MAPK) signaling pathways [1]. These latter cascades are crucial to carcinogenesis.

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Use of the visfatin inhibitor, FK-866, has been shown to induce apoptosis in tumors [12]. This further suggests that visfatin has a significant role to play in cancer biology.

We propose a potential role for the circulating adipokine visfatin in prostate cancer within the obese population. To do this we used LNCaP (androgen-sensitive) and PC3 (androgen-insensitive) human prostate cancer cell lines as well as human prostate tissue.

2. Materials and methods

2.1. Reagents

Recombinant human visfatin was purchased from Axxora (Nottingham, UK). Visfatin primary antibody was purchased from Bethyl Inc. (Montogomery, TX, USA). MAPK, β-actin primary antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). MMP-2 and MMP-9 primary antibodies were purchased from Abcam plc (Cambridge, UK). All secondary antibodies were purchased from DakoCytomation Ltd. (Cambridge, UK). Normal and tumor prostate tissue cDNA were purchased from BioChain Institute (Hayward, CA, USA).

2.2. Subjects

Following ethical approval, paraffin embedded sections (3 μm thickness) were cut from archived prostate tissue obtained from patients undergoing transurethral prostatectomy ($n = 13$; mean 75.1 years; range 57–92; SEM ±2.6). Sections were floated onto 3-aminopropyltriethoxy-silicane coated slides. All patients had histologically diagnosed prostatic adenocarcinoma, and cut sections had foci of benign and malignant tissue.

2.3. Cell culture

LNCaP and PC3 cells (ATCC, Manassas, VA, USA) were cultured as previously described [19]. Cells were seeded onto 6-well plates and cultured to pre-confluence prior to treatments. Cells were allowed to attach for 24 h and subsequently were incubated with visfatin at varying concentration ranges and time points to analyze MAPK and MMPs. For proliferation assays cells were uniformly seeded into 96-well plates cultured for 24 h and incubated with serum-free media for 16 h before treatment with visfatin.

2.4. RNA extraction and quantitative RT-PCR

RNA was extracted using Qiagen RNeasy™ Plus Mini-Kit (Crawley, UK) and RNA was reverse-transcribed into cDNA using 500 ng RNA, random hexamers and 200 units RevertAid H minus M-MuLV Reverse Transcriptase. RT-PCR of visfatin was performed using the Roche Light Cycler™ system (Roche, Mannheim, Germany). Reaction mixtures contained SYBR® Green (Roche, Mannheim, Germany) and 1.5 μl cDNA. PCR conditions consisted of denaturation at 95 °C for 1 min, 40 cycles of 95 °C for 1 s, 59 °C for 10 s and 72 °C for 15 s, followed by melting curve analysis. The primers used were: visfatin (forward: 5′-AAGAGACTGCTGGCA-TAGGA-3′, reverse: 5′-ACCACAGATACAGGCACTGA-3′); β-actin (forward: 5′-AAGAGAGGCATCCTCACCCT-3, reverse: 5′-TACATGG-CTGGGGTCTTGAA-3′). With regards to PCR for MMP analysis, protocol conditions consisted of denaturation at 95 °C for 15 s, followed by 40 cycles of 94 °C for 1 s, 60 °C for 5 s, and 72 °C for 12 s, followed by melting curve analysis. For analysis, quantitative amounts of MMP-2, MMP-9 were standardized against the housekeeping gene GAPDH. The primers used were: MMP-2 forward: 5′-TGGCAAGTACGGCTTCTGTC-3′, reverse: 5′-TTCTTGTC-GCGGTCGTAGTC-3′; MMP-9 5′-TGCGCTACCACCTCGAACTT-3′, reverse: 5′-GATGCCATTGACGTCGTCCT-3′. For all reactions a no template control and also RT-negative was used as standard. With respect to MMP analysis IGF [10 ng/ml] was also used as a positive control.

