Effects of NFκB Inhibition on Macrophage – Adipocyte – Prostate Cancer Cell Crosstalk

A thesis submitted in fulfilment of the requirements

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by

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Declaration

I confirm that I shall abide by the University of Sheffield's regulations on plagiarism and that all written work shall be my own and will not have been plagiarized from other paper-based or electronic sources. Where used, material gathered from other sources will be clearly cited in the text.

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List of Abbreviations

AR	Androgen receptor
ARO	Aromatase
AR-Vs	Androgen receptor splicing variants
ATM	Adipose tissue macrophage
BAT	Brown adipose tissue
BCA	Bicinchoninic acid
BM	Bone marrow
BMI	Body mass index
BMPs	Bone morphogenetic proteins
BPH	Benign prostate hyperplasis
BSA	Bovine serum albumin
BTZ	Bortezomib
CI	Confidence interval
CNVs	Copy number variants
CRPC	Castration resistance prostate cancer
DEX	Dexamethasone
DDR	DNA damage response
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EGF	Epidermal growth factor
ERβ	Estrogen receptor β
ET-1	Endothelin-1
FBS	Fetal bovine serum
GEPIA	Gene Expression Profiling Interactive Analysis
GO	Gene Ontology

GS	Gleason score
GTEx	Genotype-Tissue Expression
GWAS	Genome wide association studies
HFD	High-fat diet
HR	Hazard ratio
HRP	Horseradish peroxidase
IBMX	3-isobutyl-1-methylxanthine
IGF-1	Insulin like growth factor-1
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LFD	Low-fat diet
LPS	Lipopolysaccharide
MAPKs	Mitogen-activated protein kinases
M-CSF	Macrophage colony stimulating factor
MD	Mean difference
MIF	Macrophage migration inhibitory factor
MMP-9	Matrix metallopeptidase 9
NFκB	Nuclear factor kappa B
NIK	NFκB-inducing kinase
OD	Optical density
OPN	Osteopontin
PCa	Prostate cancer
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol 12-myristate 13-acetate
PPARγ	Peroxisome proliferator-activated receptor γ
PRL	Prolactin
PSA	Prostate-specific antigen
PSMB	Proteasome β

PTEN	Phosphatase and tensin homolog
PTHrP	Parathyroid hormone-related protein
PVDF	Polyvinylidene difluoride
RANKL	Receptor activator of nuclear factor kapa-B ligand
RIP	Receptor-interacting protein
SABOR	San Antonio Centre for Biomarkers of Risk of Prostate Cancer
SDF-1	Stromal-derived factor-1
SEM	Standard error measurement
SD	Standard deviation
SPOP	Speckle type Poz protein
STRING	Search Tool for the Retrieval of Interacting Genes
TAMs	Tumour associated macrophages
TBS	Tris buffer saline solution
TLRs	Toll-like receptors
TNFα	Tumour necrosis factor alpha
TNM	The tumour, node and metastasis system
TRADD	TNF-receptor-associated death domain
TRAF	TNF-receptor-associated factor
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue

Abstract

Obesity and inflammation are major risk factors of prostate cancer (PCa). Tumour associated macrophages produce various pro-inflammatory mediators that are known to regulate the activity of PCa cells and adipocytes. NFκB link inflammation to PCa and obesity. However, the role of NFκB on PCa cell - adipocyte – macrophage crosstalk remains poorly understood. Here, I hypothesised that cancer-specific inhibition of inflammation-induced canonical NFκB activation reduces obesity driven PCa tumour growth and metastasis by disrupting the crosstalk between adipocytes and PCa cells and immune cells in the tumour microenvironment. First, meta-analysis provided a preliminary evidence that confirmed that the involvement of pro-inflammatory mediators in obesity and PCa development and progression. Next, I have shown that adipogenic derived factors enhanced the growth of a panel of human and mouse PCa cells. In addition, functional *in vitro* studies confirmed that TNFα and M2 macrophage derived factors enhanced the ability of mature adipocytes to increase invasiveness of highly metastatic PCa cells, by mechanisms dependent, at least in part, on increased levels of NFκB-activating factors and/or the chemokine CCL2. I have demonstrated through bioinformatic and mechanistic analysis that the gene encoding IκBα - an essential mediator of canonical NFκB activation - is likely to regulate the interactions of adipocytes and PCa cells, and IκBα phosphorylation might be the underlying mechanism by which adipocyte-derived factors affect the behaviour of highly metastatic PCa cells. Furthermore, the inhibition of cancer-specific IκBα activation by the verified proteasome inhibitor Bortezomib significantly attenuated the ability of mature adipocytes to increase the *in vitro* growth and invasion of castration-insensitive PCa cells. Whilst the evidence from *in vivo* studies is yet to come, the present results imply that clinical approved therapeutic agents Bortezomib show promise for the treatment of PCa in obese patients. Thus, further preclinical testing of the effects of this agent – alone or in combination with chemotherapy – on PCa metastasis in obese mice are needed.

Graphical abstract

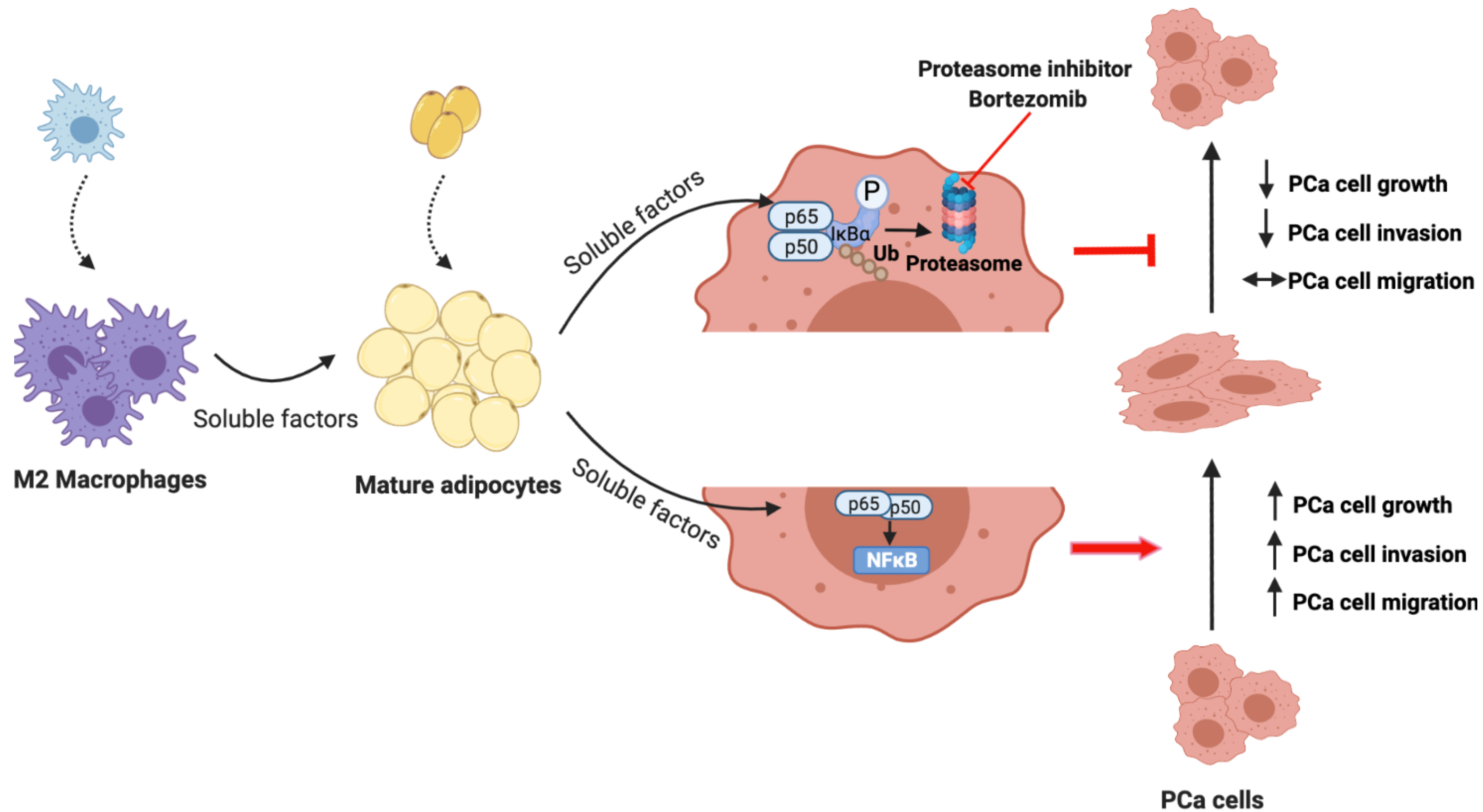


Figure. 1. **Schematic representation of the effects of inhibition of cancer-specific canonical IκBα / NFκB signalling pathway activation on prostate cancer cell – adipocyte – macrophage crosstalk.**

CHAPTER 1

GENERAL INTRODUCTION

1.1. Prostate Cancer

1.1.1. Epidemiology of prostate cancer

Prostate cancer (PCa) is the most diagnosed non-skin malignancy in men in worldwide. In the United Kingdom, PCa is the second leading cause of cancer death, it represents 26% (46,690) of all new cases of malignant cancer. The disease is age-related; with 85% of patients diagnosed are over the age of 65 years. An improved diagnosis of PCa the last 50 – 60 years together with growth in aging population have contributed to significant increase of PCa incidence in men worldwide (Siegel et al., 2018, Rawla, 2019). Asian countries, such as India, China and Japan, show the lowest prevalence rates of prostatic cancer (Bostwick et al., 2004).

The majority of PCa cases (57-63%) are diagnosed at stage I or II, and fewer cases (37-43%) at later stages (stage III of IV). 90% of patients with locoregional PCa survive the disease for 5 years or more. However, most PCa-related deaths are due to metastatic spread of the disease. 5-year survival rate amongst patients with metastatic PCa drops to 30% (Atlanta, 2003, Kirby, 2009, Key Non Parliamentary Papers Office for National, 2020). PCa preferably metastasis to the skeleton and 47% of PCa patients with bone metastasis survive for one year and 3% survive the disease for five years (Nørgaard et al., 2010).

1.1.2. Risk factors of prostate cancer

The risk of developing PCa has been attributed to a number of environmental factors (lifestyle and diet) and genetics factors (Khan et al., 2010, Attard et al., 2016). African-American men have higher risk of developing PCa compared to Caucasian men, whereas South Asian men have a lower risk (Taitt, 2018). Studies of Asian males have illustrated that diet in particular fat intake influences the risk for PCa (Akaza et al., 2011). In addition to geographic, genetic and ethnic differences, studies have also linked consumption of products rich in fatty acids such as red meat and dairy to increased risk of PCa (Giovannucci et al., 1993). The expression level of enzymes that responsible for fatty acid metabolism such as α-methyl-CoA racemase

have been found to be upregulated in PCa patients when compared to healthy individuals (Kumar-Sinha et al., 2004, Liu, 2006).

Genome wide association studies (GWAS) have associated a number of mutations with PCa initiation and progression (Benafif et al., 2018). The level of expression of a number of genes such as androgen receptor (AR) gene, NKX3.1, FOXA1 and Myc have been implicated in the initiation and progression of PCa (Jenkins et al., 1997, Gil et al., 2005, Gallucci et al., 2009, Gurel et al., 2010, Parolia et al., 2019), whereas the expression of tumour suppressor genes such as the phosphatase and tensin homolog (PTEN), TP53 and RB1 are down regulated (Hamid et al., 2019). Mutations in genes that implicated in the regulation of other cancers such as the breast cancer gene BRCA1 and BRCA2 have been associated with aggressiveness and poor clinical outcomes of PCa (Castro and Eeles, 2012), whereas mutation in genes such as HOXB13 increases the risk of hereditary PCa (Ewing et.al. 2012). A number of genes such as GSTP1, PTEN and AR play an important role in progression of hormone-dependent PCa (Ai-Jun et al., 2007, Shtivelman et al., 2014), whereas growth factors, particularly insulin like growth factor-1 (IGF-1) was found to influence the progression of PCa by activating AR under a low level of androgen (Visakorpi, 2003, Zhu and Kyprianou, 2008).

1.1.3. Diagnosis of prostate cancer

Normal and cancerous prostate cells produce prostate-specific antigen (PSA). PSA is routinely used to diagnose the development and progression of PCa in the clinic (Chen & Sawyers, 2002, Makarov and Carter, 2006). Normal PSA level is lower than 4.0ng/ml, and if elevated beyond this level, a prostate biopsy would be recommended to patients (Crawford et al, 2011). Although, serum level of PSA is affected by neoplastic processes in the prostate (Sp et al., 2013), elevated PSA levels are also observed in benign prostatic hyperplasia and as a result of inflammatory response in the prostate (Kirby, 2005).

There are several forms of PCa, and the list includes acinar adenocarcinoma, ductal adenocarcinoma, squamous cell cancer and small cell PCa (Table 1) (Jing Li Zhe, 2016). The tumour, node and metastasis (TNM) system are used to assess the size and stage of metastasis of PCa in four main stages (T1 to T4) (Figure 2A). The system indicates if the cancer has spread to the lymph nodes (N in TNM denotes node). In the lymph nodes, PCa is classified as NX (lymph nodes cannot be assessed), N0 (no cancerous cells can be found in lymph nodes), and N1 (cancer cells in nearby lymph nodes) (Montie, 1994).

Table 1． Different types of prostate cancer

	Incidence	Symptoms	PSA	Progression
Acinar adenocarcinoma	100/ 100,000 people/year	Urinary obstruction, rectal bleeding or obstruction	Positive	
Ductal adenocarcinoma	Rare (1%)	Urinary obstruction, haematuria	Normal (<4.0ng/ml) Positive	More aggressive than acinar adenocarcinoma. Metastases are more frequent
Small cell prostate cancer	<2%	Difficulty and pain while passing urine	Normal or slightly higher	More aggressive than adenocarcinomas

Metastasis is classified into two main categories, M0 and M1, with further sub classification of M1 into M1a, M1b and M1c according to the aggressiveness of the disease (Figure 2B). In the TNM staging system, T1, N0, M0 and T2a, N0, M0 are regarded as stage 1 during which the cancer is predominately located in the prostate stage 2, which includes T2b, N0, M0 and T2c, N0, M0, indicates that the tumour is increase in size; however, it is still contained with the gland. If PCa cells metastasize to seminal vesicles, it will be regarded as stage 3 (T3, N0, M0). Finally, the tumour will be assigned to stage 4 (T4, N0, M0, any T, N1, M0 or any T, any N, M1), if it has spread into nearby organs or distant tissues and organs such as the lungs or liver (RD and A, 2020).

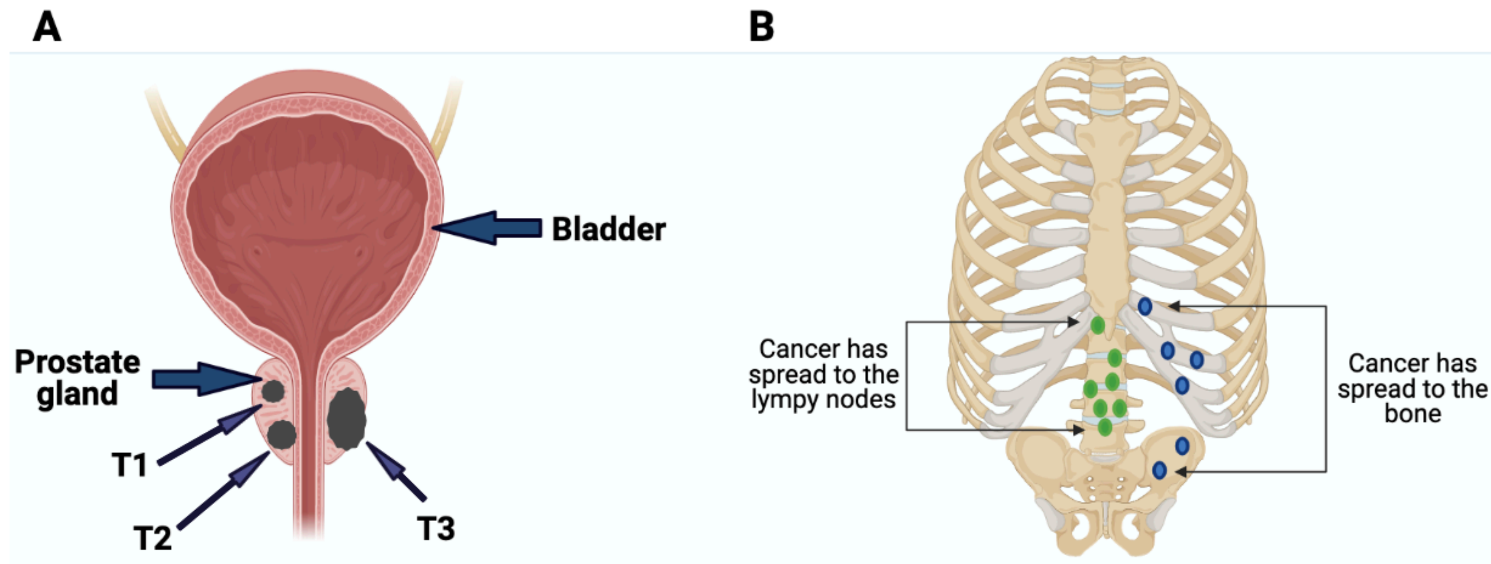


Figure. 2. The size and area of prostatic tumours according to the TNM system. A) T1: the size of prostate cancer is too small to be visualized by imaging. 2) T2: the area of cancer is confined within prostate. 3) T3: tumour break through the prostate capsule. 4) T4: tumour metastasize to other body organs nearby. B) Metastatic prostate cancer to the TNM system. 1) M0: cancer has no signs of metastases. 2) M1: distant metastasis (M1a: within lymph nodes; M1b: bones; M1c: other organs. Refer to text for details.

PCa histologic tumour grade and metastasis are also identified by the Gleason score (GS) (Bostwick, 1994). The GS range from 2 to 10 with high-grade metastatic PCa is described as 8 and over (Epstein et al., 2005).

The combination of TNM system and Gleason score is used to diagnose, monitor PCa progression and assign treatment in patients. For example, androgen deprivation therapy is recommended for patients with T1-T2a, or Gleason score 2-6. Active surveillance strategy or radiation therapy is prescribed to male with stage T2b-T2c and Gleason score 7. Treatment options for patients with 8-10 of Gleason score, or T3a stage include radical prostatectomy plus pelvic lymph node dissection (AW Partin, 1993, Chang et al., 2014, Barakzai, 2019).

1.1.4. Pathophysiology of prostate cancer

1.1.4.1. Prostate tumorigenesis

Sex hormones play an important in the initiation and progression of PCa (Peter and Donald, 2011). The levels of androgens such as testosterone and dihydrotestosterone and their receptors (AR) play a vital role in primary tumour growth and early metastasis (Debes & Tindall, 2002; Kaarbø et al, 2007). Thus, early stages of PCa can be treated with androgen deprivation therapy (Zhou et al, 2015).

AR are regarded as the major factor for hormone independency in PCa cells. The main mechanisms associated with androgen independency in PCa involve amplification, mutation and altered expression of AR and their coactivator and corepressor proteins (Balk, 2002). Therefore, inhibitors of androgen and AR synthesis and signalling, such as Dutasteride, Abiraterone and Leuprolide, are commonly used for treatment of PCa (Wu et al., 2011, Schweizer and Antonarakis, 2012, Hoda et al., 2017). Androgen independency in PCa patients is one of the major contributors to resistant to hormone therapy (Balk, 2002). Other factors include DNA damage response (DDR) (Tapia-Laliena et al., 2014) and dysregulation of expression of proteins, such as speckle type Poz protein (SPOP) and Ataxia Telangiectasis

Mutated kinase (Ide et al, 2012, Fried et al., 2016). This multi-factorial nature to hormone therapy resistance has given rationale for the use of combinational therapy such as DDR and AR inhibitors in the treatment of metastatic PCa (Karanika et al, 2014).

Adiponectin, a protein hormone involved in the regulation of fatty acid metabolism, has been implicated in the development of castration-resistant PCa. Lower levels of adiponectin or elevated expression of ERp46, a negative modulator of adiponectin receptor 1 (AdipoR1), increase the risk of castration-insensitive PCa (Pinthus et al., 2013, Duivenvoorden et al., 2015, Karnati et al., 2017). Increasing evidence has also implicated the pituitary gland hormone, Prolactin (PRL), and its receptor in prostate tumorigenesis. The level of PRL correlates with accelerated tumour growth of PCa cells (Sackmann-Sala and Goffin, 2015); thus, PRL receptors are considered as a potential target in the treatment for early PCa.

1.1.4.2. Prostate cancer bone metastasis

Most patients with advanced PCa experience complications related to metastasis to lymph node, bone and other organs including lungs, brain and liver (Gingrich et al., 1996). Among these, PCa bone metastases and skeletal-related events are common in patients with advanced disease. One- and five-year survival rate is 40% and less than 1% in patients with bone metastasis and skeletal related events such as hypercalcemia, bone loss, fracture and pain (Nørgaard et al., 2010).

PCa bone metastases, like bone metastases from other organs, follows recognized stages that includes intravasation spread of PCa cells to the bone marrow, adhesion of these cells to bone microvasculature and matrix components, extravasation of PCa cells from the vascular system and dissemination into and survival in the bone marrow environment, and interaction with stroma and host cells, particularly osteoblasts and osteoclasts (Morrissey and Vessella, 2007).

Recruitment of functional stroma coupled with the action of a plethora of hormones, cytokines and growth factors promote the production of proteases that aid with the degradation of the extracellular matrix (ECM) that in turns allows PCa cells escape the stromal compartment and facilitates their spread to the bone marrow (Ganguly et al., 2014). The process of localization and homing of prostate tumour cells to the bone marrow is poorly understood. Studies suggest that the combined action of various chemo-attractants, systematic and local hormones, pro-inflammatory cytokines and growth factors plays a critical role in the metastatic process of PCa cells to the skeleton. For example, the expression level of chemokine stromal-derived factor-1 (SDF-1/CXCL12) is elevated in metastatic PCa cells and its interaction with CXCR4 receptor has been implicated in homing and retention of hematopoietic stem and metastatic PCa cancer cells (Gupta and Duda, 2016). Furthermore, malignant prostate epithelial cells preferentially bind to bone marrow endothelial cells and the action of tethering proteins, such as vascular cell adhesion molecule 1 (VCAM-1) enhance their invasive ability in response to bone marrow endothelium (Scott et al., 2001, Zheng et al., 1999).

Bone remodelling is a balanced process of osteoclastic bone resorption and osteoblastic bone formation. The presence of PCa cells in bone disrupts this balance thereby leading to formation of cancerous disorganised bone, enhanced bone loss, or both (Morrissey and Vessella, 2007). Thus, PCa bone metastasis in patients is microscopically characterised by the presence of both osteoblastic and osteolytic lesions (Christopher and Sue-Hwa, 2005). In addition to PSA and GS, the levels of the bone turnover markers serum Alkaline Phosphatase (marker of bone turnover and osteoblast differentiation) and N-terminal telopeptide of type I collagen (marker of bone turn over and osteoclastic bone resorption) (Metwalli et al., 2014, Coleman et al., 2008) are used to diagnose and estimate the survival of PCa patients with bone metastases (He et al., 2012).

Androgen independency is also a key feature of metastatic PCa in bone (Thalmann et al., 1994, Shafi et al., 2013), and androgen deprivation therapy is known to enhance bone

remodelling, thereby exacerbating skeletal-related events induced by advanced PCa in bone (Michaelson et al., 2004, Oefelein et al., 2001).

A number of other factors are implicated in the regulation of PCa bone metastasis. PCa express bone morphogenetic proteins (BMPs) and vascular endothelial growth factor (VEGF) that are known to stimulate osteoblast activity and enhance the formation of osteoblastic lesions. A number of growth factors including IGF-1, FGF and TGFβ have been implicated in the regulation of osteoblast support for PCa cell growth and survival (reviewed in Ganguly et al., 2014). Elevated level of endothelin-1 (ET-1) has been detected in the presence of PSA and ET-1 has been found to contribute to the disruption of bone remodelling by PCa cells in bone (Rosanò et al., 2013).