2.5. Immunocytochemistry and confocal analysis

Cells were cultured onto poly-L-lysine-coated glass cover slips for 48 h and fixed with 4% paraformaldehyde. Non-specific binding was inhibited by incubating cells with 3% BSA in PBS–0.01% Triton X-100 for 1 h at room temperature. Cells were incubated with primary rabbit anti-human visfatin antibody (1:100) overnight at 4 °C. After washing with PBS–0.05% Tween, cells were incubated with secondary conjugated donkey anti-rabbit antibody, Alexa Fluor® 488 (Invitrogen, Paisley, UK). Following further washes cover slips were mounted on slides with glycerol containing 4′-6-diamidino-2-phenylindole (DAPI) nuclear marker (Invitrogen, Paisley, UK). Images were acquired using a Leica SP2 confocal laser scanning microscope system linked to a Leica DM RE7 upright microscope and a 40× oil immersion lens.

2.6. Immunohistochemical analysis

Prostate tissue sections were prepared and immunohistochemistry performed as previously described [19]; 1:200 primary rabbit anti-human visfatin antibody was used.

2.7. Proliferation assay

Cells were treated with visfatin [0–400 ng/ml] for 24 and 48 h. Cell proliferation was assayed using CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). This is a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)-based assay that quantifies the amount of formazan produced by metabolically active cells as measured by absorbance at 490 nm using a spectrophotometric plate reader, which is directly proportional to the number of living cells in culture (according to product literature).

2.8. Western blot analysis

The effect of visfatin on activation of the MAPK (ERK-1/2 and p38) as well as MMP-2 and MMP-9 protein expression in LNCaP and PC3 cells was studied using Western blotting analysis. Following treatment with visfatin at times points between 0 and 60 min for MAPK activity and at 24 h for MMP analysis, cells were lysed with Ripa buffer (containing; 0.5 M Tris–HCl, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA). Samples were centrifuged and quantified and subsequently boiled prior to use.

For MAPK analysis 40 μg of each sample was loaded; for MMP analysis 80 μg was used. Each sample were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE), and electro-blotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The PVDF membrane was then blocked by incubating with 5% bovine serum albumin (BSA; Sigma–Aldrich, Gillingham, UK) in 1 M Trizma/base, 1.54 M NaCl, 0.05% Tween 20 (tris-buffered solution (TBS) plus Tween 20 (TBS-T), pH 7.4) for 1 h at room temperature. Membranes were then incubated with primary antibody phosphorylated ERK 1/2, phosphorylated p38 (rabbit polyclonal 1:2000 diluted in 5% BSA/TBS-T), MMP-2 or MMP-9 (mouse monoclonal 1:400 diluted in 5% BSA/TBS-T) overnight at 4 °C. Membranes were then washed thoroughly for 60 min at room temperature with TBS-T (0.1%) followed by incubation with a secondary anti-mouse horseradish-peroxidase-conjugated secondary antibody (1:2000

dilution) for 1 h at room temperature. Antibody complexes were visualized using chemiluminescence (ECL; GE Healthcare, Little Chalfont, UK). To determine MAPK activity the membranes were stripped and re-probed with primary antibody for total MAPK. Band densities were measured using a scanning densitometer coupled to scanning software Scion Image™ (Scion Corporation, Maryland, USA). In addition to quantitative loading of gels, the MMP membranes were also re-probed with β-actin antibody (Cell Signaling Technology Inc., Beverly, MA, USA; 1:10,000 dilution) to determine equal protein loading. With respect to MMP analysis IGF [10 ng/ml] was used as a positive control. studies have demonstrated a role for IGF in *in vitro* prostate cancer models with respect to MMP-2,-9 [24].

2.9. Gelatin zymography

The gelatinolytic activity of MMP-2 and MMP-9 secreted into culture supernatants following treatment with 0–800 ng/ml visfatin was measured by gelatin zymography. 10 μl of culture supernatant was mixed with 10 μl zymography sample buffer and

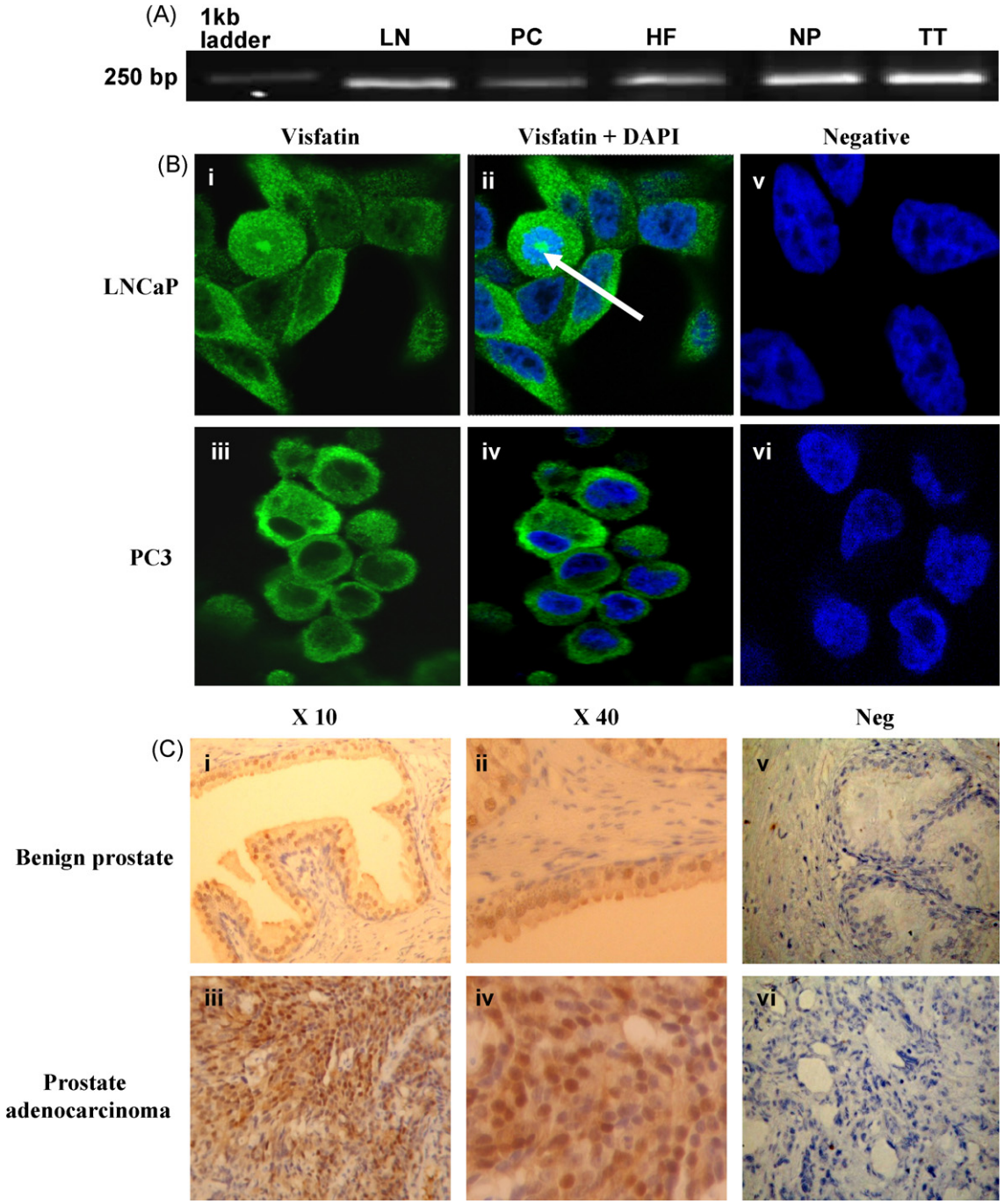

Fig. 1. Visfatin expression in LNCaP and PC3 human prostate cancer cell lines and human prostate tissue. (A) Visfatin mRNA expression in the LNCaP (LN) and PC3 (PC) prostate cancer cell lines. Human fat (HF) cDNA was used as a positive control. Visfatin mRNA was also detected in normal prostate tissue (NP) and prostate adenocarcinoma tumor tissue (TT) cDNA. (B) Immunocytochemical and confocal analysis were used to study visfatin protein expression in LNCaP and PC3 prostate cancer cell lines. Visfatin protein was detected in the cytoplasm of both cell lines (green), and following co-visualization with the DAPI nuclear marker (blue), nuclear staining for visfatin was also detected in LNCaP cells (arrowed). (C) Immunohistochemical analysis of benign and adenocarcinomatous human prostate cancer tissue. Visfatin expression was detected in the glandular epithelial cells of both benign (i and ii) and malignant tissue (iii and iv), with evidence of cytoplasmic and nuclear staining. Negative controls shown.

resolved in 10% SDS-PAGE containing 1 mg/ml of gelatin (Sigma, St. Louis, USA) under non-reducing conditions. Following electrophoresis at 4 °C, gels were washed twice for 30 min with renaturation buffer (2.5% Triton X-100) at room temperature, and then incubated overnight in incubation buffer (50 mM Tris–HCl pH 7.5, 200 mM NaCl, 10 mM $CaCl_2$, 1 μM $ZnCl_2$) at 37 °C. Characterization of MMP activity