The RANKL/RANK/OPG system plays a critical role in the regulation of osteoclast genesis and bone loss and receptor activator of nuclear factor kappa-B ligand (RANKL) induced NFκB activation is implicated in PCa induced bone cell activity and osteolysis (Wittrant et al., 2004, Marino et al., 2019). In addition, PCa cells and osteoblasts express and secrete macrophage colony stimulating factor (M-CSF) and parathyroid hormone-related protein (PTHrP) that are known to directly and indirectly promote osteoclast formation, survival and activity (Sottnik and Keller, 2013). Furthermore, bone resorption release matrix-derived factors such as FGFs, IGFs and TGFβ that further promote PCa cell – osteoblast – osteoclast interactions, thereby enhancing PCa associated bone remodelling (Ibrahim et al., 2010).

In the clinic, the anti-resorptive Zoledronic acid and Denosumab, a human monoclonal antibody for RANKL, are used to treat cancer patients with bone metastasis (Ibrahim et al., 2016). Although these anti-osteolytic drugs significantly reduce bone resorption and bone loss, their anti-tumour and anti-metastatic effects are limited and poorly understood. Recently, Radium-223, a radioactive isotope that resembles calcium, was shown to inhibit skeletal tumour growth and improve survival of PCa patients with bone metastasis (Todenhöfer et al., 2015, Coleman, 2016, Den et al., 2019).

1.2. Obesity and Prostate Cancer

Obesity is known to increase the risk of various serious diseases including cancer. Emerging evidence suggests that obesity is a major contributory factor to all aspects of PCa including initiation, progression and metastasis (Allott et al., 2013). Several lines of evidence indicate that obesity is associated with the development of various clinical features characteristic of PCa progression including high incidence, increased mortality, recurrence and metastasis, even after radical prostatectomy (Rodriguez et al., 2001, Gong et al., 2007, Keto et al., 2012, Langlais et al., 2019).

1.2.1. Introduction to Obesity

Obesity has emerged as a public health problem resulting in severe health and social difficulties for people. In the UK, around one in four adults and around on in five children are diagnosed as clinically obesity with a body mass index (BMI) greater or equal to 30. From 1993 to 2011, the prevalence and rate of obesity among adults in the UK have doubled - from 13% of men and 16% of women to 24% and 26%, respectively (NICE Clinical Guidelines, No.189, p12-14). A combination of causes and contributing factors are attributed to this rise in obesity. The list includes diet, hereditary, environmental factors, lack of physical activity and medical conditions such as hypothyroidism and Cushing’s syndrome (Causes, 2016).

Adipose tissues are endocrine organs that are classified into white (WAT) and brown adipose tissue (BAT) (Lee et al., 2014). BAT is derived from Myogenic factor 5 (Myf5)-positive progenitor cells and are often referred to as “good” fat. BAT is composed of small lipid droplets and it is metabolically active tissue that regulates energy balance through an abundance of mitochondria (Shan et al., 2013, Lee et al., 2019). BAT maintains the body temperature and generates heat through thermogenesis (Saely et al., 2011). The quantity of brown fat decreases with age, however, long-term cold exposure and exercise induces ‘browning’ of WAT (Gonçalves et al., 2017, Aldiss et al., 2018, Peres Valgas da Silva et al., 2019).

In contrast, WAT stores energy as triglycerides and thus it consists of a single large lipid droplet and fewer mitochondria than BAT. WAT is derived from Myf5-negative progenitor cells (Figure 3) (Shan et al., 2013) and act as thermal insulator and cushion for internal body organs. WAT regulates appetite and hunger through production of hormones such as estrogen and leptin (Austin and Marks, 2008).

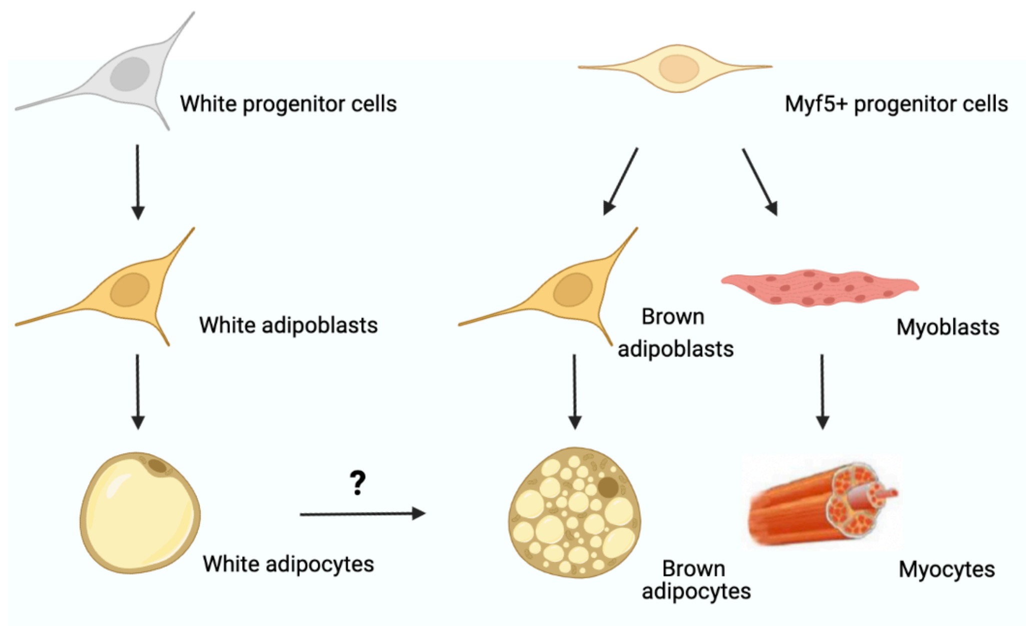


Figure. 3. Developing process of white and brown fat. It is unknown that if white adipocytes converse to brown adipocyte. Refer to text for details.

Obesity is associate with dysregulated circulating blood hormone concentrations, abnormal hormone transport, or altered hormone level of target tissues. Obese men are characterised by a reduction of testosterone levels with increasing body weight. Those alterations may play an essential role in the development of obesity and metabolic abnormalities (Figure 4) (Pasquali, 2006). Mature adipocytes, as the energy-storing cells, secrete hormones, growth factors, chemokines or pro-inflammatory molecules and adipokines such as leptin, adiponectin and resistin (Noriyuki et al., 2011, Wang et al., 2012, Victor et al., 2016). Adipokines such as adiponectin secreted from adipose tissue into the bloodstream regulate a wide range of hemostatic process including glucose and lipid metabolism and inflammation (Ghadge et al., 2018).

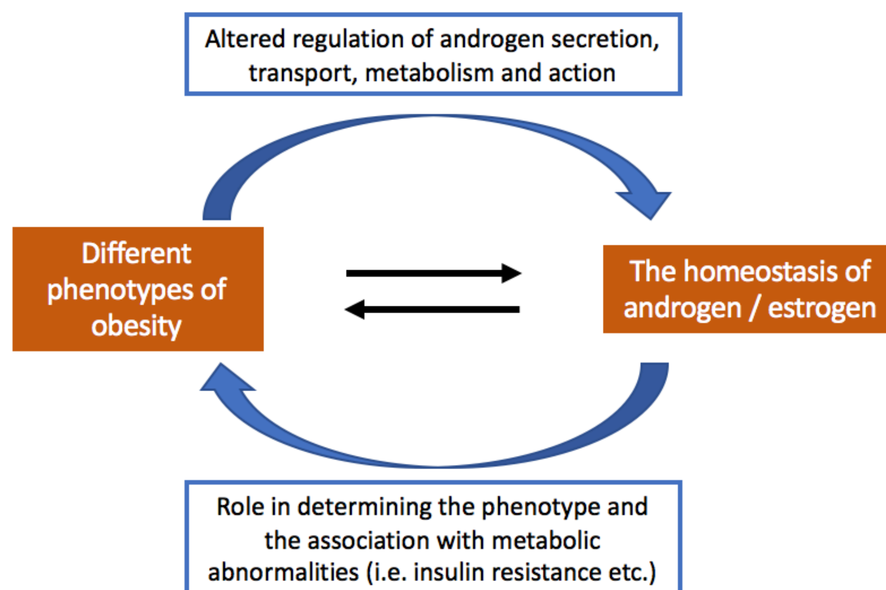


Figure. 4. **Schematic diagram of the interaction between androgen and obesity.** Refer to text for details. Adapted from Pasquali, 2006.

1.2.2. Adipogenesis in Prostate Cancer

High-fat diet (HFD) causes obesity and HFD-induced obesity is considered as a major risk factor of various cancers including PCa (Ma and Chapman, 2009; Mwangi and Zhang, 2013 Lin et al., 2015; Downer et al., 2017). Epidemiolocal studies have shown that HFD enhance PCa initiation, progression and spread directly or via suppressing the ability of the body's own immune system to fight it. HFD-derived adipose-secretory cytokines or chemokines accelerated spontaneous prostate tumour progression in the immunocompetent TRAMP mouse model, and in a xenograft mouse model of the human PCa cell line LNCaP (Yu et al., 1995, Han Jin et al., 2015). Interestingly, high-fat feeding had no effects on tumour progression in a xenograft model of patient-derived prostate tumour tissues (Lo et al., 2016).

One of the proposed mechanisms for how obesity enhances the development of PCa is the high expression levels of multiple factors that include adipose-derived cytokines, estrogens, insulin and IGF-1 (Figure 5) (Donohoe et al., 2011, G Paz-Filho 2011, Allott et al., 2013).

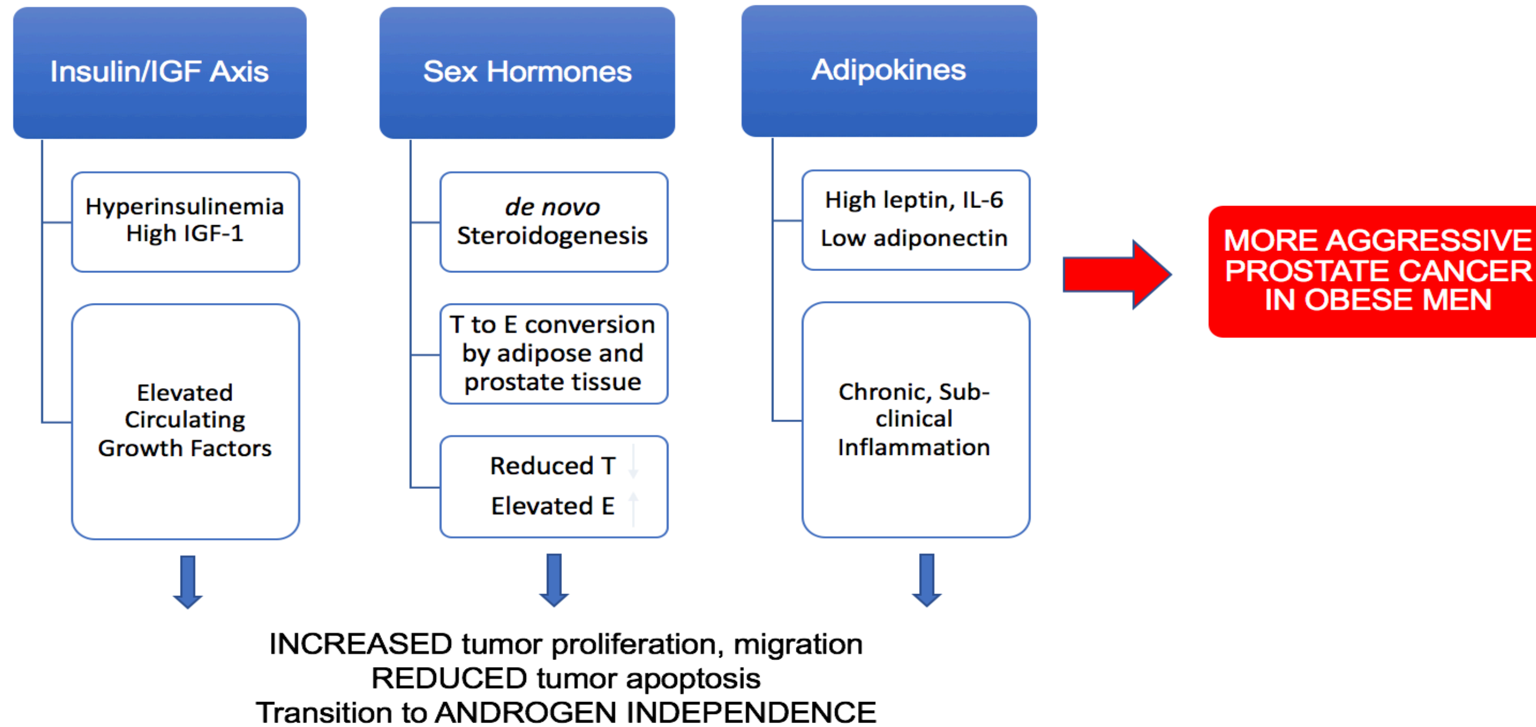


Figure. 5. Regulation of obesity and aggressive prostate cancer by growth factors, hormones and adipokines. E=estrogen; IGF=insulin-like growth factor; IL=interleukin; T=testosterone**.** Refer to text for details. Adapted from Allott et al, 2013.

The insulin / IGF signalling axis plays an important role in most stages of PCa. IGF-1 has been shown to enhance the proliferation of PCa cells in culture (Denduluri et al., 2015), and its overexpression in prostate basal epithelial layer of transgenic mice increased the risk of prostate adenocarcinoma, thereby indicating a role in PCa initiation. Conversely, inhibition of IGF-1 signalling is associated with a reduction of aggressive and metastatic behaviour of androgen-independent PCa cells to bone (Jr, 2004).

Obesity is also associated with decreased androgen levels (Pasquali, 2006). Androgen plays an important role in PCa development and influence PCa risk (Hormones, 2008). Low testosterone enhances aggressiveness of PCa (Schnoeller et al., 2013) and research shows that elderly men exhibit low levels of testosterone (McBride et al., 2016). Furthermore, treatment of patients with inhibitors of 5α-reductase, the enzyme that converts testosterone into androgen dihydrotestosterone (DHT), showed mixed results with reports of both high Gleason Grade score and reduced risk of the disease (Theoret et al., 2011).

Aromatase (ARO) is another hormone that expressed by adipose tissue mass (Cleland et al., 1983). ARO converts testosterone into estrogens in adipocytes and PCa cells (Bosland, 2005). In obese man, increased adiposity results in high ARO activity that associated with increased levels of estrogens (Eugenia and Rudolf, 2004, Graeme, 2012). A preclinical study suggests that estrogen play a role in promoting PCa development and progression (Bonkhoff and Berges, 2009).

Obesity is a state of chronic inflammation, and various pro-inflammatory cytokines influence the ability of adipocyte to proliferate, mature and influence PCa cells (Coppack, 2001, Xu et al., 2003, Stark et al., 2015). For example, leptin level is elevated in obese individuals and has been found to stimulate the proliferation of androgen-insensitive PCa cell lines including human PC3 and DU145 (Onuma et al., 2003). In contrast, reduced level of adiponectin in serum was associated with enhanced metastatic potential and reduced survival rate in PCa patients (Goktas et al., 2005, Li et al., 2010). Other studies have reported higher serum

adiponectin concentration was detected in patients with T3 stage PCa versus those with T2 stage cancer (Housa et al., 2008), thereby confirming a complex mechanistic link between obesity and PCa.

A number of preclinical studies have investigated adipocyte – PCa cell interaction. For example, factors in sera-conditioned medium from mice fed high fat diet (HFD, 40% fat) promoted the *in vitro* proliferation, migration and invasion of human DU145 PCa cells when compared cultures exposed to sera-conditioned medium collected from mice fed control diet (16% fat) (Hu et al., 2018). In contrast, the *in vitro* growth of PCa cells is inhibited by the overexpression of adiponectin (Gao et al., 2015), suggesting that adiponectin can be of benefit in the treatment of PCa. Additionally, the endogenous levels of adiponectin can also be enhanced by administration of the peroxisome proliferator-activated receptor γ (PPARγ) agonists such as thiazolidinediones or renin-angiotensin system blockers (Kusminski and Scherer, 2009, Ouchi et al., 2003) as well as by lifestyle changes in diet (Fragopoulou et al., 2010, Silva et al., 2011).

Some animal studies have demonstrated that high-fat diet induced obesity may also cause metabolic and inflammatory disorders that indirectly exacerbate cancer progression (Buettner et al., 2007, Kerstin et al., 2012). Preclinical studies showed that the expression level of key pro-inflammatory cytokines and their receptors, such as tumour necrosis factor alpha (TNFα), IL-1β, IL-6, leptin, visfatin and resistin, are significantly increased in adipose tissue of obese mice (Hotamisligil et al., 1995, Hsing et al., 2007b, Jung and Choi, 2014). Compared to lean mice, obese mice or mice fed high-fat diet intake exhibited high levels of cytokine expression that was attributed to an expansion in macrophage populations in adipose tissue (Weisberg et al., 2003). HFD and obesity are associated with elevated serum levels of inflammatory cytokines such as IL-6 (Hayashi et al., 2018) that is known to influence the progression of PCa directly or indirectly (Tewari et al., 2013, Baillargeon, 2006) by increasing the expression and secretion of NFκB-activating cytokines such as TNFα (Michalaki et al., 2004).

1.3. The NFκB signal transduction pathway

The canonical and non-canonical nuclear factor kappa B (NFκB) signal transduction pathway is involved in the regulation of a variety of biological and physiological processes including normal homeostasis, inflammation, cell proliferation and the immune response (PN, 2005; Shanmugam et.al. 2013). Phosphorylation of the cytoplasmic signalling proteins from the IKK family, namely IKKα,β,γ,ε and TBK1 represents a key of step in triggering NFκB activation (Figure 6). NFκB family of transcription factors is composed of five related proteins: NFκB1 (p105/p50), NFκB2 (p100/p52), p65/RelA, RelB and c-Rel that share a common homology domain that is essential in DNA binding and interaction with the cytoplasmic inhibitors IκBs (Figure 7 and 8). The canonical NFκB signalling pathway consists of p105 and p50, whereas the non-canonical pathway involves p100 and p52 (Figure 7 and 8) (reviewed in Oeckinghaus and Ghosh, 2009). The protein phosphorylation and subsequent proteasomal degradation play an important role in the processes for the activation of both canonical and non-canonical NFκB signalling pathways (Chen, 2005).

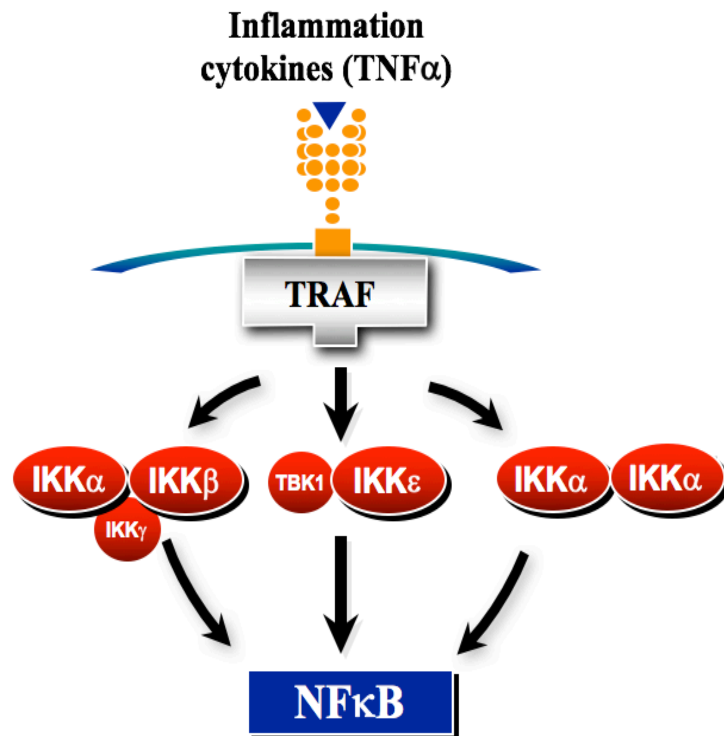


Figure. 6. NFκB signalling pathway. The left route belongs to the canonical pathway, and the middle and right route are the non-canonical pathway. Refer to text for details.

1.3.1. Canonical NFκB signalling pathway

In the absence of stimuli, the canonical NFκB dimers, p105/p50 and p65/RelA, are sequestered in the cytoplasm by the inhibitory IκB proteins. Activation of variety of receptors for inflammatory cytokines and chemokines such as TNFα, IL-1β, and RANKL leads to the sequential recruitment of adaptor proteins from the TNF-receptor-associated death domain protein (TRADD), receptor-interacting protein (RIP) and TNF-receptor-associated factor (TRAF) families (Hsu et al., 1995; Andrea et al, 2011). For example, activation of the canonical NFκB by RANKL initiates the biding of TRAF6, TAB1/2 and TAK1 to the receptor that leads to the phosphorylation of members of the IKK family of proteins, namely IKKα, IKKβ and NEMO (IKKγ, a scaffold protein NFκB essential modulator) (Darnay et al., 2007, Shi and Sun, 2018).

All evidence to date has demonstrated that phosphorylation of IκB is an essential process for canonical NFκB activation. Phosphorylation of IKKα and IKKβ and their subsequent binding to NEMO leads to the phosphorylation of IκBα at two serine sites, namely Ser32 and Ser36. This in turn results in IκB phosphorylation and subsequent proteasomal degradation that releases the p65 NFκB to translocate to the nucleus where it activates NFκB related genes (Figure 7) (Viatour et al., 2005, Jost and Ruland, 2007, Oeckinghaus and Ghosh, 2009). IκB phosphorylation and p65 nuclear translocation can also be triggered by activation of TBK1/IKKε signalling pathway (Balka et al., 2020). This additional pathway can be triggered by IL-1β in the cytoplasm, and casein kinase 2 (CK2) activity is also associated with p65 upon TNFα stimulation (Bird et al., 1997). Moreover, this phosphorylation requires IκBα degradation and is prevented by p65 binding to IκBα in unstimulated cells (Wang et al., 2000).

Activation of NFκB by pro-inflammatory and pro-apoptotic cytokines such as TNFα enhances cell survival and prevents cell death by directly triggering anti-apoptotic and tumour suppressor genes. Suppression of Phosphatase and tensin homolog (PTEN), a tumour

suppressor gene, by NFκB enhances cell survival signalling through the activation of cell multiple mechanisms (Stambolic et al., 1998). For example, activation of the phosphoinositide 3-kinase (PI3k) / Akt pathway is common in cancer cells and PTEN loss of function in human cancer cells is accompanied by activation of p65 and Akt, which consequently confers neoplastic cell survival and resistance to chemotherapy-induced apoptosis (Wang et al., 1998).

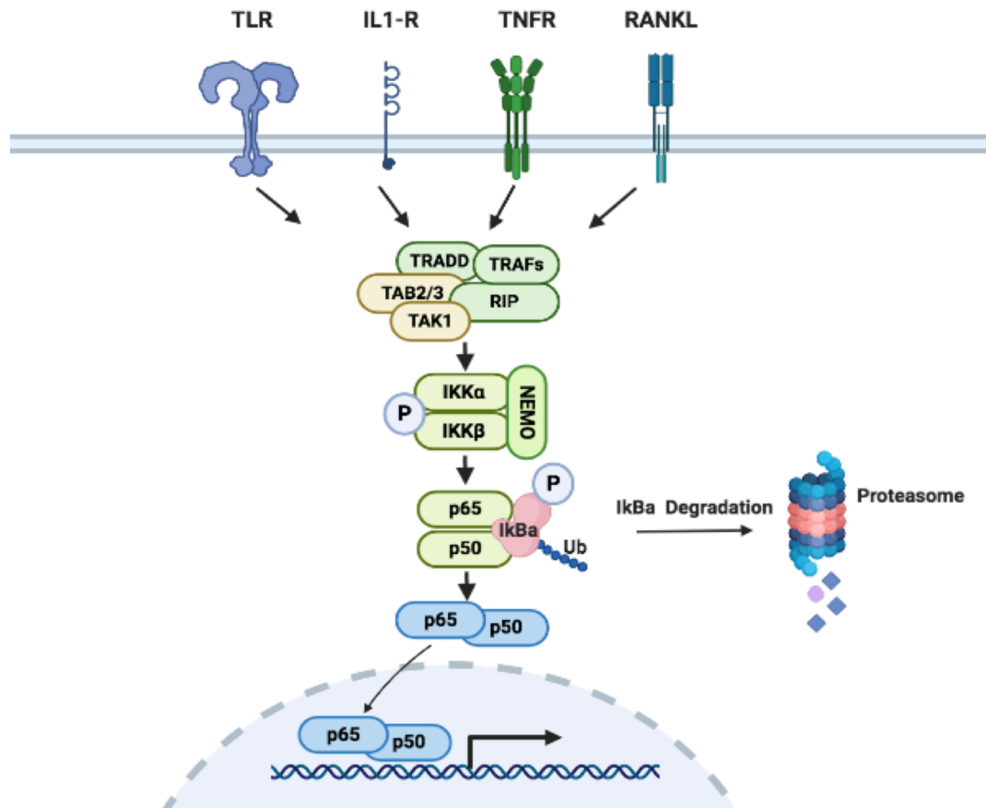


Figure. 7. The canonical NFκB pathway. Ligand binding to a receptor leads to the recruitment and activation of an IKK complex comprising IKKα and/or IKKβ catalytic subunits and two molecules of NEMO. The IKK complex then phosphorylates IκB leading to degradation by the proteasome. NFκB then translocate to the nucleus to activate target genes. Adapted from Jost and Ruland, 2007.