was determined by inhibition with 10 mM EDTA. Following incubation, gels were stained for 1 h buffer 0.25% Coomassie Brilliant Blue R-250 (in 45% methanol and 10% acetic acid), and then destained in the same buffer without Coomassie. Following de-staining, gelatinolytic activity of secreted MMP was observed as white bands against a blue background, which were quantified by measuring the band intensity [Gel Pro image analysis (Gel Pro 4.5, Media Cybernetics, USA)]. IGF [10 ng/ml] was used as a positive control.

2.10. Statistical analysis

Data are shown as mean ± SEM. Repeated measures ANOVA with post hoc Tukey's multiple comparison test was employed to calculate the significance of differences in the means between different groups; significance was determined at $p < 0.05$.

3. Results

3.1. Visfatin mRNA expression in prostate cancer cell lines and human prostate

Using RT-PCR, visfatin mRNA was detected in both cell lines studied, with agarose gel electrophoresis yielding an expected 249 base pair product. Visfatin gene expression was also present in cDNA prepared from mRNA obtained from normal and malignant human prostate tissue (Fig. 1A); sequencing analysis confirmed the identity of these products (data not shown).

3.2. Visfatin protein expression in prostate cancer cell lines and prostate tissue

Visfatin staining was demonstrated in LNCaP and PC3 cells using immunocytochemistry and confocal analysis (Fig. 1B). Visfatin staining showed a predominantly cytoplasmic distribution, although there was also evidence of nuclear staining in LNCaP cells (Fig. 1Bii arrow).

Immunohistochemistry was used to study visfatin expression in human benign and malignant prostate tissue (Fig. 1C). Visfatin

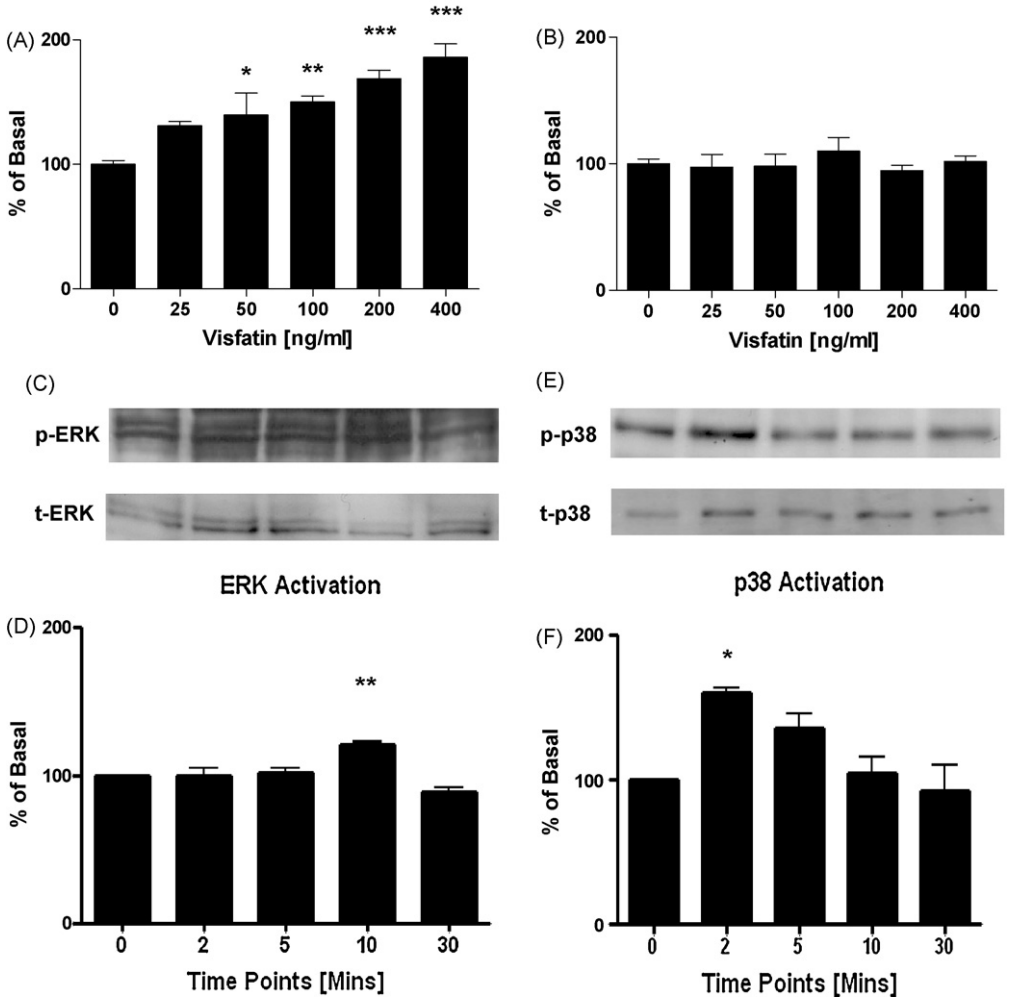

Fig. 2. (A and B) Effect of visfatin on LNCaP and PC3 cell proliferation. Effect of visfatin alone [0–400 ng/ml] on (A) PC3 and (B) LNCaP cell proliferation. 24 h data are expressed as relative to basal proliferation; $n = 4$ for all experiments, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (C–F) The effect of visfatin on MAPK (ERK 1/2 and P38) activity in PC3 cells. (C) Representative western blot showing changes in pERK/tERK expression following treatment with 400 ng/ml visfatin at varying time points of incubation 0–30 min; lanes correspond to bar chart. (D) Bar chart showing quantification of changes in ERK activation in PC3 cells. Data represent means ± SEM of three experiments; $^{*}p < 0.05$ compared to basal expression. (E) Representative western blot showing changes in p-p38/t-p38 expression following treatment with 400 ng/ml visfatin at varying time points of incubation 0–30 min; lanes correspond to bar chart. (F) Bar chart showing quantification of changes in p38 activation in PC3 cells. Data represent means ± SEM of three experiments; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to basal expression.

staining was detected in both benign and malignant tissue, with glandular epithelial cells showing high levels of expression. Interestingly, visfatin demonstrated both nuclear and cytoplasmic staining in both normal and malignant cells.

3.3. Effect of visfatin on LNCaP and PC3 cell proliferation

Visfatin treatment alone resulted in a significant concentration-dependent increase in proliferation after 24 h incubation in PC3 cells ($p < 0.001$ at 200 and 400 ng/ml) (Fig. 2A). In contrast treatment with visfatin alone had no significant effect on LNCaP cell proliferation (Fig. 2B). Given that there was no functional outcome in the LNCaP cell line no further studies were conducted.