1.3.2. Non-canonical NFκB signalling pathway

The activation of non-canonical NFκB signalling is triggered in a NEMO-independent mechanism (Xiao et al., 2004). This alternative pathway is predominately activated by the TNF family cytokines CD40L, BAFF and lymphotoxin-β. Unlike the canonical pathway, this pathway relies on the NFκB-inducing kinase (NIK) and it involves the dimerization of two IKKα subunits (Sun, 2011). The recruitment of TRAF2 and TRAF3, but not TRAF6, plays an essential role in activation of non-canonical NFκB signalling (Liao et al., 2004). Once phosphorylated by IKKα on specific serine residues located in both the N- and C-terminal regions, partial processing of p100 generates p52/RelB that is free to translocases to nucleus (Figure 8) (Jost and Ruland, 2007, Dobrzanski et al., 1993). Phosphorylation of Ser368 of RelB is essential for its dimerization and p100 stabilization (Maier et al., 2003).

The IKKε/TBK1 axis is also implicated in the activation of non-canonical NFκB signalling. IKKε and TBK1 phosphorylate c-Rel leading to its nuclear accumulation. However, this phosphorylation events are insufficient to trigger subsequent stimulation of target genes of survival and proliferation (Harris et al., 2006).

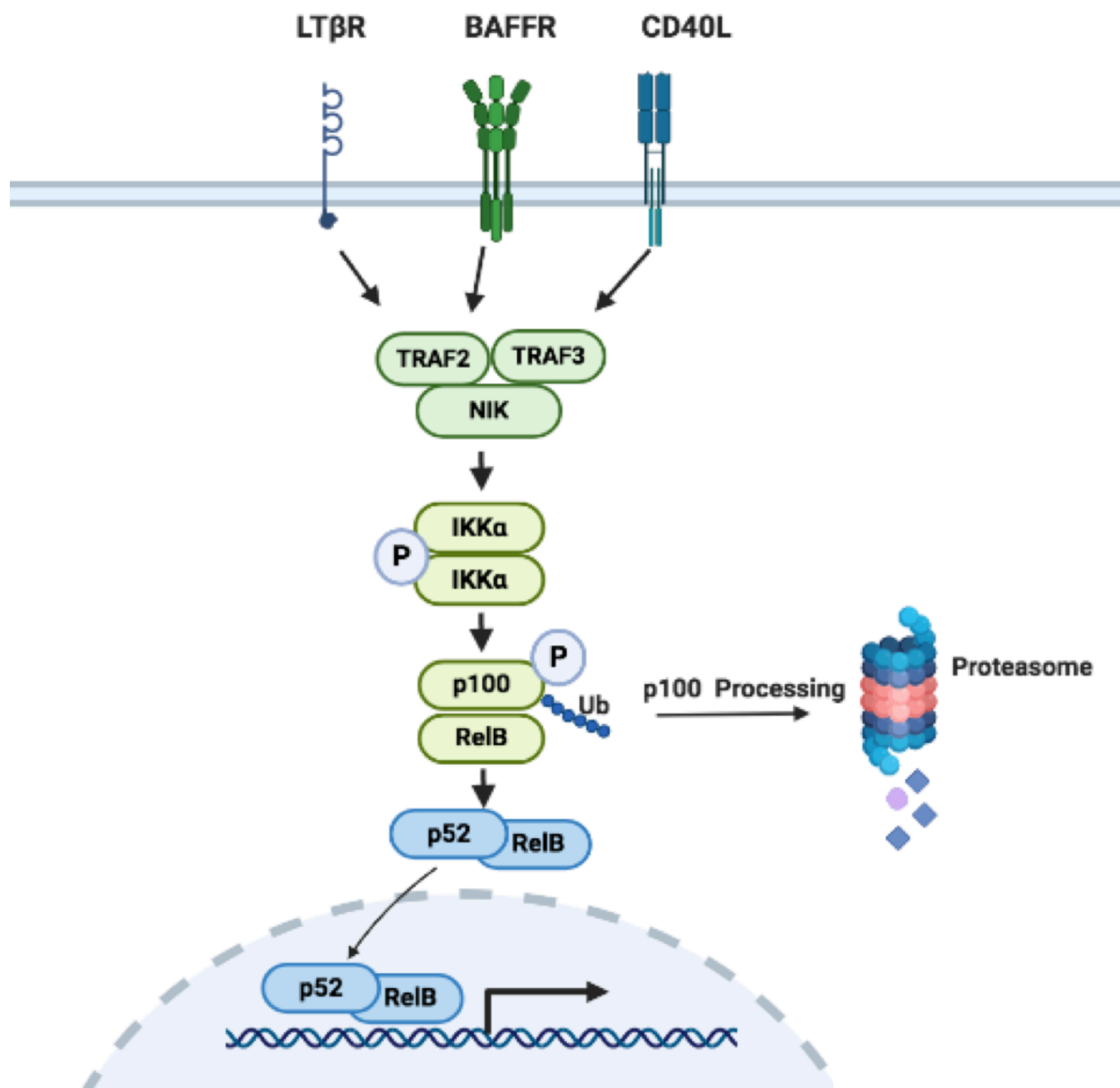


Figure. 8. The non-canonical NFκB pathway. IKKα homodimer is activated by NIK and regulate the phosphorylation and processing of the p100. Then RelB/p52 translocate to nucleus to activate target genes. Adapted from Jost and Ruland, 2007.

1.4. The role of NFκB pathway in obesity

1.4.1. The role of NFκB in high fat diet induced obesity

Obesity induces a low-grade, chronic inflammation status in response to excess nutrients and energy. A number of studies have shown that NFκB activation is regulated by high calorie- and excess nutrition-induced obesity (Tang et al., 2010; Catrysse and van Loo, 2017). NFκB activation also plays a role in regulating energy balance and provides a mechanistic link between diet and inflammation. Carlsen et al. (2009) were the first to compare NFκB expression and activity in obese and lean individuals. The data showed that NFκB activity is elevated in abdominal adipose tissue depots of obese mice when compared to controls (Carlsen et al., 2009).

The expression of the NFκB subunits p65, p68, and IκB is increased during adipocyte differentiation (Berg et al., 2004). Consistently, activation of canonical NFκB signalling by expressing a constitutively active form of IKKβ in mice resulted in insulin resistance (Arkan et al., 2005). Furthermore, activation IKKβ in the liver elevated systemic insulin resistance and glucose intolerance (Dongsheng et al., 2005). Conversely, IKKβ knockout mice exhibited high insulin sensitivity and reduced HFD-induced glucose intolerance (Arkan et al., 2005). Additionally, pharmacologic inhibition of IKKβ with salicylates reduced insulin resistance in HFD-treated mice and OB/OB obese mice (Yuan et al., 2001).

The expression of IKKε is also increased in mature adipocytes, and it has been associated with high caloric diet induced obesity, and to result in chronic low-grade inflammation and to increase levels of pro-inflammatory cytokines and angiogenic factors including TNFα, IL-6, macrophage migration inhibitory factor (MIF), and matrix metallopeptidase 9 (MMP-9) (Ghanim et al., 2004; Shoelson et al., 2007; Chiang et al., 2009). Levels of TNFα has been shown to increase in adipose tissue and plasma from obese animal (Hotamisligil et al., 1993). Consistent with *in vitro* and *in vivo* data, clinical studies have shown that NFκB activation is

significantly increased in peripheral blood mononuclear cells isolated from patients with high-fat diet when compared with lean individuals (Ghanim et al., 2004).

1.4.2. The role of NFκB in obesity-induced inflammation

Inflammation plays an important role in adipocyte differentiation. Several studies have implicated the pro-inflammatory NFκB pathway in obesity related inflammation (Kumar et al., 2004, Baker et al., 2011). Macrophages and other immune cells regulate adipocyte differentiation in health and disease (Sorisky et al., 2013, Corrêa et al., 2017), the NFκB and Toll-like receptors (TLRs) pathways are play a role in the regulation of adipocyte - macrophage crosstalk (Sica and Bronte, 2007, McKernan et al., 2020).

NFκB–dependent differentiation of monocytes into M1 or M2 plays a role in the development of inflammatory metabolic disease (Baker et al., 2011). M2 are involved in wound healing (Krzyszczyk et al., 2018), whereas M1 initiate the innate immune response to pathogens by producing NFκB-activating cytokines such as IL-1, IL-6 and TNFα (Ting et al., 2017). The pro-inflammatory M1 macrophages are highly expressed in in obese mice compared to lean mice, and adipose tissue macrophage (ATM) of the M1 type increases with the severity of obesity (Weisberg et al., 2003, Lumeng et al., 2007). Furthermore, energy accumulation leads to elevation in glucose and fatty acids. Those substrates are response for stress responses which activate pro-inflammatory signalling pathway NFκB. NFκB activation promotes the production of metabolic hormones such as leptin and adiponectin by adipocytes and ATM (Figure 9) (Ye et al., 2010). Collectively, these findings suggest that the NFκB pathway is positioned at a crossroad between energy expenditure and inflammation.

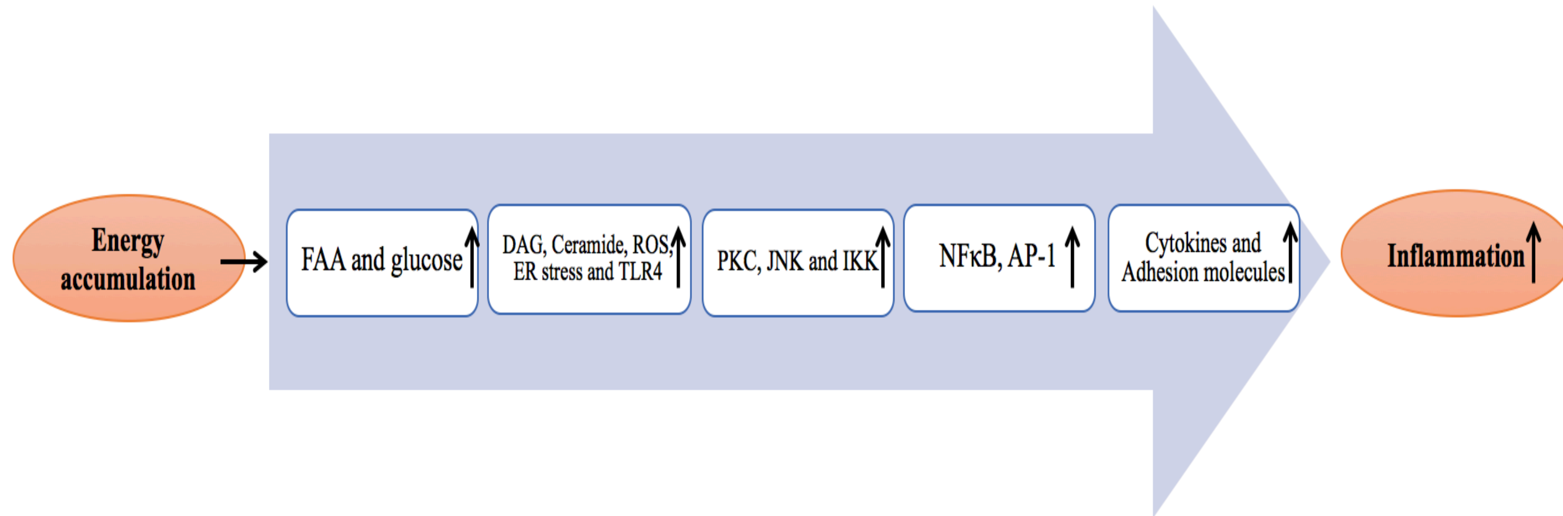


Figure. 9. Energy accumulation induces inflammation. As shown in this figure, an increase of cytokines and adhesion molecules shows in chronic local inflammation. Refer to text for details. Adapted from Ye et al., 2010.

1.5. The role of NFκB pathway in prostate cancer

A number of studies have shown that the NFκB pathway plays an essential role in the regulation of PCa cell proliferation, motility, invasion and the ability of both primary and metastatic PCa cells to influence host cell such as immune and bone cells (Jin et al., 2013, Michael et al, 2002; Inoue et.al. 2007; Shanmugam et.al. 2013), and it is implicated in PCa cells sensitivity to chemotherapy (Lumeng et al., 2007, Weisberg et al., 2003a). Studies in PCa mouse models have shown that activation of the canonical NFκB pathway by over-expressing IκBα promoted the development of early PCa, and the deficiency of PC3-specific IκBα inhibited PCa tumour growth and metastasis (Huang et al. 2001; Jin et al., 2014). Increased nuclear localization of the canonical NFκB/p65 in PCa lymph node metastasis is used as prognostic test for in patients after radical prostatectomy (Lessard et al., 2003, Ismail et al., 2004). These findings implicate the canonical NFκB signalling in both early and late metastatic human PCa.

1.5.1. The role of NFκB in prostate cancer related inflammation

Inflammation promotes the initiation and progression of PCa (Taverna et al., 2015). Numerous studies have shown that inflammation and cancer are molecularly linked by NFκB and the level of pro-inflammatory NFκB-activating cytokines such as IL-1, IL-6 or TNFα are upregulated in PCa (reviewed in Staal and Beyaert, 2018). In addition, the transcription regulatory element of PSA is enhanced by both exposure to the NFκB-activating TNFα and over-expression of NFκB (Chen & Sawyers, 2002).

Macrophages is one of the most abundant types of immune cells in the tumour microenvironment. Both tumour associated macrophages (TAMs) M1 and M2 phenotype are known to promote PCa tumour growth and metastasis (Lanciotti et al., 2014). NFκB activation is stimulated by cytokines released by macrophages in the tumour microenvironment (Hallam et al., 2009). M1 macrophage secrete pro-inflammatory factors such as TNFα, IL-1β and IL-6

and IL-12 in the tumour microenvironment, whereas M2 macrophage secrete other factors that include IL-4, IL-10 and TGFβ (Atri et al., 2018). Additionally, NFκB activation favors the polarization and lineage commitment of macrophages towards the activated form of TAMs-M2 phenotype, which is known to tolerate and even foster tumour growth (Hagemann et al., 2008).

Secretion of the pro-inflammatory cytokine TNFα, IL-6, IL-1β (Gao et al., 2014, Stijn et al., 2015, Braune et al., 2017) and chemokine CCL2 (Rajasekaran et al., 2019) by adipocytes is implicated in macrophage polarization and recruitment to adipose tissue. HFD-induced obesity reduces the ratio of M2 to M1 in mice (Lumeng et al., 2007). Although the role of macrophage in the PCa with obesity is still require further study, research study has shown that macrophages are recruited to PCa tumour via CCL2 (Jamie et al., 2012) and CCL2 levels increase in sera of obese mice inoculated with human LNCaP PCa cells (Huang et al., 2012).

1.5.2. The role of NFκB in regulation of androgen receptor expression in prostate cancer

AR expression is a key determinant of PCa initiation and progression (Brian and David, 2001, Montgomery et al., 2001), and it plays a vital role in the development of the resistant state of hormone insensitivity in PCa patients (Charlie et al., 2003). NFκB activation is implicated in the regulation of AR activity, protein and mRNA in PCa cells. AR expression can be altered NFκB activation and expression (Zhang et al., 2009). For example, increased NFκB signaling in the human hormone-dependent PCa cell line LNCaP is associated with enhanced AR signaling that was abolished by anti-androgen treatment (Altuwaijri et al., 2003). In addition, NFκB expression is inversely correlated with expression estrogen receptor β (ERβ), and ERβ-knock down in PCa cells enhanced the expression of the key canonical NFκB signaling pathway protein IKKβ (Paul et al., 2015). Consistently, NFκB inhibitors decreased AR expression and activation, PSA secretion, and reduced tumour growth in androgen deprivation resistant PCa xenograft models (Zhang et al, 2009). Research studies illustrated that AR activation was inhibited by blocking NFκB signaling *in vitro*, and NFκB activation enhanced

the progression of castration resistance PCa (CRPC) by maintaining high levels of nuclear AR *in vivo* (Jin et al., 2008a, Zhiyong et al., 2009, Chandrasekar et al., 2015). NFκB activation increased the expression of androgen receptor splicing variants (AR-Vs) in PCa cells with the relative abundance of AR-V7 was found in both PCa cell lines and in human prostate tissues (Jin and Yun, 2016). Conversely, inhibition of NFκB signaling reduced ARVs expression and delayed development of CRPC (Jin et al., 2015). Together, these findings suggested that enhanced activation and expression of canonical NFκB signalling is implicated in the regulation of AR expression and activity in PCa, and thus inhibition of key components of this pathway represents a novel therapeutic strategy for the treatment of both androgen dependent and androgen-insensitive PCa (Zhang et al., 2009).

1.5.3. The role of NFκB in obesity related prostate cancer

Obesity and HFD contribute to PCa progression. Preclinical studies have demonstrated that prostate tumour growth and levels of insulin and IGF-1 in serum are reduced in mice fed with low-fat diet (LFD) compared to mice fed HFD (L-López et al., 2017). Clinical trials show that PCa patients have a favourable prognosis with LFD (Van Patten et al., 2008).

An *in vitro* proof-of-concept experiment part of a randomized clinical trial has shown that pre-exposure LNCaP to serum from PCa patients on fat restriction diet decreased the PCa cells growth (Aronson et al., 2010). Furthermore, increased apoptosis was observed in androgen-independent LNCaP cells treated with serum from men in low-fat, high-fibre diet (Tymchuk et al., 2001). Conversely, NFκB was found to be upregulated in mice bearing PCa cells and fed moderate fat / carbohydrate diet, and high level of NFκB activation in these mice was attributed to low survival rate (Mavropoulos et al., 2009). Oxidative stress has been implicated in prostatic disease (Udensi and Tchounwou, 2016), and NFκB plays an essential role in stress response (Tilstra et al., 2011). Over-expression of NFκB enhances NADPH oxidase activity (Maloney et al., 2009), and HFD intake elevated levels of the canonical IκB/p65 NFκB axis

and NADPH oxidase in the prostate of transgenic NFκB-Luc-Tag mice (Vykhovanets et al., 2011), thereby implicating NFκB activity in HFD related oxidative stress.

The NFκB pathway plays an important role in the immune response through its regulation of expression and secretion of pro-inflammatory cytokines, such as TNFα, IL-1, IL-6 and IL-8 (Ting et al., 2017). In the PCa immunocompetent mice model, HFD enhanced tumour growth and a combination therapy of the anti-inflammatory NFκB inhibitor celecoxib and anti-IL-6 receptor antibody inhibited tumour growth in obese mice (Hayashi et al., 2018). Collectively, findings to date suggest that inhibition of inflammation-induced NFκB activation in obese mice could be of value in the treatment of PCa.

1.6. NFκB as potential target for treatment of prostate cancer

In patients with early PCa, radical prostatectomy and radiation therapy are currently and routinely used in the clinic (D'Amico et al., 1998). Androgen deprivation is the standard treatment of care for patients with advanced PCa after primary therapy (Carroll et al., 2015). Nevertheless, most patients eventually progress to castration resistance PCa within 2-3 years (Harris et al., 2009), and eventually die of hormone refractory PCa (Tomioka et al., 2008). Despite significant improvement in the efficacy of chemotherapeutic agents, survival rate among patients with advanced PCa remains poor.

NFκB is implicated in all aspects of PCa in particular metastasis and chemo-resistance (Yamamoto and Gaynor, 2001). Thus, agents that directly or indirectly inhibit NFκB activation – alone or in combination with chemotherapy - may of value in the treatment of castration- and chemo-resistant PCa in obese patients (Figure 10) (Verzella D, 2016).

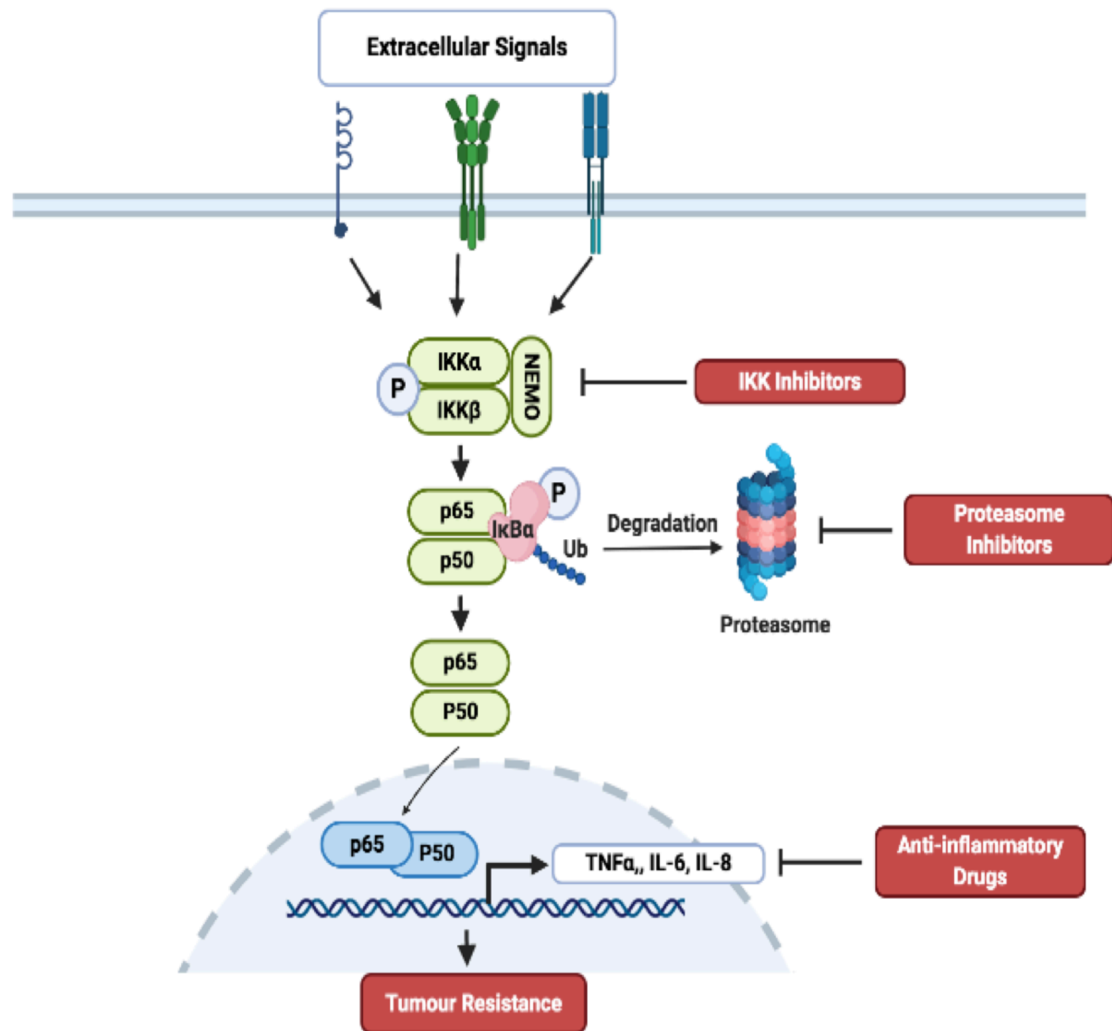


Figure. 10. The potential target of NFκB signalling pathway for the treatment of advanced prostate cancer. Refer to text for details. Adapted from Verzella D, 2016.

1.6.1. IKK inhibitors in the treatment of prostate cancer

In pro-inflammatory NFκB signalling pathway, IKKs are well recognized as key regulatiors and act as point for many extracellular agents that active this pathway (Liu et al., 2012). For instance, IKKα has been implicated as a key regulator of oncogenesis and driver of the metastatic process in PCa (Shukla et al., 2015). Therefore, selective inhibitors of IKKα are expected to be of therapeutic value in the treatment of advanced PCa. The naturally occurring plant-derived anti-cancer agent, apigenin has been shown to exert anti-tumour effects by binding directly and inhibiting the kinase activity of IKKα, leading to significant suppression of NFκB/p65 activation in the human castration-insensitive PC3 PCa cells (Shukla et al., 2015).

In addition, IKKβ is a key regulator of canonical NFκB signaling. Numereous studies have identified and tested small-molecules inhibitors IKKs that exhibit higher selectivity for IKKβ over IKKα (Prescott and Cook, 2018). IKKβ inhibitors have denmonstrated efficacy in various pre-clinical models of cancer (Prescott and Cook, 2018). For instance, the selective IKKβ inhibitor BMS-345541 inhibited invasion and induced apoptosis in human PC3 cells by a mechanism depdedent on inhibition of IκBα phosphorylation and nuclear translocation of p65 (Ping et al., 2016).

Notwithstanding these findings, inhibitors of IKK have not been successfully developed for clinical use. One potential reason for this lack of clinical success is that IKK inhibition is associated with side effects that include off-target toxicities and immunosuppression (Garber, 2006, Prescott and Cook, 2018).

1.6.2. Proteasome inhibitor in the treatment of prostate cancer

The ubiquitin-proteasome pathway plays a key role in cellular homeostasis, and is essential to multiple metabolic processes, including osteoblast differentiation and bone formation (Garrett et al., 2003). The ubiquitin-proteasome cascade is responsible for the degradation of unneeded or damaged intracellular proteins by proteolysis, a chemical reaction that breaks peptide bonds (Adams, 2004a). Proteasomal activity is also vital for programmed cell death, cell growth and division (Hannes, 1997, Reed, 2006), and thus it leads to degradation of various proteins.

NFκB activation is induced and maintained by the ubiquitin-proteasome system, which facilitates the degradation of its inhibitory protein, IκB (Collins et al., 2016). Proteasome inhibitors block NFκB-mediated transcription in tumour cells and enhance their susceptibility to chemotherapy and radiation therapy (Manasanch and Orlowski, 2017). Inhibition of NFκB-dependent transcription was the underlying rationale behind the development of the first clinically-approved proteasome inhibitor, Bortezomib (BTZ). It was approved by the Food and Drug Administration for the treatment of relapsed or refractory multiple myeloma (Robert et al., 2006, Mateos et al., 2006). BTZ is a small molecule that reduce tumour cell proliferation and induce apoptosis by inhibiting the 20s protelytic sites. The 20s subunit β5 is the target for BTZ and is associated with chymotrypsin-like activity (Figure 11) (Adams, 2004, Tanaka, 2009, Kubiczkova et al., 2014). The NFκB activation trigger a cascade of signal transduction events that phosphorylate and ubiquitinate IκB, thereby to the degradation of intracellular proteins, including p53 and NFκB inhibitor IκB (Figure 12) (Chen et al., 1996; Paramore and Frantz, 2003, Adams, 2004; Landis-Piwowar et al., 2006; Tanaka, 2009).

In PCa, treatment of mice bearing human LNCaP-Pro5 tumours with BTZ resulted in up to 70% reduction in tumour growth (Williams et al., 2003). Intersetingly, administration of BTZ had no effect on the formation of osteolytic lesions but it reduced tumour growth in mice bearing PCa

bone lesions (Whang et al., 2005). *In vitro*, BTZ induced marked apoptosis and increased expression level of Caspase-3 in the human DU145 PCa cells (Zheng et al., 2015).

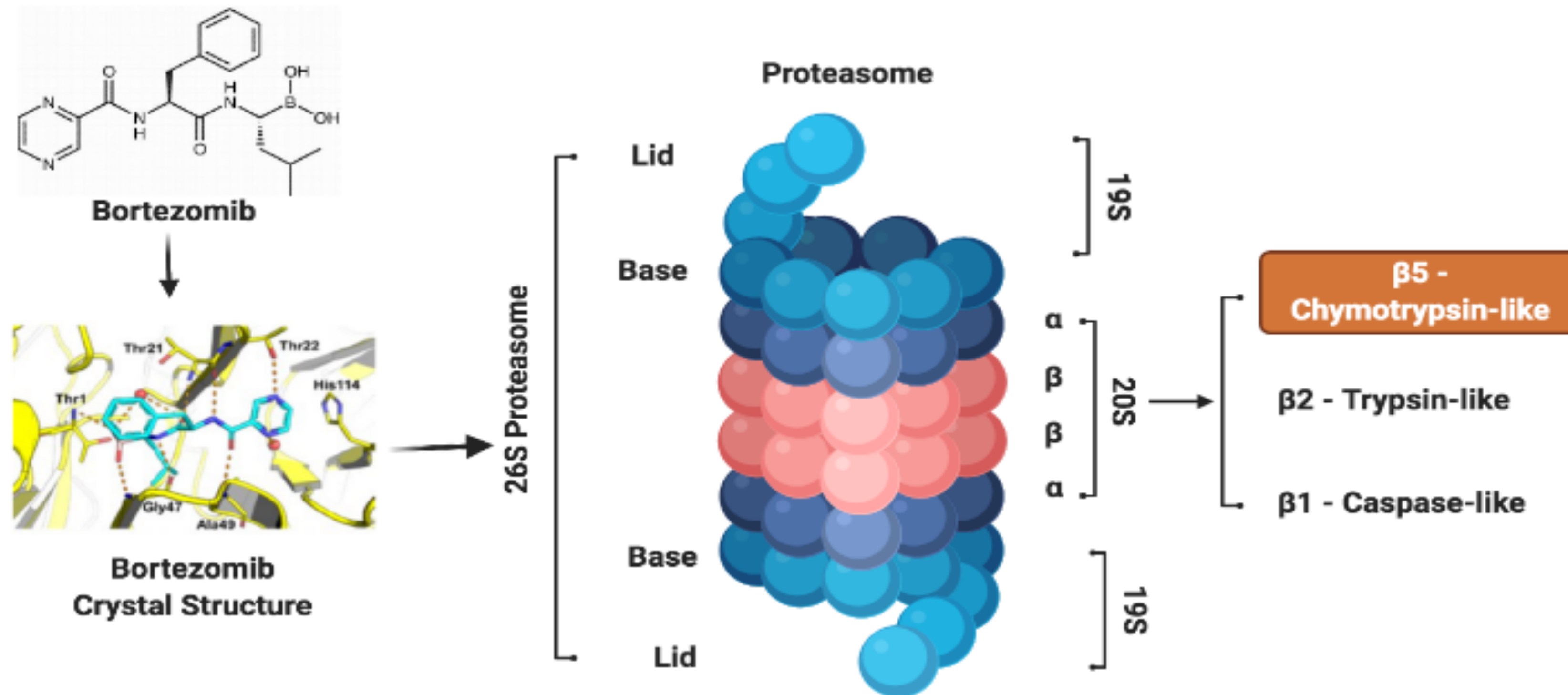


Figure. 11. Schematic diagram of 26s proteasome. Positions of subunits of proteasome indicated in this figure. The 19S RP is shown in green, the 20S CP comprised of alpha- and beta-type subunits (blue and pink, respectively) is also presented. Refer to text for details. Adapted from Adams, 2004 and Tanaka, 2009.

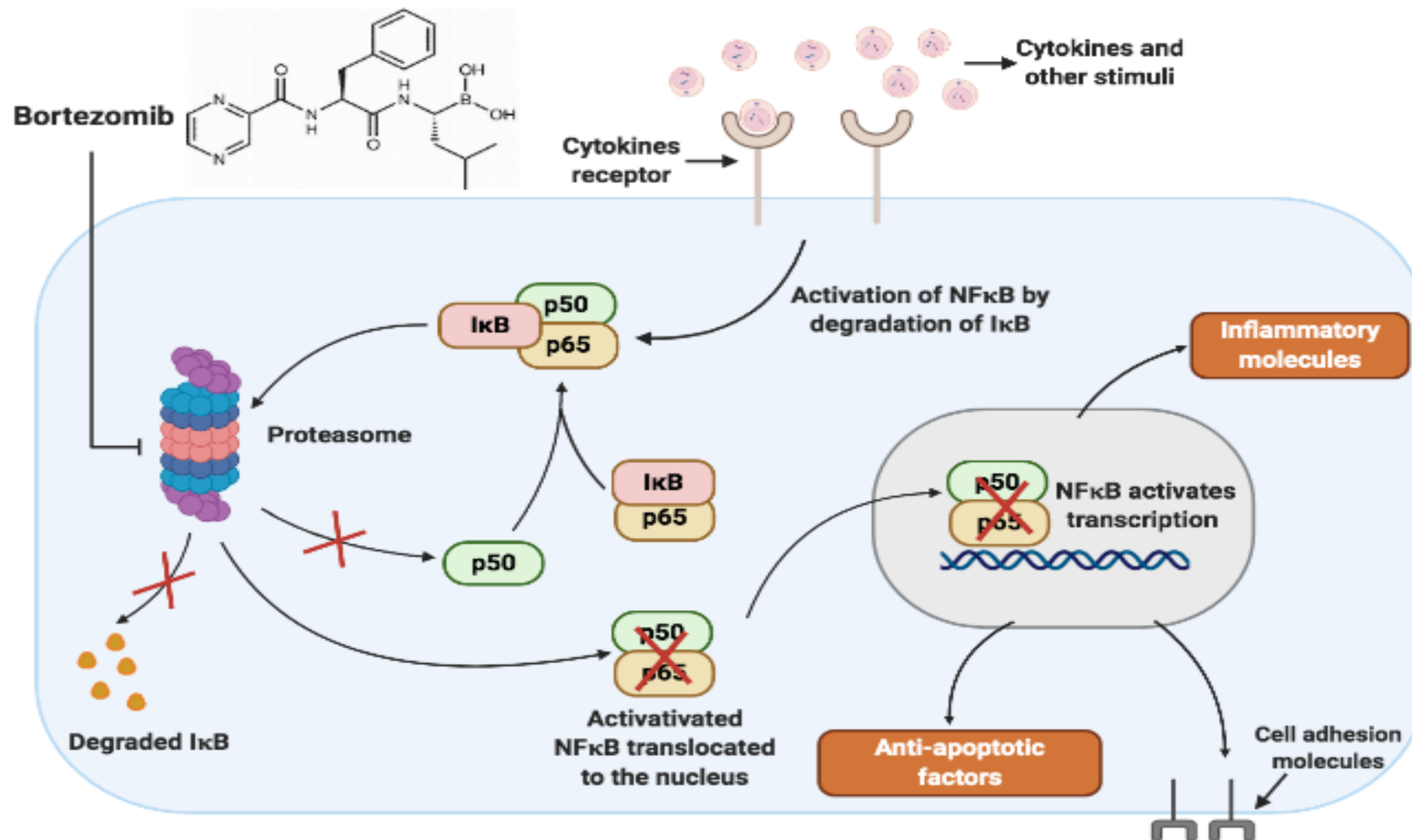


Figure. 12. **The relationship of proteasome, NFκB and BTZ.** By inhibiting the proteasome, bortezomib inhibits the activation of NFκB and consequent events that can supress tumour cell survival. Refer to text for details. Adapted from Paramore and Frantz, 2003.

In a clinical trial, BTZ stablized PSA but induce neurotoxicity in PCa patients with early PSA recurrence (Kraft et al., 2011). Furthermore, BTZ increased IL-6 and IL-8 expression in PC3 cells (Manna et al., 2013a), these effects were attenuated by inhibition of IKKα enzymatic activity (Manna et al., 2013b). Taken together, these data suggest that proteosomal inhibition by agents such as BTZ – alone or in combination with other NFκB inhibitors – may be of value in the treatment of metastatic PCa.

1.6.3. IκB inhibitors in the treatment of prostate cancer

Parthenolide, a sesquiterpene lactone, is the most abundant active phytochemicals that derived from the plant feverfew (Pareek et al., 2011). It has been used in folk chinese medicine as anti-inflammatory and anti-hyperalgesic agents (Hall et al., 1979), and in clinical use for the treatment of rheumatoid arthritis and migraines (Johnson et al., 1985, Jain and Kulkarni, 1999). Parthenolide inhibits canonnical NFκB signaling at the level of IκB and / or by direct interaction with of p65 NFκB (Bork et al., 1997, Hehner et al., 1998). In addition, parthenolide inhibits signal transducers and activators of transcription 3 (STAT3) after activation by IL-6 (Sobota et al., 2000), and c-jun N-terminal kinase (JNK) after activation by TNFα (Siyuan et al., 2004).

In the androgen-insensitive PCa cell line, parthenolide restored sensitivity to docetaxel chemotherapy and augmented efficacy of docetaxel chemotherapy *in vivo* (Shanmugam et al., 2006). Parthenolide is also cytotoxic to prostate tumour initiating cells (cancer stem cells) (Kawasaki et al., 2009). In additon, pathenolide enhanced radiosensitivity of the PCa cell lines DU145 and PC3 via inhibiting NFκB pathway. However, it also acivates the PI3K/AKT prosurvival pathway in androgen-insensitive PCa cell lines. The activated Akt kept a low level in DU145 cells when compared to PC3 cells due to the PTEN function. Thus, the presence of PTEN enhances the radiosenstitization effect of parthenolid by suppressiong the partialy activated p-Akt (Sun et al., 2007). In the treatment of TRAMP and C57BL/6J mice, parthenolide selectively exhibits radiosenstivity in prostate tumour tissues, while protecting

normal prostate tissues from radiation-induced damage (Morel et al., 2017). In addtion to monotherapy with parthenolide, the combined treatment of parthenolide and paclitaxel significantly increase chemosensitivity to the paclitaxel-induced apoptosis of cancer cells (Nikhil et al., 2000, Sohma et al., 2011). Taken together, experimental data showed that parthenolide protect normal prostate cells and tissues from radio- or chemo-agents induced apoptosis, whereas in PCa cells, it inhibit the proliferation and enhance radio- and chemo-sensitivity. Therefore, due to anti-tumour potency and anti-inflammatory nature of parthenolide, it could be a promised therapeutic agent for obese patients with PCa.

1.7. Aims

The canonical and non-canonical NFκB signalling pathway plays an important role in PCa, adipocyte differentiation and inflammation. In this project, I hypothesised that the pro-inflammatory NFκB signalling pathway is implicated in the regulation of macrophage - adipocyte - PCa cell interactions, and NFκB inhibition reduces the ability of adipocyte to influence the metastatic spread of PCa cells in the presence of tumour-associated macrophages.

This hypothesis will be tested by:

1. carrying out meta- and bioinformatic analysis of the scientific literature to assess evidence for correlation between expression and activity of key components of the canonical and non-canonical NFκB signalling pathway, obesity and PCa progression and metastasis.
2. conducting western blot analysis to confirm the expression of key components of the canonical NFκB pathway in a panel of human and murine PCa cells with different metastatic abilities. The list includes the hormone-dependent human LNCaP, C4-2 and C4-2B and castration-insensitive human PC3 and DU145 and mouse RM1 cells. This analysis also included undifferentiated and mature mouse adipocytes.
3. Establishing if derived factors from undifferentiated and mature mouse 3T3-L1 adipocytes exposed to M2 macrophage conditioned medium affect the *in vitro* growth, migration and invasion of a panel of the hormone-dependent and castration-insensitive PCa cells described.
4. testing the effects of knockdown and selective pharmacological inhibition of the canonical NFκB signalling pathway affects the *in vitro* growth, migration and invasion of the hormone-dependent and castration-insensitive cells described in the presence of macrophage- and/or adipocyte-derived factor.

5. identification and characterisation of pro-inflammatory NFκB-activating mediators in conditioned medium from mature adipocytes and tumour associated macrophage.

CHAPTER 2

MATERIALS & METHODS

2.1. Preparation of Test Compound

The proteasome inhibitor, Bortezomib was purchased from Millennium Pharmaceuticals (Cambridge, MA, USA). For *in vitro* and *in vivo* experiments, the compound was prepared according to manufacturer's instructions. Bortezomib was prepared at 50mM in dimethyl sulfoxide (DMSO). Serial dilutions of the tested compounds were pre-prepared and kept at -20°C. Concentrations in motility studies used were determined by cell viability experiments, such that the concentration(s) that had no effect on viability at a given time point was used.

2.2. Tissue Culture

2.2.1. Cell culture condition

Cell culture was carried out in a Class II laminar flow cabinet under sterile conditions. All solutions were warmed at 37°C before use. Plasticware supplied by various manufactures predominately Corning (UK) and Thermofisher (UK) was sprayed with 70% (v/v) industrial methylated spirit prior to use. All cultures were incubated at 37°C supplied with 5% CO_2 and 95% humidity. Phase-contrast microscopy was used routinely during the culture period in order to assess cell confluence and/or contamination of cultures.

2.2.2. Tissue culture media

Human PC3 and LNCaP PCa cells, mouse RM1 PCa cells and mouse 3T3L1 pre-adipocytes were cultured in DMEM + GlutaMAX supplemented with 10% fetal bovine serum (FBS) (Life Technologies^{TM}, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (standard DMEM). Monocytes THP-1 cells were cultured in RPMI + GlutaMAX supplemented with 10% FBS, 100 U/ml penicillin and 100 μg /ml streptomycin (standard RPMI).

2.2.3. Cancer cell lines

Human PCa cell lines used include: LNCaP (androgen sensitive adenocarcinoma cell line derived from PCa lymph node metastasis in a male patient; AR+ and PSA+); C4-2B4

(androgen insensitive cell line derived from LNCaP lymph node metastasis after injection in castrated nude mice; AR+ and PSA+); DU145 (androgen-insensitive cell line derived from a central nervous system metastasis of primary prostate adenocarcinoma origin; AR+ and PSA-); PC3 (androgen-insensitive neuroendocrine cell line derived from PCa bone metastasis in male patient; AR- and PSA-). Mouse PCa cell lines used include: RM1 (androgen-insensitive cell line derived from C57BL/6 mice using the mouse prostate reconstitution model). Frozen cells in cryogenic vials (stored at -80°C in 10% DMSO in FBS) were retrieved from storage and defrosted in a 37°C water bath. Prior culturing, cells were washed by adding 4 ml standard DMEM to the cell suspension. Cells were spun at 1000rpm for 5 min, supernatant was removed, and cell pellet was re-suspended in 1 ml standard DMEM. All cancer cells used in this study were cultured in 25 cm^2 flasks and passaged every 48-72 hours at a ratio of 1:3 – 1:10. Briefly, culture medium was removed, the cells washed in PBS (Lonza, USA) and were detached by addition of trypsin (Invitrogen, USA). The detachment process was inactivated by adding twice the volume of standard medium to the cells, transfer the cell suspension to a fresh sterile 15 ml tube and then centrifuge at 1000 rpm for 5 minutes. The supernatant was discarded, and 1 ml standard medium was added to cell suspension. A fraction of the suspension was also transferred to a 25 cm^2 flask containing 10 ml standard medium.

2.2.4. Adipocyte cell line

Mouse 3T3-L1 cells are pre-adipocyte cell line purchased from ATCC (Manassas, VA, USA). To generate mature adipocytes, 3T3-L1 cells were plated at 2×10^4 cells/ml (24 well-plate) or 2×10^5 cells/ml (6 well-plate) in standard DMEM. Media was refreshed for 48 hours until the culture reaches approximately 100% confluency. Adipocyte differentiation was initiated by incubating the confluent 3T3-L1 cells in adipogenic medium containing 0.5mM 3-isobutyl-1-methylxanthine (IBMX), 1μM dexamethasone (DEX) and 1μg/ml insulin. This was considered to be Day 0 of culture. After 48 hours, cells were transferred to maintenance medium containing 1μg/ml insulin in standard DMEM and media was refreshed every 48 hours. Culture

was terminated in day 10 (HubLE Methods: https://www.huble.org/huble-methods/huble-method-001/).

2.2.5. Human monocyte-macrophage cell lines

Human THP-1 cells are monocytic-macrophage cell line that is derived from an acute monocytic leukaemia patient and was purchased from ATCC (Manassas, VA, USA). Non-adherent THP-1 were cultured in 1mM sodium pyruvate contained standard RPMI. THP-1 monocytes are differentiated into adherent M0 macrophage incubation with 5ng/ml phorbol 12-myristate 13-acetate (PMA) for 48 hours followed by 24 hours incubation in 1% growth medium. M0 Macrophages were polarized in M1 macrophages by incubation with 20 ng/ml of IFN-γ (R&D system, #285-IF) and 10 pg/ml of Lipopolysaccharide (LPS) (Sigma, #8630) for 72 hours. Macrophage M2 polarization was obtained by incubation with 20 ng/ml of IL-4 (R&D Systems, #204-IL) and 20 ng/ml of IL-13 (R&D Systems, #213-ILB) for 72 hours.

2.2.6. Preparation of conditioned medium

2.2.6.1. Macrophage conditioned medium

M1 and M2 Macrophages generated as described above were incubated with standard RPMI media, and conditioned medium were collected after 24 hours. All conditioned media was filtered through a 0.2 μm filter and stored at -20°C.

2.2.6.2. Adipocyte conditioned medium

3T3-L1 cells were differentiated into mature adipocytes as we described above. 16 hours prior to collection of conditioned medium, the maintenance medium (1μg/ml insulin) was replaced with DMEM medium containing 1% FBS (1% standard DMEM) for 16hrs, then the conditioned medium (CM) was collected. The conditioned medium collected on day 3, 7, 10 or 12 were labelled as Adipocytes ^{CM}, Mature Adipocytes ^{CM}, and Adipo ^{CM} respectively. CTRL ^{CM} was collected from empty well with no 3T3-L1 cells present.

To obtain conditioned medium from mature adipocytes exposed to pro-inflammatory mediators, mature adipocytes were treated with 1ng/ml TNFα on day 9 for 24 hours, and then this medium was replaced with 1% standard DMEM. After 16 hours, the conditioned medium (Adipo $^{CM/TNF\alpha}$) was collected. Similar procedure was used to obtain conditioned medium form mature adipocytes stimulated by macrophage conditioned medium (100% v/v) from M0, M1 and M2 macrophage (generated as described below). The conditioned medium from these cultures were labelled as follows Adipo $^{CM/M0}$, Adipo $^{CM/M1,}$ Adipo $^{CM/M2}$. All conditioned medium should be filter through a 0.2 μm filter and stored at -20°C. Conditioned medium collection from mouse 3T3-L1 mature adipocytes primed with pro-inflammatory cytokine are summarised in Table 2.

Table 2. The collection of conditioned medium from culture of adipocytes

Conditioned Medium	Collection
CTRL ^{CM}	The culture of 1% standard DMEM with no 3T3-L1 cells present
Adipocyte ^{CM}	The culture of adipocytes was stopped on day 3
Mature Adipocytes ^{CM}	The culture of adipocytes was stopped on day 7 and day 12
Adipo ^{CM}	The culture of adipocytes was stopped on day 10
Adipo $^{CM/TNF\alpha}$	Mature adipocytes were stimulated by 1ng/ml TNFα on day 9, and the culture was stopped on day 10
Adipo $^{CM/M0}$, Adipo $^{CM/M1}$, Adipo $^{CM/M2}$	Mature adipocytes were stimulated by macrophage conditioned medium on day 9, and the culture was stopped on day 10

2.2.7. Characterization of mature adipocyte by Oil red staining

Oil Red O staining was carried out to assess the differentiation of 3T3-L1 cells into mature adipocytes (Aldridge et al, 2013).

2.2.7.1. Cell fixation

To terminate culture, media was removed, and mature adipocytes were washed twice with PBS. Cells were then fixed in 500µl (24-well plate) of 4% (v/v) paraformaldehyde in 10x PBS for 30 minutes with gentle rotation at room temperature.

2.2.7.2. Cell staining

Following fixation, cells were washed three times with distilled water and incubated for 5 minutes in 60% isopropanol (3 parts of 100% isopropanol and 2 parts of distilled water). This was followed by an incubation in 500µl of Oil Red O working solution (Appendix 1.1) for 30 minutes and with gentle rotation at room temperature. The Oil Red O working solution was removed, and the cells were washed 2-5 times with distilled water. Oil Red O-Stained cultures were photographed using Leica DMI4000B microscope with 40x objective.