3.4. Effect of visfatin on MAPK activity

Visfatin treatment of PC3 cells resulted in a significant activation of ERK1/2 after 10 min at a treatment dose of 400 ng/ml which corresponded to the maximal proliferative effect of visfatin ($p < 0.05$) (Fig. 2D). There was also a significant increase in p38 activity after 2 min incubation with visfatin [400 ng/ml] ($p < 0.05$) and subsequently rapidly returning to basal levels after 10 min (Fig. 2F). We observed no significant effect of visfatin on MAPK activity in the LNCaP cell line (data not shown).

3.5. Effect of visfatin/IGF-1 on MMP-2 and MMP-9 expression

mRNA expression of both MMP-2 and MMP-9 was significantly increased following treatment with all doses of visfatin studied ($p \leq 0.05$); the greatest increases were seen in MMP-9 expression. Addition of 10 ng/ml IGF-1 alone also significantly increased both MMP-2 and MMP-9 expression (Fig. 3).

The effect of visfatin on MMP-2 and MMP-9 protein expression was studied using Western blotting. We found that the changes in mRNA expression of these MMPs were also associated with corresponding significant increases in protein expression ($p \leq 0.05$) (Fig. 4A and B).

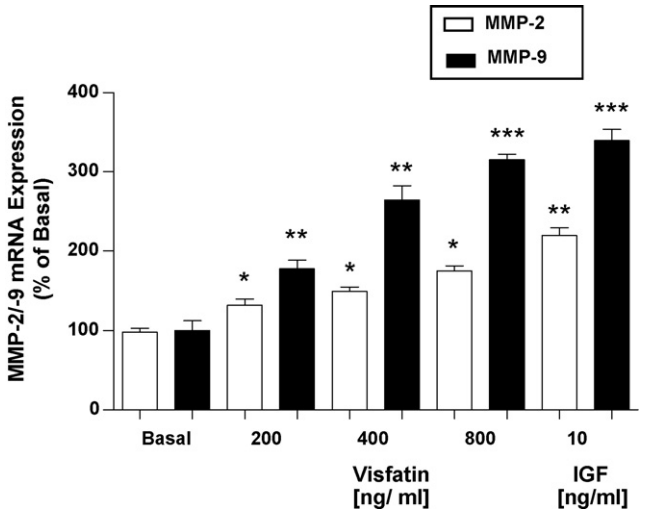

Fig. 3. The effect of visfatin/IGF-1 on MMP-2 and MMP-9 mRNA expression in PC3 cells. Bar chart showing quantification of changes in MMP-2/-9 mRNA expression in PC3 cells. Data represent means ± SEM of three experiments; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared to basal expression.

No significant effects on MMP-2 or MMP-9 mRNA or protein expression were detected in LNCaP cells following treatment with visfatin or IGF-1 (data not presented).

3.6. Effect of visfatin on secreted MMP-2 and MMP-9 activity

The effect of visfatin ± IGF-1 on the activity of secreted MMP-2 and MMP-9 by PC3 cells was studied using gelatin zymography. Visfatin induced a concentration-dependent increase in MMP-2 activity that was significant at concentrations over 400 ng/ml ($p \leq 0.05$); 10 ng/ml IGF-1 alone also significantly induced MMP-2 activity ($p < 0.001$) (Fig. 4C and D). MMP-9 also showed a dose-dependent increase in activity following treatment with visfatin which was significant at concentrations over 400 ng/ml ($p < 0.001$). IGF-1 alone also significantly increased MMP-9 activity ($p < 0.001$)