2.2.7.3. Quantification

Intracellular lipid vesicles in Oil Red O-stained mature adipocytes were quantified as described in datasheet of Lipid (Oil Red O) Staining Kit (BioVision, #K580-24) (https://www.biovision.com/documentation/datasheets/K580.pdf). Briefly, cells were washed 3 times with 60% isopropanol for 5 minutes with gentle rotation. The Oil Red O stain was extracted by adding 100% isopropanol (250µl per well of 24-well plate), and 200µl of extraction volume was used to measure absorbance at 492nm using a SpectraMax M5® microplate reader (Molecular Devices, California, USA).

2.2.8. Measurement of cell viability

The viability of cancer cells and adipocytes was measured by AlamarBlue® assay (Invitrogen, Carlsbad, California, USA). AlamarBlue® assay contains an oxidation-reduction indicator that is used to evaluate changes in metabolic function. Briefly, Resazurin (oxidized, non-fluorescent, blue state) in Alamar Blue® assay is reduced to resorufin (fluorescent, pink state) by reductase enzymes such as mitochondrial reductases, diaphorases or flavin reductases (Figure 13) (Rampersad, 2012, Csepregi et al., 2018).

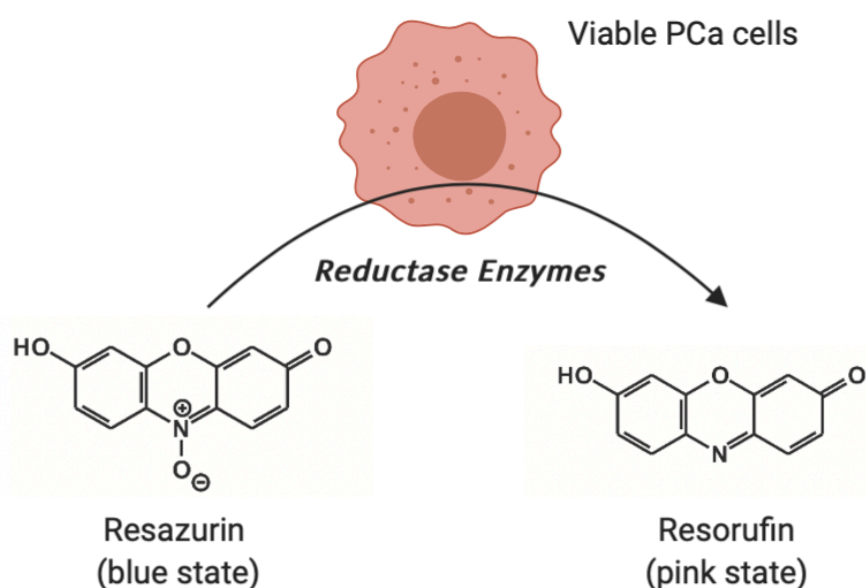


Figure. 13. Alamar Blue reaction equation. Reductases enzymes of viable cells reduce resazurin resulting in the form of fluorescent metabolic product resorufin**.** Refer to text for details. Adapted from Csepregi et al., 2018.

The PCa cells LNCaP (3 x 10^3 cells/well) were seeded in 96-well plates in 100 µl of 1% standard DMEM and C4-2B4 (3 x 10^3 cells/well) and PC3, DU145 (1 x 10^3 cells/well) and RM1 (2 x 10^3 cells/well) were seeded in 96-well plates in 100 µl of standard DMEM. After 24 hours, cells were treated with 20% CM (mix CM at 1:5 ratio with serum free DMEM medium). In addition, PCa cells that primed with CTRL ^{CM} were used as a control with a final volume of 100 µl. At 24-96 hours, Alamar Blue® reagent (10% v/v, without replacing the medium) was added, and after a 3-hour incubation, fluorescence was measured using a SpectraMax M5® microplate reader (Molecular Devices, California, USA) with excitation of 530nm and emission of 590 nm. To eliminate background fluorescence, wells without cells were included as blanks and the average absorbance value from these wells was subtracted. Cell viability was presented as percentage of controls (Figure 14).

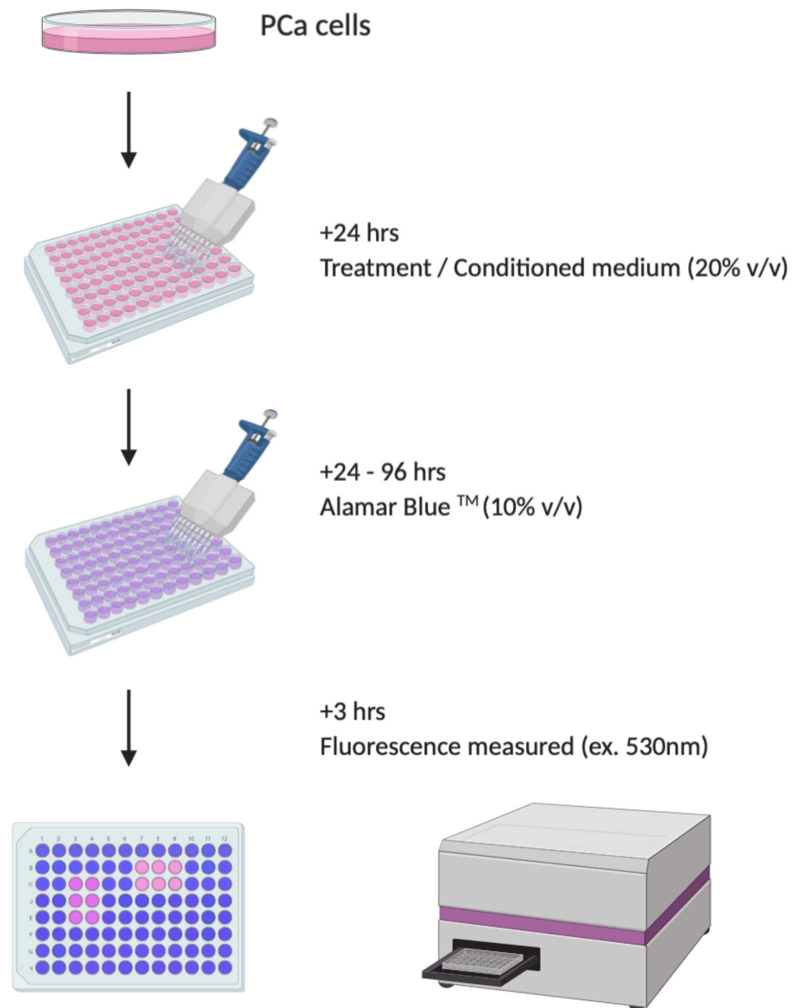


Figure. 14. Cell viability assessed by Alamar Blue ™ assay. PCa cells are plated in 96-well plates. After 24 hours, cells are treated with conditioned medium (20%v/v). After the incubation period, cells are exposed to Alamar Blue ™ (10%v/v) for 3 hours. Fluorescence is measured using SpectraMax M5® microplate reader.

2.2.9. Assessment of cell motility

2.2.9.1. Cell migration

Wound healing assay was conducted to study cell migration (Lampugnani, 1999). After introducing a wound, it could be healed with an increased concentration of growth factors at the wound margin through a combination of proliferation and migration (Perlman Zachary et al., 2004).

To study the effects of conditioned medium on the migration of PCa cells, PC3 cells ($2x10^5$ cells / well) were cultured to confluence in 24-well plates in standard DMEM. After 24 hours, a wound was created in a cell monolayer using a p200 pipette tip and cell debris were removed by washing the cell monolayer with serum free medium. Cultures were treated with conditioned medium (20% v/v) in 1% standard DMEM. Cells were also treated with 10ng/ml of the cell cycle and cell differentiation inhibitor Mitomycin-C (Sigma-Aldrich, Missouri, USA) for 30 minutes (Elgass et al., 2014, Honor et al., 2016). Cell migration over a period of 24 hours was recorded using a Leica AF6000LX inverted microscope with 10X, capturing images at 4 positions from each sample every 30 minutes. Image J software was used to analyse the time-lapse images and to quantify cell migration (Figure 15).

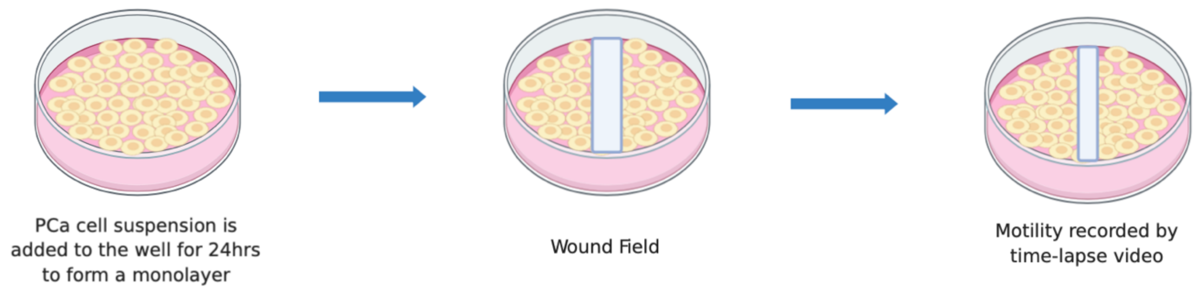


Figure. 15. **Wound healing assay.** The steps of wound healing migration assay consist in forming a monolayer, introducing a wound by scratching the monolayer and analysing migration (wound healing behaviour) from cells recorded by time-lapse video.

2.2.9.2. Cell invasion

The Transwell cell invasion assay was performed to measure the invasion of cancer cells (Justus et al., 2014). Tumour invasion of basement membrane is one of the most important steps that results in the tumour metastasis. To investigate the invasive capacity of basement membrane of tumour cells *in vitro*, Matrigel is used as alternative substrate to mimic the extracellular matrices. The Matrigel-coated Transwell insert act as the barriers to tumour cell invasion (Hall and Brooks, 2014). Briefly, Corning® CoStar® Transwell inserts (8 μM) were coated with 75 μl Corning® Matrigel® Basement membrane matrix (Discovery Labware, Massachusetts, USA) and incubated for 1.5-2 hours at 37°C and 5% CO_2. PCa cells were seeded on top of the filter membrane at a concentration of $5x10^4$ in 200μl serum free medium. Conditioned medium from control (CTRL ^{CM}, 20% v/v) or TNFα stimulated (Adipo $^{CM/TNF\alpha}$, 20% v/v) or M2 macrophage conditioned medium-stimulated mature adipocytes (Adipo $^{CM/M2}$, 20% v/v) in 500μl standard DMEM was used as chemoattractant gradients. The human LNCaP and PC3 cells were incubated in Transwell insert for 72 hours and mouse RM1 cells were incubated in Transwell insert for 48 hours. Cells on the top surface of the filters were wiped off with cotton swabs. The membranes were fixed in 100% ethanol for 5 minutes, stained with 1% eosin for 1 minute, washed by distilled water and stained with haematoxylin for 5 minutes. After washing with distilled water, the membrane was removed by scalpel, mounted with Faramount aqueous mounting medium (Dako North America, California USA), covered with a 13mm cover glass (VWR International, Pensilvania, USA) and cells were visualized using Pannoramic 250 Flash III Slide Scanner (3D Histech, Budapest, Hungray) with 40x. Image J software was used to analyse the images and to quantify cell invasion (Figure 16).

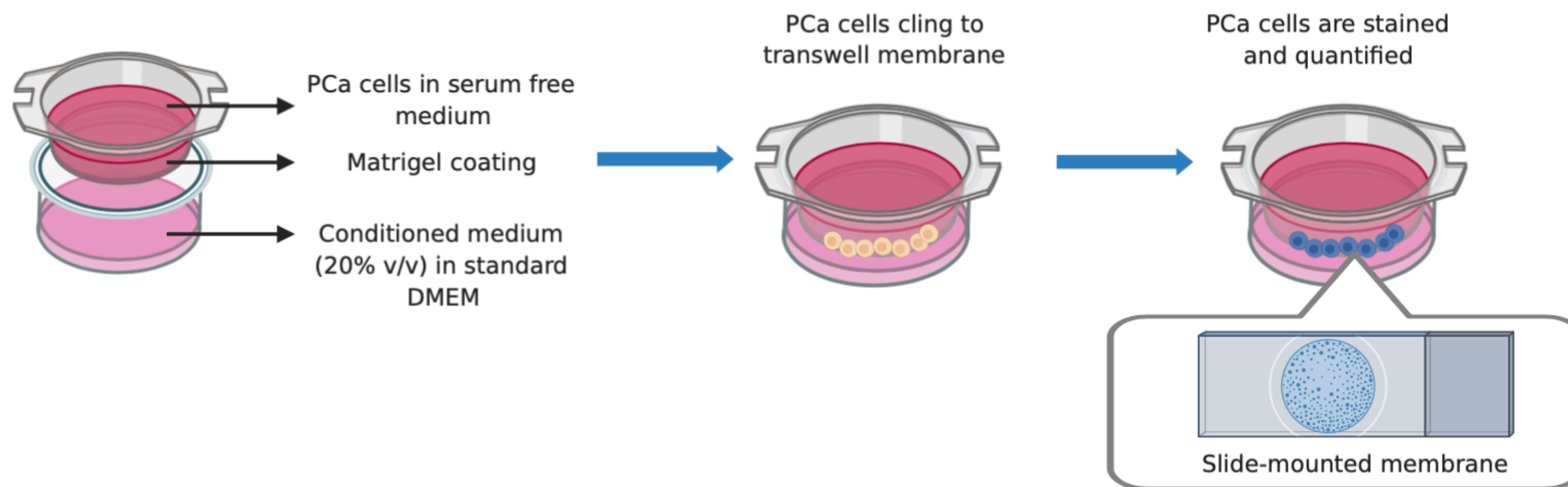


Figure. 16. **Transwell invasion assay.** Cells were seeded in the upper part of the Transwell insert. Invasive cells penetrated basement membrane and were attached to the bottom of the Transwell membrane. After 72 hours, invading cells were stained with Haematoxylin / Eosin.

2.3. Western blot analysis

2.3.1. Preparation of cell lysates

Cultures were plated in standard DMEM to 80% confluency in 6- or 24-well plates at 200 x 10^3 and 20 x 10^3 cells/well, respectively. Cultures were then treated as desired; medium was removed, cells were washed with ice-cold PBS for 5-minute incubation in 100µl (6-well plate) or 50µl (24-well plate) of RIPA lysis buffer (Appendix 1.2) containing 2% (v/v) protease inhibitor and 0.4% (v/v) phosphatase inhibitor on ice. Cells were gently scraped and transferred to an Eppendorf tube and centrifuged at 13.3g for 10 minutes at 4°C. The supernatant fraction was transferred to fresh Eppendorf tubes and stored at -20°C until further use.

2.3.2. Measurement of protein concentration

The bicinchoninic acid (BCA) Pierce protein assay (Pierce, USA) was carried out to quantify protein concentration, according to manufacturer's instruction. Briefly, BCA reaction involves the alkaline cuprous ion ($Cu^{2+)}$ reacting with proteins (Biuret reaction) to form a purple-coloured complex with Cu^+ (Protein + Cu^{2+} = Cu^+). This coloured complex produced from this reaction increases in proportion over an increasing of protein concentrations (Smith et al., 1985).

A standard curve was generated by serial dilutions of bovine serum albumin (BSA) (0-2000 µg/µl). In a 96-well plate, 10 µl of standard BSA dilutions and protein samples diluted 1:5 in distilled water were added in duplicates. 200 µl of copper (II)-sulphate (diluted 1:50 in BCA) were added in each well and the plate was incubated for 25-30 minutes at 37°C. The absorbance was then measured at 562 nm using a SpectraMax M5® microplate reader (Molecular Devices, California, USA) and protein concentration in sample was calculated from the BSA standard curve.

2.3.3. Gel electrophoresis

Gel electrophoresis was performed to study cell signalling in PCa cells and adipocytes. Briefly, Criterion^{TM} XT BioRad (12% Bis-Tris) pre-cast gels were placed into a vertical electrophoresis tank filled with 1xTGS running buffer (Appendix 2.2). Cell lysates (50-100 μg) were mixed with 7 μl of 5X sample loading protein buffer (Appendix 2.2), heated at 95°C for 5 minutes and loaded into the gel (Figure 17). The loading buffer denatures the negative charging proteins in samples. Kaleidoscope pre-stained standard (Bio-Rad, UK) and Magic Marker XP western standard (20-220kDa, Lifetechnology, Novex, UK) were used. Gels were run at constant voltage of 180V.

2.3.4. Electrophoretic transfer

The resolved proteins were transferred from the polyacrylamide gel to polyvinylidene difluoride (PVDF) membrane (GE Healthcare Amersham^{TM} Hybond^{TM}-P membrane, UK). The PVDF membrane was cut to the size, activated in 100% methanol and equilibrated in transfer buffer for 10 minutes (Appendix 1.3). A blotting sandwich (pre-soaked blot paper, membrane, polyacrylamide gel, pre-soaked blot paper) was prepared, placed in the transfer cassette and run at a constant voltage of 21V for approximately 7 minutes (Figure 17).

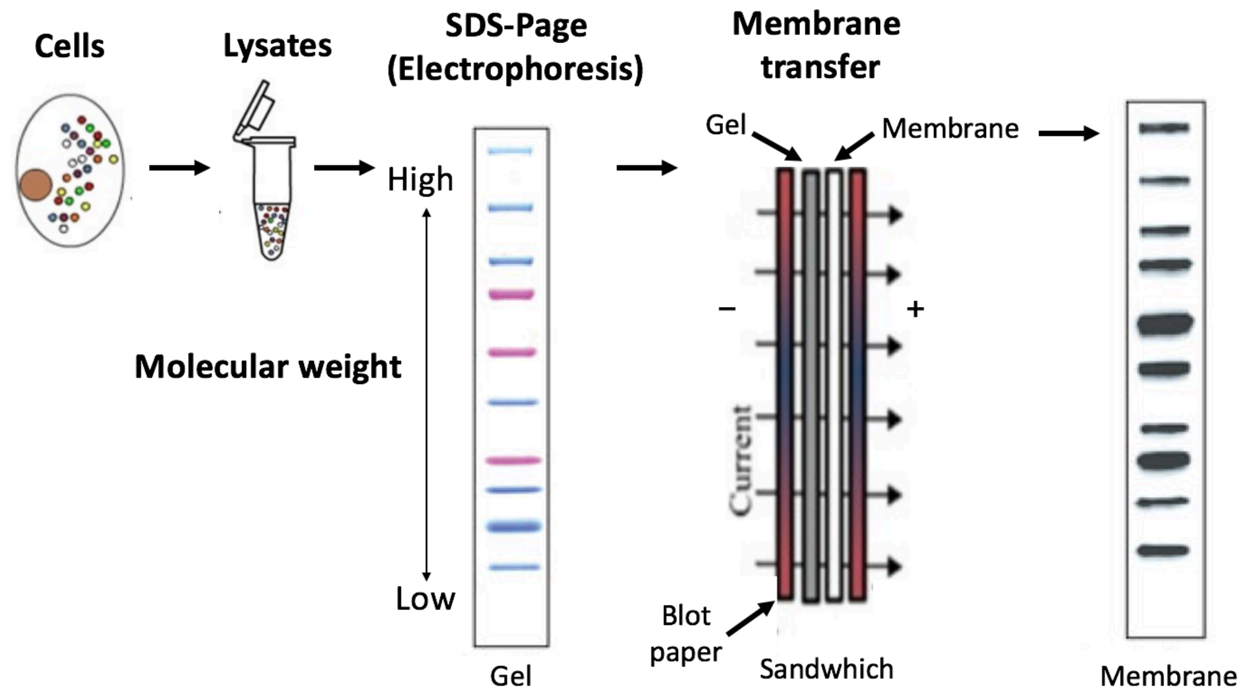


Figure. 17. Gel electrophoresis and electrophoretic transfer. Gel electrophoresis is used to separate native or denatured proteins. The proteins are then transferred to a membrane for detection using antibodies specific to the target proteins.

2.3.5. Immunostaining and antibody detection

An appropriate secondary antibody labelled with an enzyme (horseradish peroxidase (HRP) or alkaline phosphatase (AP)) was used to interact with an appropriate primary antibody that was chosen specifically for the studied protein. The probed protein was visualized by a chemiluminescent or chromogenic method (Figure 18) (Mahmood and Yang, 2012).

Briefly, the PVDF membrane was incubated in 50ml blocking buffer (Appendix 2.2) for 1 hour at room temperature with gentle shaking/rolling to ensure blocking of the remaining binding surface and non-specific binding sites. The membrane was then washed 3 times in Tris Buffer Saline solution (1xTBS) supplemented with 0.1% (v/v) Tween 20 (TBST) for 10 minutes. The membrane was incubated with the appropriate primary antibody (diluted 1:1000 in 3% BSA in TBST) overnight at 4°C with continuous rocking/rolling. The membrane was then washed 4 times in TBST for 15 minutes and incubated with the enzyme-conjugated secondary antibody peroxidase AffiniPure donkey Anti-Rabbit (Jackson Immunoresearch Laboratories, USA) at a concentration of 1:5000 in 5% w/v dried non-fat milk in TBST for 1 hour at room temperature with gentle rocking. The membrane was washed three times with TBST and visualised. The chemiluminescent substrates (Clarity ™ Western ECL Substrate, Bio-Rad, UK) were spread on blots and the light emission was detected using a Syngene Genegenome Bio Imaging System. The band intensity was visualised using ChemiDoc™ Imaging System and quantified using Image Lab Software (Bio-Rad, UK).

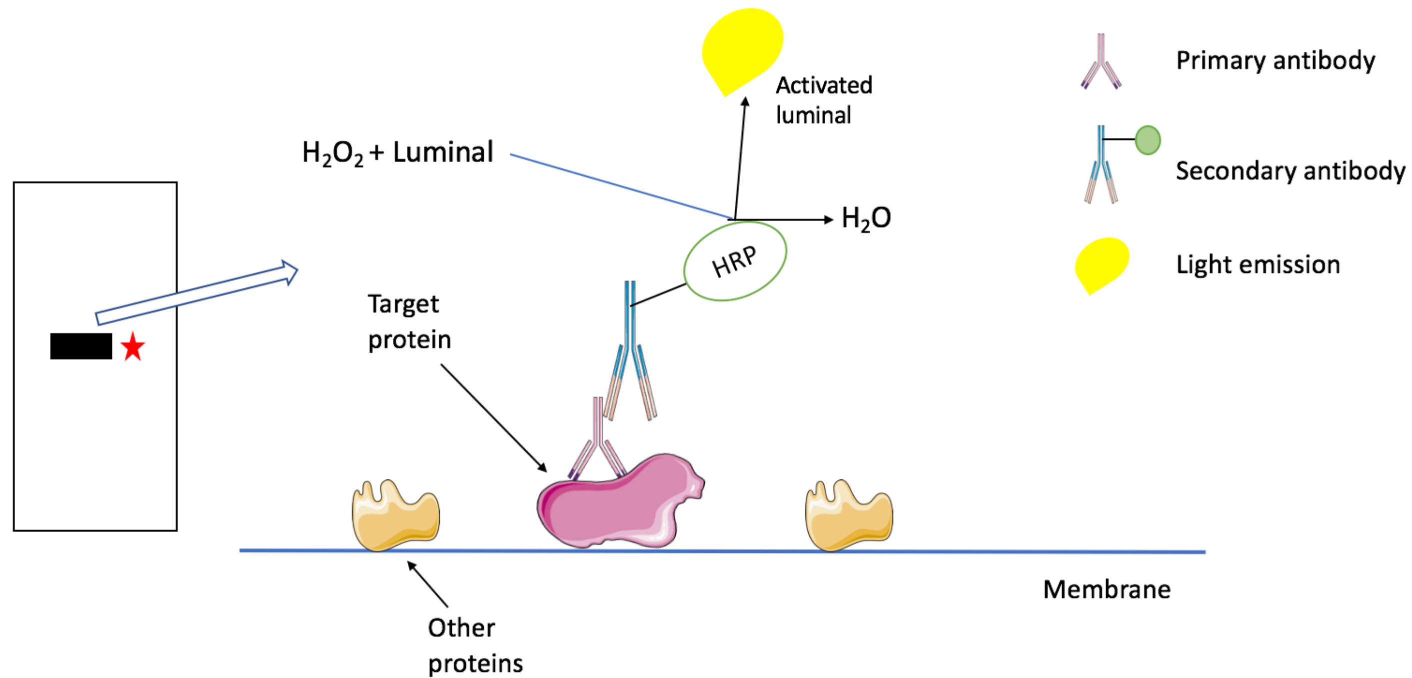


Figure. 18. Protein detection. To probe the different primary antibody with close molecular size, the membrane was incubated in stripping buffer (Appendix 2.3) at 58°C for 5min in order to break strong antibodies binding and remove all antibodies. Then blocked-in blocking buffer for 30min and detected with a different primary antibody and processed as described above.

2.4. Microarray analysis of adipocyte and macrophage derived factors

Conditioned medium was collected as described above (section 2.2.6.2). Protein expression in the conditioned medium was measured with the Proteome Profiler Mouse XL Cytokine Array Kit by R&D systems, according to manufacturer's instructions. The arrays were analysed following chemiluminescent detection on a Chemidoc Imaging System (BioRad), according to manufacturer's instructions.

2.5. Gene expression analysis

Online tools with publicly available databases were applied for the analysis of protein expression and genetic alteration of obese patients with PCa, including cBioPortal tool (http://www.cbioportal.org, data accessed: 18/02/20) (Cerami et al., 2012, Gao et al., 2013), Search Tool for the Retrieval of Interacting Genes (STRING) (https://string-db.org/) (Snel et al., 2000), and Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn/index.html) (Tang et al., 2017).

The online tool STRING was used to construct an interactome map of deregulated genes Protein interaction and functional association between the components of NFκB pathway was investigated using curated interaction obtains from Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) from STRING database. The indicated network properties include: Nodes: number of proteins in the network; Node Contents: protein structures are sketched in the nodes; Edges: number of interactions; Nodes and edges are colored according to type of evidence. The colored nodes and edges are used as a visual aid to identify which node get involved with which biological process and KEGG pathway.

To analyse the copy number variants of gene encoding NFκB proteins in PCa progression, cancer studies were accessed using online tool cBioPortal. Briefly, database was selected and combined based on PCa stage: The PROSTATE (Eur Urol 2017; MSKCC/DFCI 2018; TCGA PanCancer Atlas 2018) for studying primary tumour stage (1572 patients total) and the

Metastatic Prostate Cancer Project (Provisional, November 2019) in combination with the Metastatic Prostate Cancer Project (SU2C 2019) database for studying metastasis tumour stage (474 patients total). cBioPortal was also used to generate Kaplan-Meier survival curves.

The GEPIA was used to compare the gene expressions encoding key NF B proteins between prostate tumour and health control tissue with the dataset Prostate Adenocarcinoma (TCGA, The Cancer Genome Atlas). In addition, to explore the correlation of genetic alteration of NFκB proteins and adipogenesis, the key interactives were analysed using GEPIA based on the database Prostate Adenocarcinoma (TCGA, PanCancer Atlas) and Genotype-Tissue Expression (GTEx) database.

2.6. Systemic review – Meta-analysis

Meta-analysis is a quantitative, epidemiological study design that assesses the effectiveness of interventions in experimental setting. The outcomes of meta-analysis provides an estimate of treatment effectiveness as well as risk factor of disease (Haidich, 2010).

2.6.1. Literature search

A protocol was created according to the purposed hypothesis and searches of meta-analysis were conducted in the following databases: Medline, Web of Science and PubMed.

The search strategy and keywords for Medline and Web of Science in the protocols were used to search an extensive list of keywords related to PCa, obesity, inflammation and NFκB signal transduction pathway. Search strategies that developed for Medline and Web of Science can be found in Appendix 3. These terms, their combination and alternative keywords were used to search titles, abstracts as well as full text. Acquired research articles were curated managed and duplicates were removed using EndNote X9 (Clarivate Analytics, 2016).

2.6.2. Inclusion and exclusion criteria

Human clinical trials, animal intervention experiments *in vivo* and *in vitro* studies were included in the systematic review and meta-analysis. Then, studies that reported the role of obesity in cancers other than PCa were excluded. Only studies that focused on pro-inflammatory mediators other than components of the canonical and non-canonical NFκB signalling pathway were excluded. Additionally, studies that reported the role of NFκB or adipocytokine expression in obesity in non-PCa models or settings were excluded.

2.6.2.1. Types of intervention

Human studies that examined the serum level of adipokines and IL-6 in patients with PCa were included. *In vivo* studies that examined the effects of HFD-induced obesity on protein expression level of pro-inflammatory cytokines were included. *In vitro* studies that examined the influence of adipokines on PCa cell behaviour were included.

2.6.2.2. Outcomes measures

Eligible outcomes include cell growth, cell migration, changes in visceral tissue pro-inflammatory cytokine and adipocytokine expression levels.

2.6.3. Data collection and analysis

2.6.3.1. Selection of studies

Studies were initially screened by title and abstract for an extensive list of keywords. The full text of eligible studies was then further screened and figures and tables that contain relevant data were selected. The exclusion criteria and the stage at which the studies were excluded were noted in result section (Chapter 3). The meta-analysis was performed if there were at least 2 studies of the same design addressing the same parameters in section 2.6.2.

2.6.3.2. Data extraction

Items obtained from each included study include author's name, publication year, experimental design, sample size, outcome measures. Mean and standard deviation (SD) or standard error measurement (SEM) were extracted from each relevant figure or table using online tool WebPlotDigitizer (https://apps.automeris.io/wpd/). For studies that reported mean ± SEM, SD was calculated using the following formula ($SEM = SD/\sqrt{N}$).

2.6.3.3. Data analysis

Data was summarised, comparison between subgroups was performed and forest plots were generated using Review Manager (RevMan Version 5). Outcomes of the eligible studies consist of continuous variable. Forest plots provide a graphical depiction of its mean difference (MD) and their corresponding 95% confidence interval (CI) (Zlowodzki et al., 2007).

To identify variation in outcomes between studies, statistical heterogeneity was measured using the Cochrane's Q statistic and the I^2 statistic and Chi square test. The Chi square test was used to determine the degree of significance between studies. A high correlation between two sets of data is suggested by a low value of Chi square. I^2 cut-offs is 25%, 50% and 75% that represent the low, moderate and considerable heterogeneity, respectively (Higgins and Thompson, 2002). In the random-effect or fixed-effect model of MD measure, outcomes are weighted with the heterogeneity parameter and the inverse of their variance (Haidich, 2010). Random-effect models were used for initial calculation with the ratio >50% in the I^2 statistics. Fixed-effect models were used when no heterogeneity or low heterogeneity was observed (DerSimonian and Kacker, 2006, Higgins et al., 2003).

2.7. Statistical analysis

Where appropriate comparison among groups was performed using analysis of variance (ANOVA) followed by Bonferroni post-hoc test using GraphPad Prism 7. A student's T-test was used to determine the significance between two groups using GraphPad Prism 7. A p-

value of 0.05 or below was considered statistically significant, and a p-value of 0.01 or below highly statistically significant.

CHAPTER 3

ROLE OF NFκB IN ADIPOCYTE – PROSTATE CANCER CELL CROSSTALK: A META-ANALYSIS

3.1. Summary

Numerous studies have shown that obesity and inflammation play an important role in PCa initiation, progression and metastasis. In this project, we hypothesised that obesity regulates PCa progression by modulating the pro-inflammatory NFκB signalling pathway. In this chapter, this hypothesis was tested by performing a meta-analysis that examined the association between PCa progression in obese patients and the expression of NFκB and levels of its activating cytokines and adipocytokines in human studies. Additionally, my analysis examined the association between levels of adipocyte-derived NFκB-activating mediators and adipocytokines and the behaviour of PCa cells in obese animals and *in vitro*.

A search of PubMed, Web of science, and Medline was conducted and a systematic review and meta-analysis of 2646 studies were performed. After reviewing the title and abstract for content, 2313 articles were deemed irrelevant studies, and an additional 325 papers were excluded after examination of the content of full text. Eight English-language studies involving human studies, animal intervention experiments (*in vivo*) and *in vitro* studies were included in the present meta-analysis. No *ex vivo* studies were identified. The data were analysed from June 1st through October 20th, 2019. The effect estimates with 95% CI were calculated using a random-effect or fixed-effect model based on I^2 statistic (section 2.6.3.3.). The search strategy contained the following 3 concepts linked together with the AND operator: NFκB; AND prostate cancer; AND obesity. Two reviewers reviewed independently all article titles / abstracts and full texts. Comparisons of NFκB-activating expression and mediators in (A) co-culture of PC3 PCa and adipocytes; (B) co-culture of PC3 PCa and adipokines; (C) prostatic tissue from obese and lean patients, and (D) benign prostate hyperplasia (BPH) and PCa tumours were carried out.

My meta-analysis of pooled mean difference from 2 *in vitro* studies revealed that exposure to adiponectin or leptin significantly increased the *in vitro* migration of the castration-insensitive human PC3 cells, compared to control group. The analysis detected one study that showed

that co-culture of human PCa cells with bone marrow (BM) adipocytes affect the expression level of tumour-derived IL-1β. The analysis of pooled mean differences from 3 *in vivo* studies indicated that mRNA expression of TNFα, IL-1β and p65/RelA NFκB significantly increased in the prostatic tissue from obese mice, compared to prostatic tissue from lean mice. Further analysis of one of these studies revealed that HFD-induced obesity increased the serum expression level of leptin in the prostatic tissue. In human, analysis of pooled mean difference from 2 studies showed no significant difference in serum level of IL-6, leptin and adiponectin in PCa patients and BPH group.

Overall, the results of meta-analysis of the included studies in this chapter suggested that adipokines are implicated in the orchestration of the *in vitro* motility of highly metastatic human castration-insensitive PCa cells, and increased expression of NFκB-induced pro-inflammatory cytokines plays a role in HFD-induced obesity in mice. Notwithstanding these findings, further human, *in vivo* and *in vitro* studies are needed to confirm the role of NFκB in the regulation of obesity driven PCa.

3.2. Introduction

In the UK, PCa is the most common malignancy in elderly people, and obesity is a common public health problem in this demographic. A number of studies have shown that the growth and metastatic spread of PCa cells is affected by factors derived from mature adipocytes in adipose tissues in the prostate and site of distant metastases (Tang and Lu, 2009; Huang et al., 2011; Moreira et al., 2015).

Chronic inflammation is implicated in all aspects of PCa and obesity, and adipocyte-derived factors such as adipokines as well as pro-inflammatory cytokines and chemokines have been reported to enhance the behaviour of PCa cells by stimulating the expression and activity of classical pro-inflammatory signalling pathways, particularly NFκB (Tang and Lu, 2009; Huang et al., 2011). Additionally, the levels of classical pro-inflammatory NFκB-activating mediators such as TNFα and IL-1β have been found to be elevated in patients with increased level of PSA (Michalaki et al., 2004). Notwithstanding these findings, the role of NFκB and its activating- and activated-factors in the interaction between PCa cells and adipocytes has not been investigated.

In this chapter, I carried out a meta-analysis to examine the association between the expression and levels of NFκB and its activating cytokines and adipocytokines and PCa progression in human studies. My analysis also investigated the association between the expression and levels of NFκB-activating mediators and adipocytokines and metastatic behaviour of PCa cells *in vitro* and in obese animals.

3.3. Results

3.3.1. Included studies

Using the search strategy that shown in Appendix 3, a search of PubMed, Web of science, and Medline was conducted on June 1st, 2018, and updated on September 30th, 2019. A systematic review and meta-analysis of studies in 2646 papers published from January 1st, 1973, to September 30th, 2019, were performed. I have identified 1115 papers in “Medline”, 29 papers in “Web of Science” and 2025 papers in “PubMed”. A detailed schematic of the search strategy and outcomes are shown in figure 19. After removal of duplicates, 2646 papers were identified from the three independent searches. The exclusion criteria were applied, and 2313 papers were deemed irrelevant studies after reviewing the title and abstract for relevant content. Additionally, a further 325 papers were removed after examination of the full text of articles. The remaining included English-language papers (n=8) were categorised into human studies, animal intervention experiments (*in vivo*) and *in vitro* studies. No *ex vivo* studies were identified. As shown in figure 19, 2 human clinical studies and 3 *in vivo* and 3 *in vitro* preclinical studies were identified for the meta-analysis.

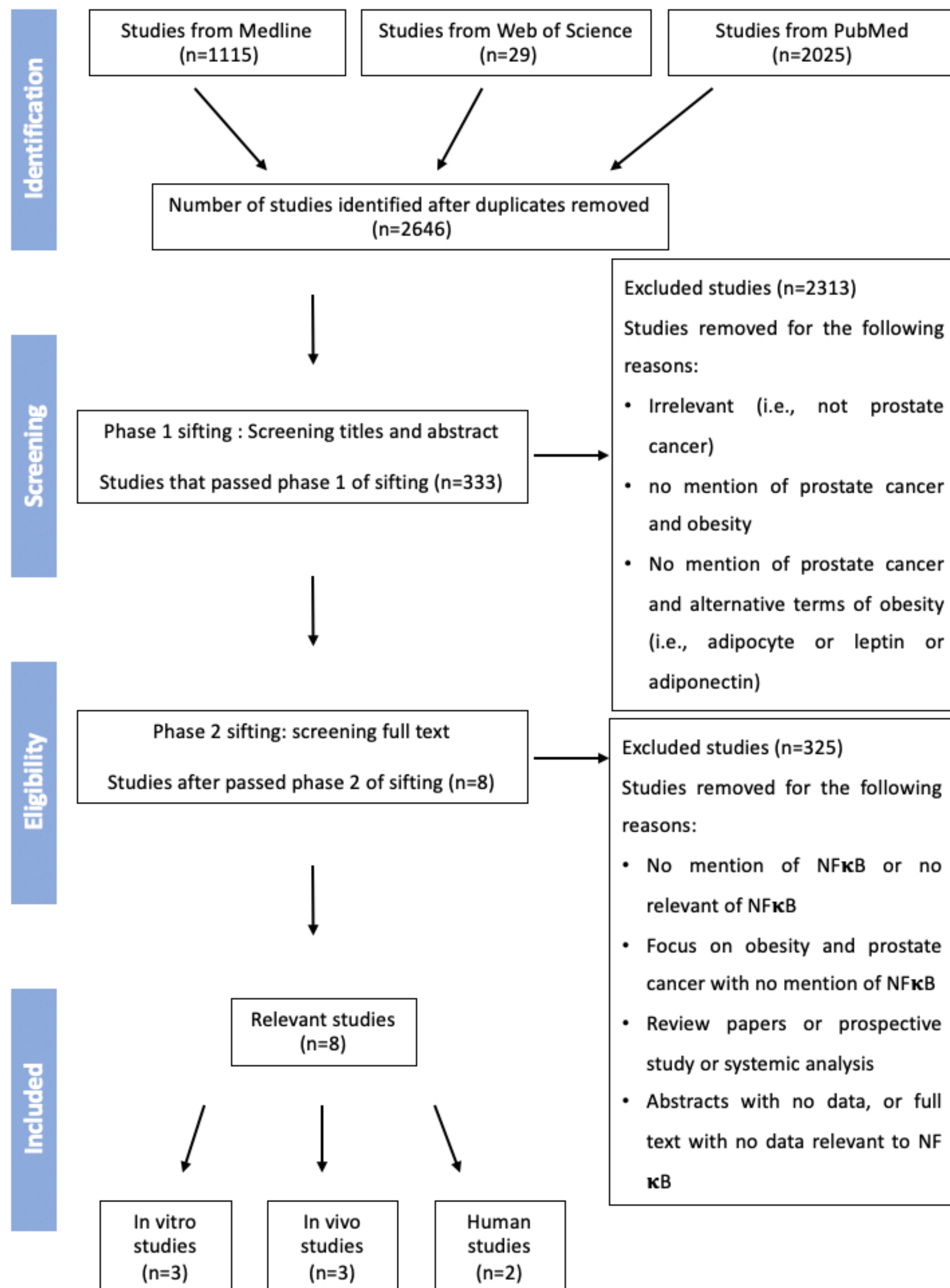


Figure. 19. ***Flow chart indicating the process that followed to screen the relevant studies.***

3.3.2. Reported outcomes

Next, the following comparisons were carried out:

(1) The level of NFκB-activating mediators in

a. culture of PC3 PCa cells was compared to that in co-cultures with BM adipocytes

b. obese prostatic tissue was compared to that in lean prostatic tissue

c. BPH was compared to PCa tumours

A preclinical research study performed by Herroon, 2019 showed that the mRNA expression level of the pro-inflammatory factor IL-1β was significantly increased in cocultures of human castration-insensitive PC3 cells and BM adipocytes when compared to culture of PC3 alone.

Three independent animal studies performed by Vykhovanets et al., 2011, Laurent et al., 2016 and Hayashi et al., 2018 showed that the mRNA expression of a number of pro-inflammatory cytokines and NFκB subunit were significantly increased in the prostatic tissue of mice fed with HFD when comparing with mice fed with regular diet.

One human study performed by Tewarl et al., 2013 showed that the serum expression level of IL-6 and adipokines significantly increased in patients with PCa than that in patients with BPH. However, the human study performed by Baillargeon et al., 2006 showed that there is no significant difference in serum expression level of pro-inflammatory cytokine and adipokines in PCa patients when comparing with BPH group.

(2) The effects of level of adipocyte-derived factors on the metastatic behaviour of PCa cells

Two independent preclinical studies performed by Tang and Lu, 2009 and Huang et al., 2011 indicated that exposure to leptin and adipocytokine significantly enhanced the migration of human PC3 PCa cells *in vitro*.

3.3.3. Statistical analysis

The meta-analysis was performed if there were at least 2 studies of the same design addressing the same parameters in section 2.6.2. Forest plots provide a graphical depiction of effect size estimates and their corresponding 95% CI. MD were extracted or calculated with 95% CI when adequate number of experiments were provided as previously described (Zlowodzki et al., 2007). To identify variation in outcomes between studies, statistical heterogeneity was measured using the Cochrane's Q statistic and the I^2 statistic. I^2 cut-offs is 25%, 50% and 75% indicates the low, moderate and considerable heterogeneity (Higgins and Thompson, 2002). Random-effect models were used for initial calculation with the ratio >50% in the I^2 statistics. Fixed-effect models were used when no significant heterogeneity was observed (DerSimonian and Kacker, 2006, Higgins et al., 2003).

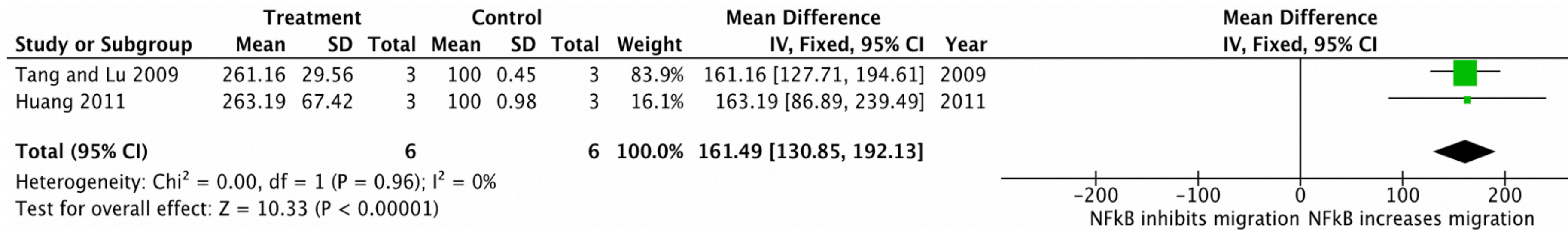
3.3.4. In vitro interventions

My analysis identified studies that reported the *in vitro* effect of exogeneous adiponectin and leptin on the motility of human castration-insensitive PC3 cells, and *in vitro* secretion of pro-inflammatory cytokines by human castration-insensitive PC3 cells in the presence and absence of mature adipocytes in culture. Data from *in vitro* studies that included experiments with three biological repeats were included.

The two included *in vitro* studies that carried out by Tang and Lu, 2009 and Huang et al., 2011 investigated the effects of obesity-related factors adiponectin and leptin on the motility of highly metastatic PCa cells. The effect estimates with 95% CI were calculated using a fixed-effect model. As shown in figure 20A, the pooled mean difference of these two included *in vitro* studies indicated that exposure to adiponectin or leptin increased the migration of the castration-insensitive human PC3 cells, compared to control group. The analysis showed significant difference between treatment and control group (MD [CI] =161.49 [130.85, 192.13], I^2=0% ($p<0.00001$)).

Next, the *in vitro* study performed by Herroon, 2019 tested the effect of adipocytes on expression levels of PCa tumour-derived pro-inflammatory mediator. The effect was estimates with 95% CI were calculated using a fixed-effect model. As shown in figure 20B, the pooled mean difference of this *in vitro* study revealed that the secretion of IL-1β by PC3 cells was augmented at the mRNA levels in the presence of BM adipocytes (MD [CI] = 3.59 [1.37, 5.81], (p=0.0002)). However, there was insufficient evidence to support significant differences between the intervention and control group.

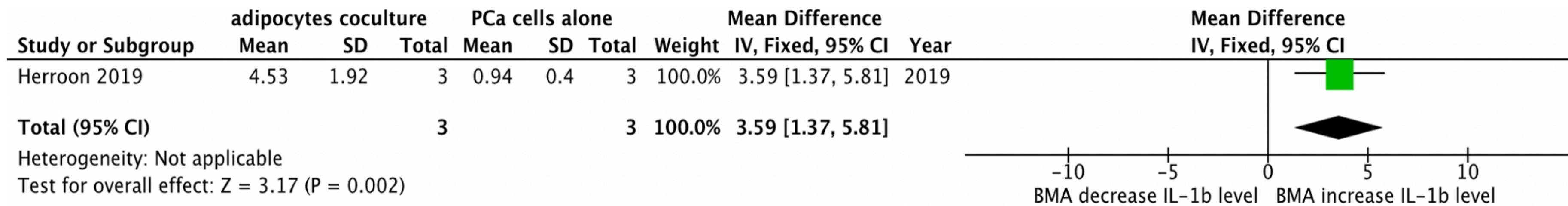
A



Study or Subgroup	Treatment Mean	Treatment SD	Treatment Total	Control Mean	Control SD	Control Total	Weight	Mean Difference IV, Fixed, 95% CI	Year
Tang and Lu 2009	261.16	29.56	3	100	0.45	3	83.9%	161.16 [127.71, 194.61]	2009
Huang 2011	263.19	67.42	3	100	0.98	3	16.1%	163.19 [86.89, 239.49]	2011
Total (95% CI)			**6**			**6**	**100.0%**	**161.49 [130.85, 192.13]**	

Heterogeneity: $Chi^2 = 0.00$, df = 1 (P = 0.96); $I^2 = 0\%$
Test for overall effect: Z = 10.33 (P < 0.00001)

B



Study or Subgroup	adipocytes coculture Mean	adipocytes coculture SD	adipocytes coculture Total	PCa cells alone Mean	PCa cells alone SD	PCa cells alone Total	Weight	Mean Difference IV, Fixed, 95% CI	Year
Herroon 2019	4.53	1.92	3	0.94	0.4	3	100.0%	3.59 [1.37, 5.81]	2019
Total (95% CI)			**3**			**3**	**100.0%**	**3.59 [1.37, 5.81]**	

Heterogeneity: Not applicable
Test for overall effect: Z = 3.17 (P = 0.002)

figure. 20. Forest plots indicating the effects of NFκB activation on biological behaviour or protein levels of PC3 cells. The forest plots were calculated as mean difference +/- standard deviation. The number of samples, weight of each study and the heterogeneity (presented as I^2) are all mentioned in the tables next to their corresponding forest plots. (A) Forest plot showing the effects of adipokine-induced NFκB activation on the migration of PC3 cells. Two studies were used, with a total number of 12, I^2=0% (p<0.00001). (B) Forest plot showing the effects of BM adipocytes on mRNA expression level of IL-1β of PC3 cells. One study was used, with a total number of 6.

3.3.5. In vivo interventions

My analysis identified *in vivo* studies that examined the roles that HFD-induced obesity and chronic inflammation play in the development of PCa by measuring the levels of adiponectin, leptin and pro-inflammatory cytokines expression in prostatic tissue of mice bearing PCa.