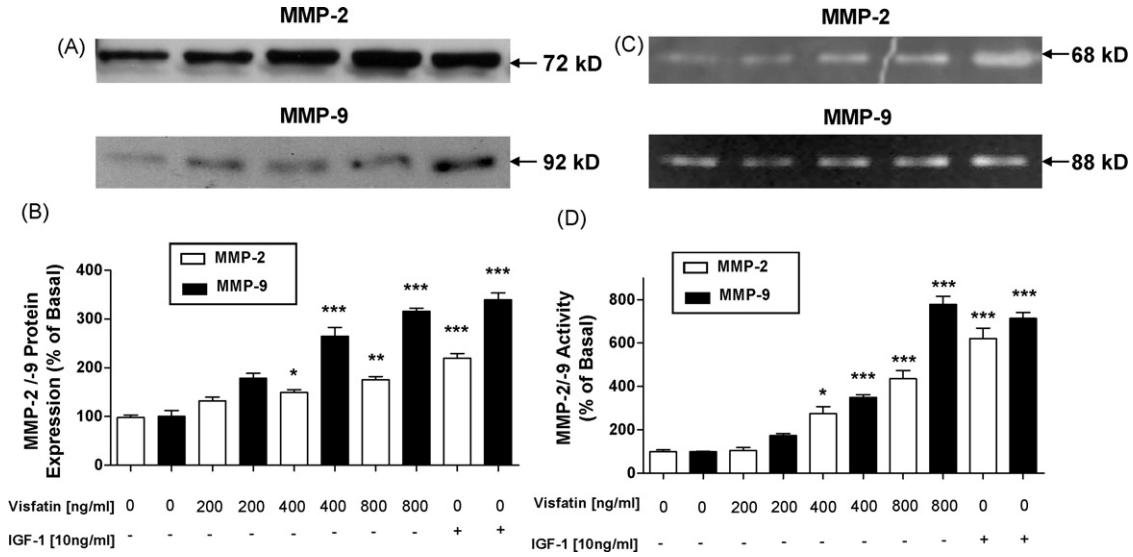

Fig. 4. The effect of visfatin/IGF-1 on MMP-2 protein expression and secreted MMP-2 activity in PC3 cells. (A) Representative western blot showing changes in MMP-2/-9 expression following treatment with 0–800 ng/ml visfatin or 10 ng/ml IGF-1; lanes correspond to bar chart. (B) Bar chart showing quantification of changes in MMP-2/-9 expression in PC3 cells. Data represent means ± SEM of three experiments; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared to basal expression. (C) Representative zymography gel showing changes in MMP-2/-9 activity following treatment with 0–800 ng/ml visfatin or 10 ng/ml IGF-1; lanes correspond to bar chart. (D) Bar chart showing quantification of changes in MMP-2/-9 activity in PC3 cells. Data represent means ± SEM of three experiments; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared to basal expression.

(Fig. 4C and D). Again, visfatin had no significant effect on LNCaP cell MMP activity (data not shown).

4. Discussion

Obesity is gaining recognition as an important risk factor for high grade prostate cancer and also an increased risk of prostate cancer mortality compared to the non-obese population [4]. There is growing evidence to suggest that adipokines may play an important role in mediating this association, and we have investigated the possible role of the adipokine visfatin on prostate cancer progression.

In addition to its identification as a circulating adipokine, visfatin has been identified as an intracellular enzyme (NMPRTase) and thus we investigated the expression of visfatin in the established prostate cancer cell lines LNCaP and PC3 and human prostate cancer tissue. This is the first study to demonstrate visfatin expression in these prostate cancer cell lines and tissue, however, its expression in both normal and tumor tissues other than the prostate [9,14,25] has been demonstrated. Previous studies have shown nuclear and cytoplasmic staining for visfatin in both adrenal cells and fibroblasts which is thought to be dependent on the stage of the cell cycle [18]. In our study, we detected both cytoplasmic and nuclear staining for visfatin which is in accordance with these findings. Immunohistochemical analysis did not identify any significant difference between cancerous and benign sections of tissue. Further quantitative studies are needed to assess the importance of this observation. Although we demonstrated comparable gene expression as well as intracellular visfatin protein levels in this study, we report clear differences in the proliferative response of the two different prostate cancer cell lines to visfatin. Visfatin-induced increases in cell proliferation were observed in PC3 cells but not LNCaP cells, suggesting that circulating visfatin can exert differing effects based on cell characteristics. One of the key differences in the characteristics of the two cell lines used is the androgen status of these cells. PC3 cells represent an *in vitro* model for androgen-insensitive prostate cancer, and thus do not express the classical intracellular androgen receptor (AR) as compared to the androgen-sensitive LNCaP cells. The difference in response to visfatin between the two cell lines raises the possibility of androgen or androgen receptor status being of potential significance in the modulation of prostate cancer cell biology by visfatin. It is also a possibility that the absence of androgen in the treatment media may have influenced the results seen in the 'androgen-sensitive' LNCaP cell line. Future studies to strengthen these findings in this cell line could therefore involve comparing visfatin exposure in the presence and absence of androgen. The possibility of an interaction between AR and visfatin in prostate cancer cells is also an area for future research but falls beyond the remit of this paper.

The effect on proliferation was demonstrated in a concentration-dependent manner and perhaps more importantly at levels of visfatin similar to those that have been demonstrated in the obese population [11]. This provides novel evidence that elevated levels of circulating visfatin may influence the growth of prostate cancer tissue.

Given that visfatin treatments increased cell proliferation, we investigated two common MAPKs implicated in cell viability/apoptosis. ERK1/2 is generally associated with mitogenesis and therefore inversely related to apoptosis [6]. We demonstrate an increase in ERK1/2 activity after 10 min which is consistent with our functional studies. In keeping with our results, ERK activation has been previously demonstrated in PC3 cells following incubation with leptin, also an adipokine [7]. Other groups have also demonstrated visfatin as an activator of ERK1/2 in other tissues including endothelial and smooth muscle cells [17,28].

Interestingly we also found a significant increase in p38 activation after 2 min. p38 has been broadly associated with apoptosis induction [6]. There is also evidence that p38 increases cell proliferation in prostate cancer in response to external stimuli and also its over-expression is implicated in increased cell proliferation [22]. Furthermore p38 has also been shown to play an important role as a mediator in the up regulation of MMP-2 in prostate cancer [13]. Our data lends support to the existing data that p38 activation may have proliferative effects dependant on the stimulant applied, in this case visfatin, and also that it plays a significant role in mediating factors responsible for prostate cancer progression.

MMP-2 and -9 are important proteases that are involved in the breakdown of the extracellular matrix under both physiological and pathological conditions [1,23]. More specifically, they degrade type IV collagen, the major structural component of basement membranes and are crucial to the process of cell migration, invasion and metastasis. Both MMP-2 and -9 were highly expressed in prostate cancer tissue [29] and circulating levels of MMP-2 and -9 have also been correlated with bone metastases and tumor grade in prostate cancer patients [15]. Not only were the effects of visfatin more notable in the PC3 prostate cancer cell line, but they also induced significant increases in MMP-2 and -9 expression and gelatinolytic activity. In conjunction with their aforementioned effects on proliferation and apoptosis, it is of great interest that both ERK 1/2 and p38 have been implicated in the regulation of MMP-2 and -9. Visfatin induced ERK 1/2 phosphorylation has been shown to regulate the production and activity of MMPs and play a role in dysregulated angiogenesis [1] while p38 has also been shown to influence MMP-2 in prostate cancer [13]. One can speculate from these findings that a possible mechanism by which visfatin influences prostate cancer progression may be via an increase in MMP production and activity via visfatin induced MAPK phosphorylation.

Our findings, particularly the increased gelatinolytic activity seen with visfatin treatment, allows us to tentatively speculate that the elevated circulating visfatin levels associated with obesity may promote prostate cancer progression by enhancing the capability of metastasis. The apparent increase in metastatic sensitivity of the PC3 cell line supports to the theory that obesity is an important risk factor for prostate cancer disease progression and mortality. Further investigation of visfatin in prostate cancer biology and investigation into the mechanisms by which visfatin drives cell proliferation is required before any pathological or therapeutic conclusions can be drawn.

In conclusion our novel data have demonstrated a clear difference between androgen-sensitive and androgen-insensitive prostate cancer cell lines with regards to visfatin induced cell proliferation, metastatic and angiogenic potential. This potential role for visfatin in prostate cancer may well be a significant contributory factor in the increased aggression of prostate cancer in obese individuals.

Conflict of interest

The authors have no conflicts of interest to declare.

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