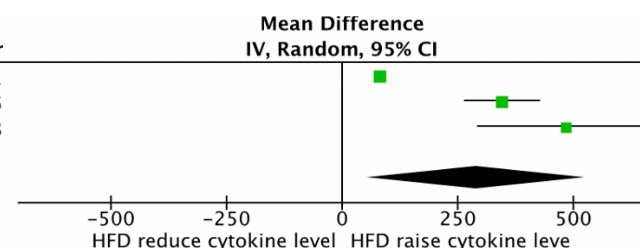
The 3 included *in vivo* studies by Vykhovanets et al., 2011, Laurent et al., 2016 and Hayashi et al., 2018 examined the role of adipogenesis in the regulation of the mRNA expression of pro-inflammatory cytokines and NFκB subunit in prostatic tissue from mice fed with either HFD or regular diet. The 3 *in vivo* studies included 75 mice in total. The effect estimates with 95% CI were calculated using a random-effect model. The pooled mean difference from these 3 animal intervention studies indicated that mRNA expression of IL-1β and TNFα and p65/RelA increased in prostatic tissue from obese mice than that of lean mice. The analysis showed significant difference between HFD and CD group (MD [CI] =290.96 [54.49, 527.44], I^2=96% (p=0.02)) (Figure 21, panel A).

Additionally, the *in vivo* study (Laurent et al., 2016) examined the expression of obesity-related factors adiponectin and leptin in prostatic tissue. This animal intervention study included 10 mice in total. The effect was estimates with 95% CI were calculated using a fixed-effect model. The figure 21B confirmed that expression level of adiponectin decreased in prostatic tissue of obese mice, when compared to lean mice (MD [CI] = -25.48 [-26.19, -24.77], (p<0.00001)). As shown in figure 21C, the expression level of leptin also increased in prostatic tissue from these HFD-fed mice (MD [CI] = 102.89 [79.39, 126.39], (p<0.00001)). However, there was insufficient evidence to support significant differences between the obese and lean group.

A

Study or Subgroup	obese group Mean	SD	Total	lean group Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI	Year
Vykhovanets 2011	181.7	26.2	24	100	0.0709	24	36.1%	81.70 [71.22, 92.18]	2011
Laurent 2016	445.7	92	5	100	0.201	5	34.7%	345.70 [265.06, 426.34]	2016
Hayashi 2018	584.6	292.9	9	100	0.000007	8	29.2%	484.60 [293.24, 675.96]	2018
Total (95% CI)			**38**			**37**	**100.0%**	**290.96 [54.49, 527.44]**	

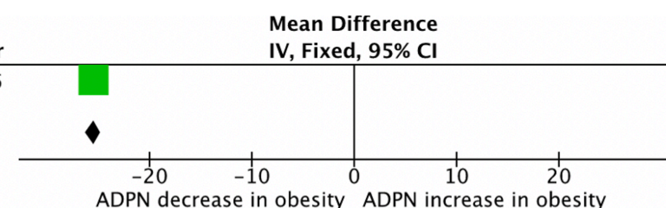
Heterogeneity: $Tau^2 = 40288.56$; $Chi^2 = 57.10$, df = 2 (P < 0.00001); $I^2 = 96\%$
Test for overall effect: Z = 2.41 (P = 0.02)



B

Study or Subgroup	obese group Mean	SD	Total	lean group Mean	SD	Total	Weight	Mean Difference IV, Fixed, 95% CI	Year
Laurent 2016	74.52	0.79	5	100	0.2	5	100.0%	-25.48 [-26.19, -24.77]	2016
Total (95% CI)			**5**			**5**	**100.0%**	**-25.48 [-26.19, -24.77]**	

Heterogeneity: Not applicable
Test for overall effect: Z = 69.91 (P < 0.00001)



C

Study or Subgroup	obese group Mean	SD	Total	lean group Mean	SD	Total	Weight	Mean Difference IV, Fixed, 95% CI	Year
Laurent 2016	202.89	26.81	5	100	0.221	5	100.0%	102.89 [79.39, 126.39]	2016
Total (95% CI)			**5**			**5**	**100.0%**	**102.89 [79.39, 126.39]**	

Heterogeneity: Not applicable
Test for overall effect: Z = 8.58 (P < 0.00001)

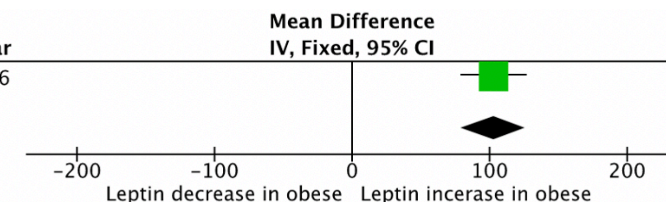


Figure. 21. Forest plots indicating the effects of adipogenesis on NFκB activation in prostatic tissue of obese mice model. The forest plots were calculated as mean difference +/- standard deviation. The number of samples, weight of each study and the heterogeneity (presented as I^2) are all mentioned in the tables next to their corresponding forest plots. (A) Forest plot showing the effects of obesity on pro-inflammatory cytokine-induced or NFκB subunit-induced activation. Three studies were used, with a total number of 75, I^2=96% (p=0.02). (B) Forest plot showing the effects of obesity on adiponectin-induced NFκB activation. One study was used, with a total number of 10. (C) Forest plot showing the effects of obesity on leptin-induced NFκB activation. One study was used, with a total number of 10.

3.3.6. Human study interventions

The meta-analysis identified human clinical trials that reported the role of pro-inflammatory cytokine and adipokines in PCa progression. Two included human studies (n=440 patients) were carried out part of the San Antonio Centre for Biomarkers of Risk of Prostate Cancer (SABOR) (Baillargeon, 2006, Tewari et al., 2013). The effect estimates with 95% CI were calculated using a random-effect or fixed-effect model.

First, the two included human studies performed by Baillargeon et al., 2006 and Tewarl, 2013 examined the role of the pro-inflammatory cytokine IL-6 in the progression of PCa. The findings of these studies were broadly contradictory, Tewari et al., 2013 indicated that IL-6 enhanced the progression of PCa, whereas Baillargeon et. al., 2006 showed that IL-6 level was reduced in PCa patients. As shown in figure 22A, the meta-analysis of pooled mean difference from these 2 human studies suggests no statistical difference in serum level of IL-6 between PCa patient and BPH groups (MD [CI] = -3.89 [-61.80, 54.03], I^2=89% (p=0.09)).

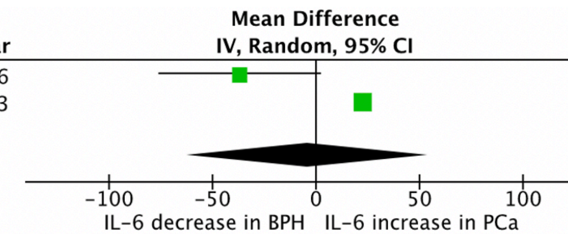
The 2 human studies also investigated the role of the obesity-associated adipokines adiponectin and leptin in the progression of PCa. The meta-analysis of serum level of adiponectin in PCa patients is shown in figure 22B. The pooled mean difference from adiponectin expression level of human studies showed no significant difference between PCa patients and BPH groups (MD [CI] = -49.09 [-141.44, 43.25], I^2=100% (p=0.30)).

The meta-analysis of serum level of leptin in PCa patients from the same clinical trial is shown in figure 22C. The meta-analysis of pooled mean difference from the 2 human studies revealed no statistical difference in serum level of leptin between PCa patients and BPH group (MD [CI] =9.54 [-5.58, 24.66], I^2=90% (p=0.22)).

A

Study or Subgroup	prostate cancer Mean	SD	Total	BPH Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI	Year
Baillargeon 2006	70.9	128.6	125	107.8	180.5	125	44.5%	−36.90 [−75.75, 1.95]	2006
Tewari 2013	34.08	13.41	95	11.52	7.31	95	55.5%	22.56 [19.49, 25.63]	2013
Total (95% CI)			**220**			**220**	**100.0%**	**−3.89 [−61.80, 54.03]**	

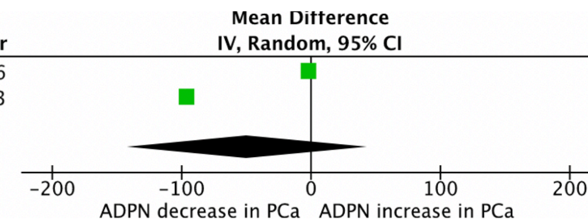
Heterogeneity: $Tau^2 = 1570.05$; $Chi^2 = 8.94$, df = 1 (P = 0.003); $I^2 = 89\%$
Test for overall effect: Z = 0.13 (P = 0.90)



B

Study or Subgroup	prostate cancer Mean	SD	Total	BPH Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI	Year
Baillargeon 2006	17.9	10.6	125	19.9	13.2	125	50.0%	−2.00 [−4.97, 0.97]	2006
Tewari 2013	18.64	20.23	95	114.87	13.22	95	50.0%	−96.23 [−101.09, −91.37]	2013
Total (95% CI)			**220**			**220**	**100.0%**	**−49.09 [−141.44, 43.25]**	

Heterogeneity: $Tau^2 = 4435.43$; $Chi^2 = 1052.00$, df = 1 (P < 0.00001); $I^2 = 100\%$
Test for overall effect: Z = 1.04 (P = 0.30)



C

Study or Subgroup	prostate cancer Mean	SD	Total	BPH Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI	Year
Baillargeon 2006	11.1	11.7	125	8.62	7.4	125	54.4%	2.48 [0.05, 4.91]	2006
Tewari 2013	55.48	40.26	95	37.51	23.13	95	45.6%	17.97 [8.63, 27.31]	2013
Total (95% CI)			**220**			**220**	**100.0%**	**9.54 [−5.58, 24.66]**	

Heterogeneity: $Tau^2 = 107.86$; $Chi^2 = 9.90$, df = 1 (P = 0.002); $I^2 = 90\%$
Test for overall effect: Z = 1.24 (P = 0.22)

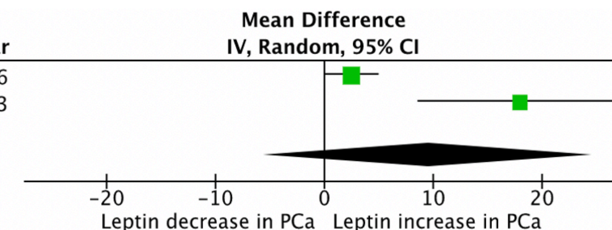


Figure. 22. Forest plots indicating the effects of adipocyte-derived factors on the promotion of PCa. The forest plots were calculated as mean difference +/- standard deviation. The number of samples, weight of each study and the heterogeneity (presented as I^2) are all mentioned in the tables next to their corresponding forest plots. (A) Forest plot showing the association of serum IL-6 level and incidence of PCa. Two studies were used, with a total number of 440, I^2=89% (p=0.90). (B) Forest plot showing the association of adiponectin level and incidence of PCa. Two studies were used, with a total number of 440, I^2=100% (p=0.30). (C) Forest plot showing the association of leptin level and incidence of PCa. Two studies were used, with a total number of 440, I^2=90% (p=0.22).

3.4. Discussion

In the UK, PCa is the most common non-skin cancer in elderly population. Approximately 90% of men with PCa survive the disease for five years. However, the survival rate of patients with distance metastases drops to 30% (Kirby, 2009). Obesity induces a low-grade, chronic inflammation status and chronic inflammation is associated with increased risk of PCa in clinically obese individuals (MacLennan et al., 2006, Gucalp et al., 2017). Furthermore, a number of studies have reported that adipocyte-derived factors enhanced the growth and motility of PCa cells (Tymchuk et al., 2001, Onuma et al., 2003, Tokuda et al., 2003, Moreira et al., 2015, Hu et al., 2018), thereby suggesting that PCa development and metastasis are affected by obesity-associated adipokines.

Inflammation is a major risk factor of PCa and obesity (Kumar et al., 2004, Taverna et al., 2015, Baker et al., 2011). The pro-inflammatory signalling pathway NFκB plays a role in both PCa and obesity (Berg et al., 2004, Carlsen et al., 2009, Jin et al., 2014). In this chapter, I hypothesised that NFκB implicated in obesity-driven PCa by regulating the crosstalk between adipocytes and PCa cells in prostatic tissues and distant metastasis. There are number of classical pro-inflammatory cytokines, such as TNFα, IL-1β and CD40, that are likely to play a key role in the regulation of PCa cell – adipocyte crosstalk. In this chapter, I showed evidence from meta-analysis of *in vivo* studies that further confirms the role of chronic inflammation in obesity-driven PCa. My analysis revealed that the level of the classic pro-inflammatory cytokine TNFα was significantly increased in prostatic tissue from obese mice, thereby indicating an association between PCa development and TNFα level of expression in the prostate. Moreover, my analysis implicated other factors that classically associated with NFκB activation in the regulation of PCa cell – adipocyte interaction in *in vitro* and *in vivo* studies. The *in vitro* study by Herroon et al. (2019) reported that BM adipocytes enhance IL-1β expression and secretion by PCa cells (Herroon et al., 2019). The *in vivo* study performed by Hayashi et al. (2018) detected a significant association between the level of IL-1β and

obesity in prostatic tissue from mice (Hayashi et al., 2018). Due to the lack of sufficient number of studies carried out in this area, my meta-analysis using the present search strategy failed to find any research study that implicates other important pro-inflammatory NFκB -activating factors in the regulation of PCa cell – adipocyte interaction. A good example of this is CD40 ligand (CD40L), a member of the TNF receptor superfamily that is known to activate NFκB and to contribute to the pathogenesis of PCa (Palmer et al., 2004). In models of obesity, CD40L plays a complex role in the regulation of insulin resistance (Poggi et al., 2011, Wolf et al., 2012).

Chronic inflammation is a complex process, and growing evidence have shown that factors that indirectly activate or are produced as a result of NFκB activation, such as IL-6, are upregulated in PCa and adipose tissue of obese mice (Malinowska et al., 2009, Braune et al., 2017). The meta-analysis of *in vivo* study has detected an association between high expression level of IL-6 and obesity in mice model (Hayashi et al., 2018). In contrast, the data from the included human studies by Baillargeon et al. (2006) or Tewari (2013) failed to detect any significant association between the level of IL-6 and high grade and stage PCa progression in patients (Baillargeon, 2006, Tewari et al., 2013).

The expression of key components of the canonical and non-canonical NFκB signalling pathway by PCa cells and adipocytes is important for the development of PCa and obesity (Michael et al, 2002; Inoue et.al. 2007; Tang et al., 2010; Shanmugam et.al. 2013; Jin et al., 2013; Catrysse and van Loo, 2017). My analysis revealed an association between the expression of the canonical p65/RelA NFκB in prostatic tissue and obesity in mice (Vykhovanets et al., 2011). Taken together, these findings confirm the role of NFκB and its related cytokines TNFα, IL-1β and IL-6 in the development of PCa in the obesity models described.

Adipokines that are produced by adipose tissue contributes to the development of inflammation and PCa (Karnati et al., 2017). The meta-analysis conducted in this chapter

revealed few studies that examined the correlation of levels of adiponectin and leptin in patients with PCa. An inverse association between the levels of adiponectin and insulin resistance and risk of cancer have now been extended to PCa. Karnati and colleagues (2017) showed that the serum level of adiponectin is lower in PCa patients when compared with the normal healthy controls and those with BPH (Karnati et al., 2017). This is confirmed by the included two human studies (Baillargeon et al., 2006; Tewarl, 2013) which indicated that the level of adiponectin in serum is lower in patients with high grade and stage PCa.

Conversely, meta-analysis of data from *in vitro* studies revealed that exposure to adiponectin or leptin significantly increases the *in vitro* motility of the castration-insensitive human PC3 cells (Tang and Lu, 2009, Huang et al., 2011). This effect was previously attributed to increased expression of $\alpha 5\beta 1$ or $\alpha v\beta 3$ through the activation of AMPK or Akt, which in turn induces NFκB activation in PCa cells (Tang and Lu, 2009, Huang et al., 2011). The analysis of included *in vivo* studies (Laurent et al., 2016) showed an increase in the expression of leptin in prostatic tissue from obese mice when compared with that from normal mice. However, of the two human studies investigated, there is no significant association between the serum levels of leptin and the progression of PCa (Baillargeon et al., 2006; Tewarl, 2013).

One of the key limitations of the present meta-analysis is the small sample size and number of the included studies. There was also a lack of sufficient studies that examined the expression and activity of NFκB in the progression of PCa to late stages in models of obesity. Thus, in the forthcoming chapters, I plan to investigate the hypothesis that derived factors from mature adipocytes primed with pro-inflammatory NFκB activating cytokines enhances PCa cell behaviour and attempt to provide evidence to support the hypothesis that inhibition of NFκB in high metastatic human or mouse PCa cells may be of value in the management of obesity-driven advanced PCa.

CHAPTER 4

ESTABLISHMENT AND CHARACTERIZATION OF *IN VITRO* MODEL OF MOUSE MATURE ADIPOCYTES

4.1. Summary

Obesity increases the risk of PCa, and a number of studies have reported that systematic and local adipocyte-derived factors enhance the growth of PCa cells in obese patients, animal models and culture (Ribeiro et al., 2010, Parekh et al., 2010, Moreira et al., 2015, Narita et al., 2019). Notwithstanding these findings, the mechanism(s) by which adipocytes influence the metastatic behaviour of PCa cell is poorly understood, and how this process is affected by inflammation has not been thoroughly investigated. In this chapter, I have successfully generated mature adipocytes from mouse pre-adipocytes 3T3-L1 cells to study the crosstalk between adipocyte - PCa cells – macrophages and I used this model to test if and how adipocyte-derived factors influence the *in vitro* growth of a panel of mouse and human PCa cells.

My experiments showed that mouse pre-adipocytes 3T3-L1 cells begun to visibly express characteristic of mature adipocytes that include shaped cell morphology and lipid droplets on day 7. On day 10 and day 12, staining and quantification of lipid droplets confirmed the differentiation of 3T3-L1 pre-adipocytes into mature adipocytes. Furthermore, conditioned medium from 10-day-old mature adipocytes significantly enhanced the *in vitro* growth of a panel of human and mouse PCa cells that include both hormone-dependent human LNCaP and C4-2 and castration-insensitive human PC3 and DU-145 and mouse bone-seeking RM1 cells. Protein microarray analysis of the levels of adipocyte-derived factors in conditioned medium from 10-day old mature adipocytes suggested that these effects were associated with significant increase in the level of adipocyte-derived NFκB-mediated pro-inflammatory mediators, pro-migratory tumour-associated factors, chemokines and adipokines.

Collectively, the results of this chapter showed that mature adipocytes secrete a complex array of pro-inflammatory mediators, pro-migratory tumour-associated factors, chemokines and adipokines that enhance the *in vitro* growth of both hormone-dependent and castration-insensitive PCa cells. Thus, strategies that successfully disrupt adipocyte-PCa cell crosstalk

can be of value in the reduction of the PCa cell growth at both primary and distant metastatic sites such as the skeleton.

4.2. Introduction

Obesity is characterised by significant increase in the number of mature adipocytes in WAT (Vishvanath and Gupta, 2019). Adipocyte differentiation and maturation are accompanied by the accumulation of oil-rich lipid droplets, high level of adipocytokine production and enhanced expression of various adipocyte markers and transcription factors including FABP4, C/EBPβ, δ, and α, PPARγ, ADD1/SREBP1 (Moseti et al., 2016). Excessive adipocyte differentiation is also linked to glucose and insulin sensitivity, which are notably coupled with an increase in the expression of insulin receptor, IRS-1 and GLUT-4 (Yu-Hua et al., 2004, Jackson et al., 2017). A series of other genes and proteins such as fatty acid synthase and glycerol-2-phosphate dehydrogenase have also been implicated in regulation of mature adipocyte differentiation and maturation (Schmid et al., 2005, Klemm et al., 2001).

Obesity increases the risk of PCa aggressiveness and mortality, and a number of studies have shown that local and systemic adipocytokines such as leptin both directly and indirectly enhance the growth of PCa cells (Tokuda et al., 2003; Hsing et al., 2007). Consistently, *in vitro* studies have confirmed that derived factors from adipocytes stimulate the growth and metastatic behaviour of PCa cells (Onuma et al., 2003). Whilst these studies implicate obesity in the progression of PCa, little is known about if disruption of adipocyte – PCa cell interactions affect the metastatic behaviour of PCa cell.

4.3. Aim

The aim of this chapter is to develop an *in vitro* model to study adipocyte – PCa cell – macrophage crosstalk, and to use this model of mature adipocytes and their precursors to confirm if and to demonstrate how and which adipocyte-derived pro-inflammatory mediators influence the growth of PCa cells *in vitro*.

These aims were achieved by:

- successful generation of mature adipocytes from mouse 3T3-L1 pre-adipocytes
- identification, characterization and quantification of the differentiation of mature adipocytes *in vitro*
- testing effects of adipocyte-derived factors on the viability of mouse and human PCa cells *in vitro*
- quantification of levels of adipocyte-derived NFκB-mediated pro-inflammatory cytokines, pro-migratory tumour-associated factors, chemokines and adipokines in conditioned medium from mature adipocytes.

4.4. Results

4.4.1. Successful in vitro generation of mature adipocytes

In this chapter, mature adipocytes were first generated from the mouse pre-adipocyte cells 3T3-L1 as described in the Material and Methods (section 2.2.1.1). These cultures were cultured in adipocyte differentiation medium (1µg/ml insulin, 0.5mM IBMX, 1µM DEX) and characterized as described in figure 23. Adipocyte maturation was visualised, confirmed and quantified using Oil Red O staining (section 2.4.). The quantification of Oil Red O staining was optimised and absorbance was measured with optical density (OD) of 492nm. The presence of mature adipocytes in mouse 3T3-L1 pre-adipocyte cells was first detected on day 7. As seen in figure 24, adipocyte maturation was significantly increased by 1.5-fold on day 7, as compared to OD in day 0 ($p<0.01$). A further 6- and 12-fold increases in OD were achieved in day 10 and 12, respectively ($p<0.0001$). Representative images of mature adipocytes that show spindle-shaped cell morphology and accumulation of intracellular lipid vesicles from the experiments described are shown in figure 25.

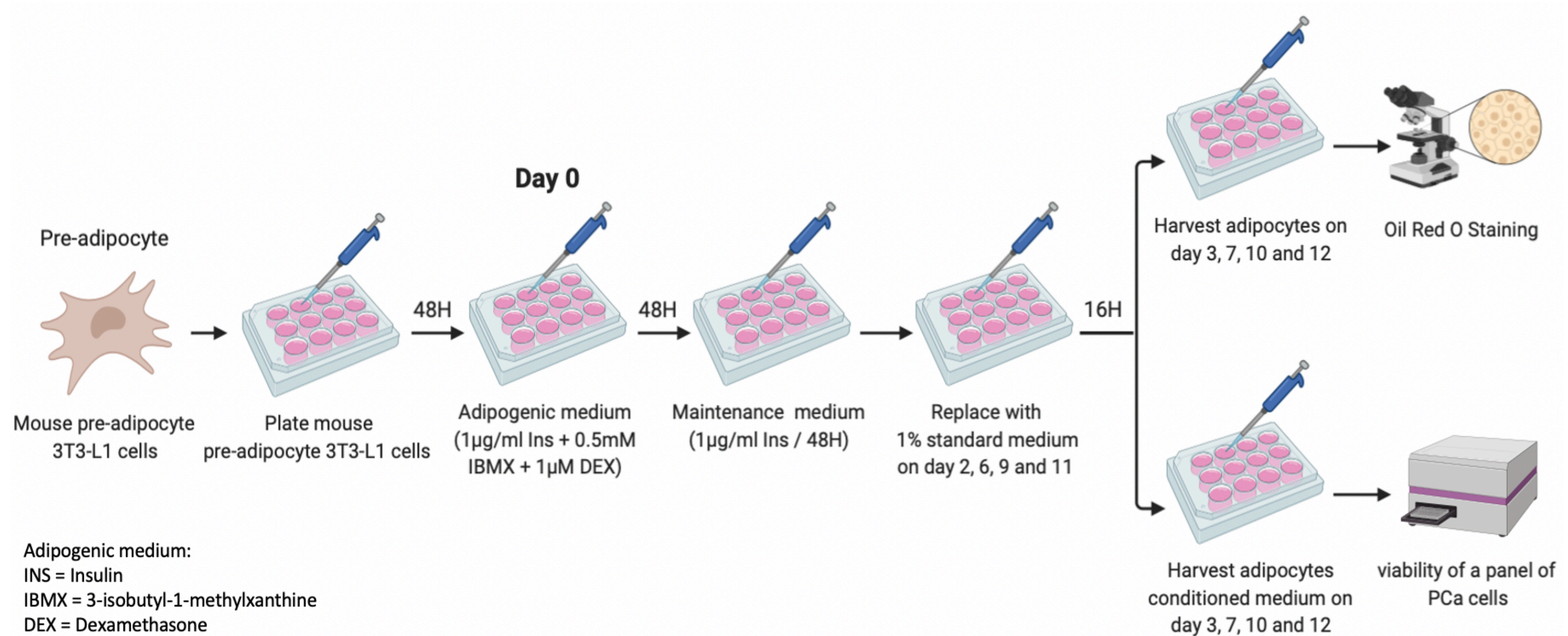


Figure. 23. Schematic of generation and characterisation of mature adipocytes.

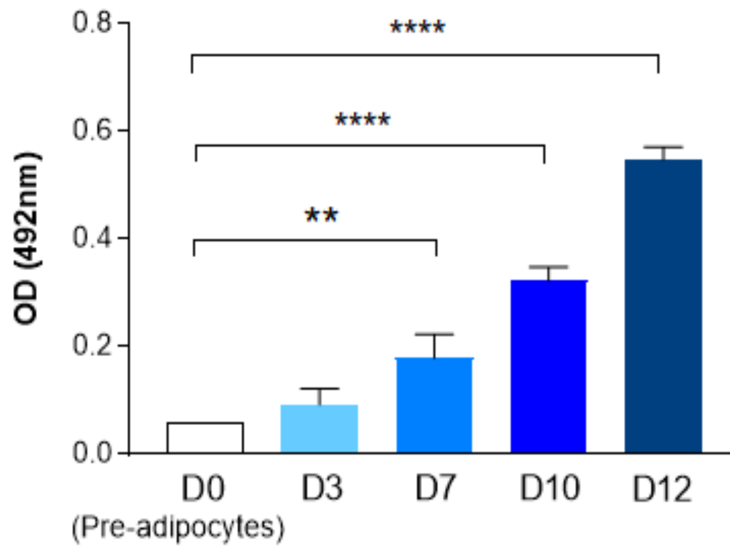


Figure. 24. *The in vitro differentiation of mature mouse adipocytes*. *Quantification of oil red staining in mouse 3T3-L1 cells cultured in the presence of adipocyte differentiation medium for indicated period. Oil red staining and de-staining was performed as described in Materials and Methods (section 2.4). Results are from three independent experiments (N = 3). Data were analysed using one-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

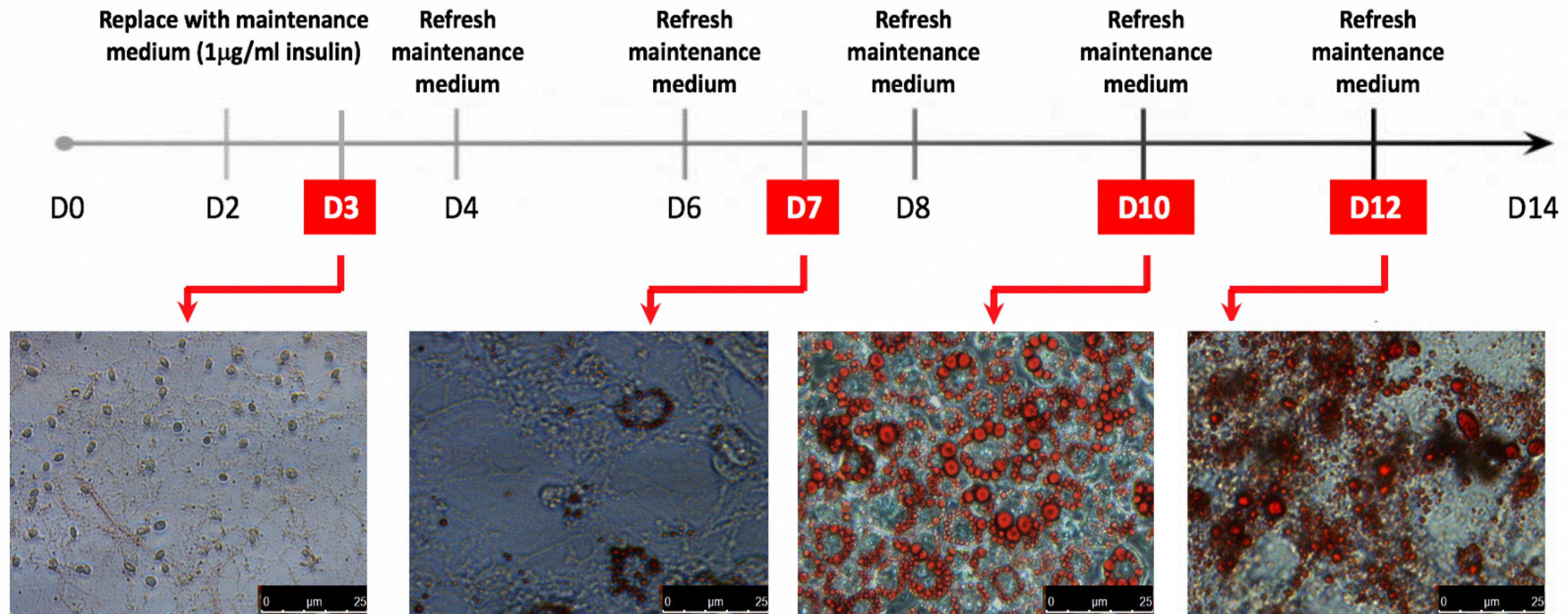


Figure. 25. Timeline of differentiation and maturation of mouse adipocytes. Mouse 3T3-L1 cells were cultured in adipocyte differentiation medium (section 2.2.1.1) and mature adipocytes were identified by oil red staining as described in materials and methods.

4.4.2. Adipocyte-derived factors enhance the in vitro growth of prostate cancer cells

Previous studies have reported that primary PCa tumour growth is enhanced in obese mice and individuals (Narita et al., 2019). In this chapter, I used my model of mature adipocytes and their precursors (figures 23 – 25) to examine the effects of adipocytes-derived factors in conditioned medium on the growth of the androgen dependent primary human LNCaP cells. Conditioned medium was collected from pre- and mature adipocytes under different conditions after 3, 7, 10 and 12 days in culture. Cell number was measured by AlamarBlue assay as described in section 2.2.8.

My results showed that exposure of human LNCaP cells to conditioned medium from 10- but not 3-, 7- and 12-day-old mature adipocytes (Mature adipocyte ^{CM}, 20% v/v) significantly increased cell number, compared to cultures treated with control medium (CTRL ^{CM}, 20% v/v) (Figure 26). Figure 27 shows representative images of LNCaP cells after 24 hours in presence of adipogenic conditioned medium collected from mature 3T3-L1 adipocytes after 10 days in culture.

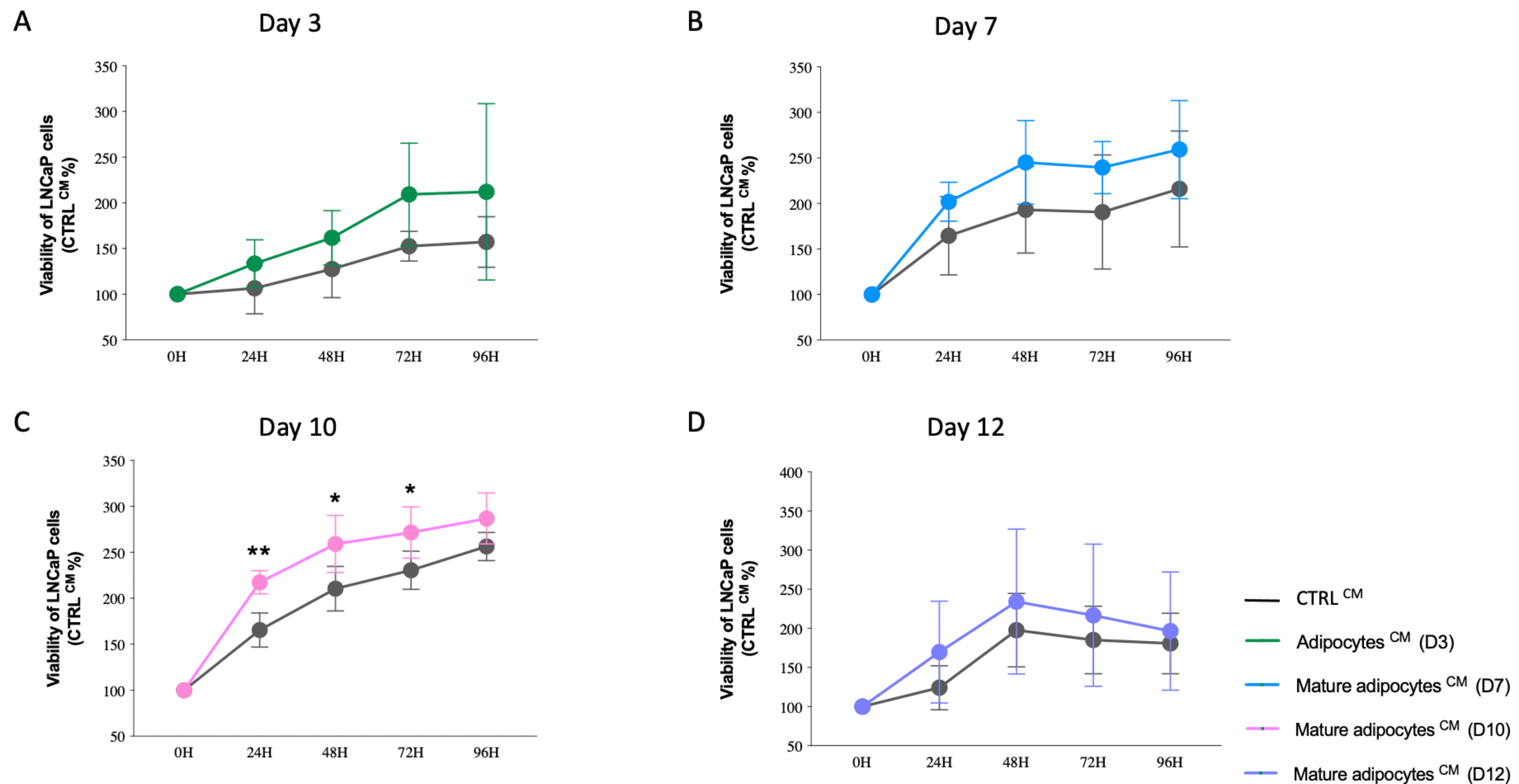


Figure. 26. **Adipocyte-derived factors from day 10 enhanced the growth of human LNCaP PCa cells *in vitro*.** LNCaP cells were plated in 96-well plates and after 24 hours 1% DMEM medium was replaced with adipogenic conditioned medium collected from D3, D7, D10 and D12. Cell viability was assessed every 24hrs (24-96hrs) by Alamar Blue. Results are from three independent experiments (N=3). Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ±SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

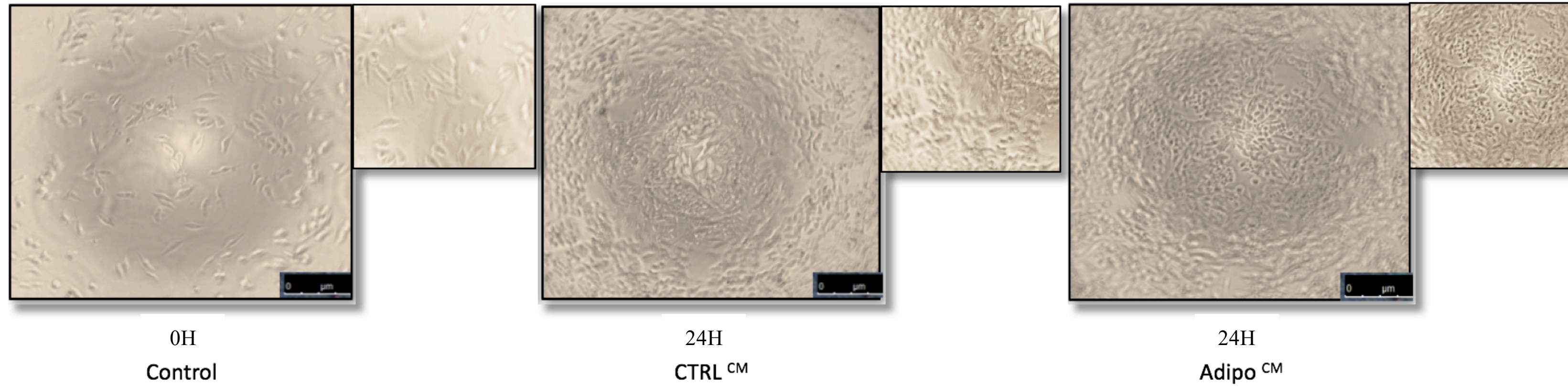


Figure. 27. Representative images of LNCaP cells after 24 hours in the presence of D10s obtained from 3T3-L1 cultured in standard DMEM and adipose medium for 10 days. Magnifications is 20x. Conditioned medium used 20% v/v.

Next, I tested the effects of conditioned medium from mature adipocyte (Adipo ^{CM}, 20% v/v) obtained from 10-day-old adipocytes on the growth of a panel both hormone-dependent and castration- resistant human and mouse PCa cell lines with different abilities to metastasise to organ such as lymph nodes and the skeleton. The list includes the metastatic sub-clone of human LNCaP, C4-2, and the castration-resistant human DU145 and PC3 PCa cells and mouse osteotropic RM1 PCa cells.

As shown in figure 28 (panel A), the addition of adipogenic conditioned medium (Adipo ^{CM}, 20% v/v) significantly increased the growth of hormone-dependent human LNCaP PCa cells by 72.8% (24 hours), 102.8% (48 hours), 76.8% (72 hours) and 63.8% (96 hours) respectively when compared to control group (CTRL ^{CM}, 20% v/v) ($p<0.05$). In contrast, the cultures exposed to conditioned medium that collected from mature adipocytes (Adipo ^{CM}, 20% v/v) failed to affect the cell growth of hormone-dependent C4-2 PCa cells as when comparing the cultures of CTRL ^{CM} (20% v/v) (Figure 28, panel B).

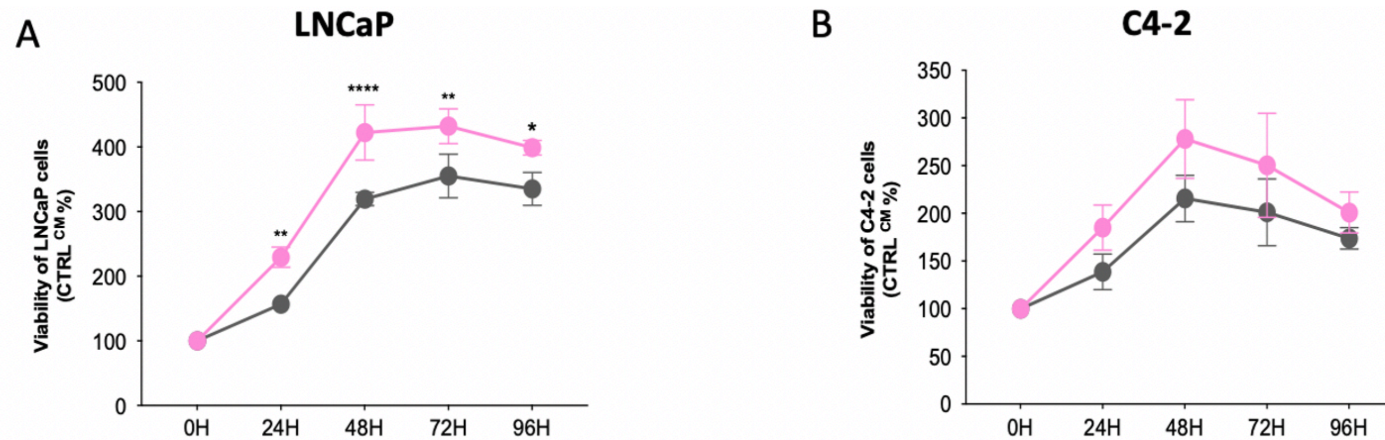


Figure. 28. **Derived factors from mature adipocyte enhanced the growth of hormone-dependent human PCa cells *in vitro*.** Hormone-dependent human LNCaP and C4-2 PCa cells were plated in 96-well plates and after 24 hours standard DMEM medium was replaced with either adipogenic conditioned medium collected from D10 or control treatment. Cell viability was assessed every 24hrs (24-96hrs) by AlamarBlue. Results are from three independent experiments (N=3). Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ±SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Furthermore, the viability in cultures exposed to adipogenic conditioned medium (Adipo ^{CM}, 20% v/v) for 72 hours indicated that castration-insensitive human DU145 cells showed a significant increase in growth (2.1-fold increase, $p<0.05$) (Figure 29, panel A). Additionally, the addition of adipogenic conditioned medium (Adipo ^{CM}, 20% v/v) increased the growth of castration-insensitive human PC3 PCa cells tested by 2.2-fold, 1.9-fold and 1.8-fold after 48, 72 and 96 hours respectively when compared to control group (CTRL ^{CM}, 20% v/v) ($p<0.05$) (Figure 29, panel B). Similarly, the conditioned medium obtained from mature adipocytes (Adipo ^{CM}, 20% v/v) showed a significant increase in the growth of mouse osteotropic RM1 PCa cells by 2.9-fold after 72 hours when compared to control group (CTRL ^{CM}, 20% v/v) ($p<0.05$) (Figure 29, panel C).

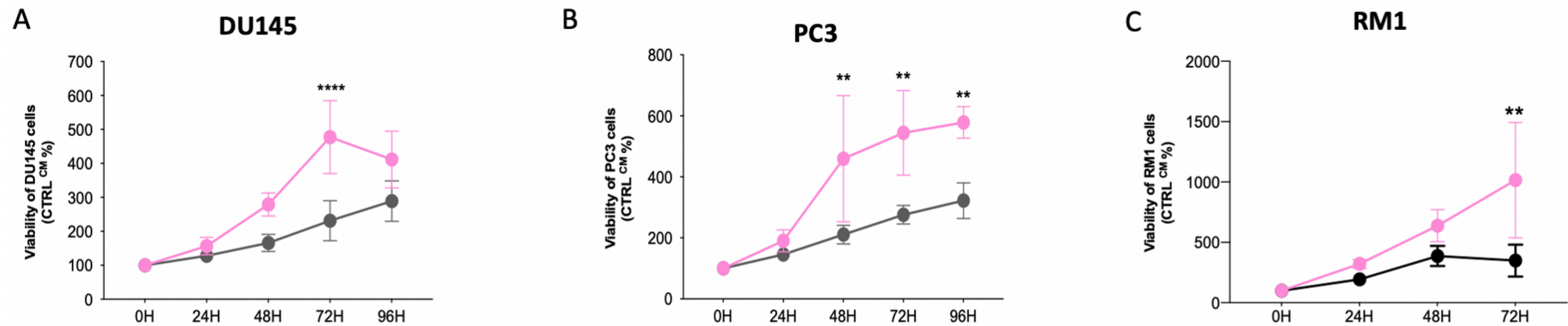


Figure. 29.Derived factors from mature adipocyte enhanced the growth of human castration-insensitive and mouse osteotropic PCa cells *in vitro*. Human castration-insensitive DU145 and PC3 and mouse osteotropic RM1 PCa cells were plated in 96-well plates and after 24 hours standard DMEM medium was replaced with either adipogenic conditioned medium collected from D10 or control treatment. Cell viability was assessed every 24hrs (24-96hrs) by Alamar Blue. Results are from three independent experiments (N=3). Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ±SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Further analysis of cell viability in cultures exposed to adipogenic conditioned medium (Adipo ^{CM}, 20% v/v) for 72 hours revealed that the highly metastatic mouse RM1 PCa cells exhibited the significant increase in growth by 2.4-fold when comparing with the growth of hormone-dependent LNCaP PCa cells ($p<0.05$) (Figure 30). However, the comparative analysis of the growth of a panel of PCa cells showed that exposure to conditioned medium from the mature adipocytes equally enhanced growth of hormone-dependent human C4-2 and castration-insensitive DU145 and PC3 PCa cells when compared to growth of human LNCaP cells (Figure 30).

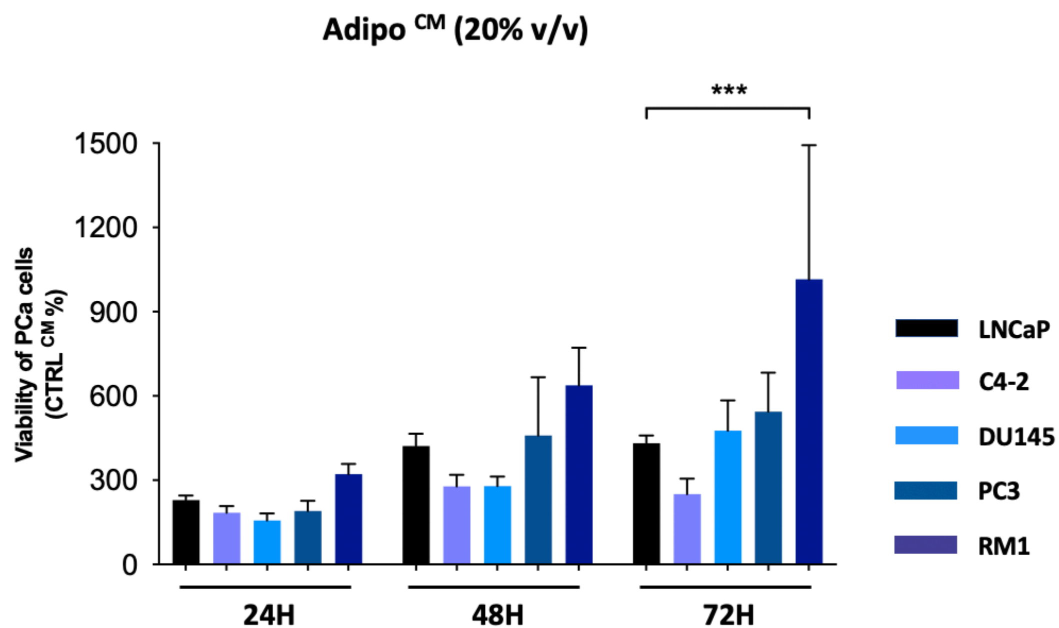


Figure. 30. Derived factors from mature adipocyte significantly improved the growth of mouse osteotropic RM1 cells in vitro when comparing to the growth of human LNCaP cells after 72 hours *in vitro*. A panel of PCa cells were plated in 96-well plates and after 24 hours standard DMEM medium was replaced with either adipogenic conditioned medium collected from D10 or control treatment. Cell viability was assessed by AlamarBlue. Results are from three independent experiments (N=3). Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ±SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.4.3. Mature mouse 3T3-L1 cells increased expression level of pro-inflammatory factors

To explore the mechanism by which adipocyte-derived factors affect the *in vitro* growth of PCa cells, I utilized protein micro-array analysis to identify and quantify the levels of the adipocyte-derived factors expressed in the conditioned medium from mature adipocytes and their precursors. Using the Mouse XL Cytokine Array Assay, I analysed the levels of 111 pro-inflammatory cytokines, growth factors and soluble proteins that are known to regulate PCa and adipocytes.

First, I detected significant increase in the expression level of the adipokines adiponectin and resistin ($p<0.001$) in conditioned medium from mature mouse 3T3-L1 adipocytes when compared with the control group (Figure 31, panel A). My analysis also showed a significant increase in the expression level of the pro-tumour factors pentraxin-3 and Lipocalin-2 ($p<0.001$) in conditioned medium from mature mouse 3T3-L1 adipocytes when compared with the control group (Figure 31, panel B).

Maturation of mouse 3T3-L1 pre-adipocytes also significantly enhanced the expression level of a panel of pro-inflammatory cytokines and angiogenesis in conditioned medium ($p<0.05$). The list includes M-CSF, VCAM-1, Chitinase-3-like protein 1 and various members of the CCL and CXCL families of chemokines (figure 32, panel A). Additionally, as can be seen in figure 32 (panel B), a number of angiogenic factors most notably VEGF, HGF, IGFBP-6, coagulation factor III and members of the MMP family of proteases, namely MMP-2, -3 and -9 increased in the conditioned medium collected from mature mouse adipocytes.

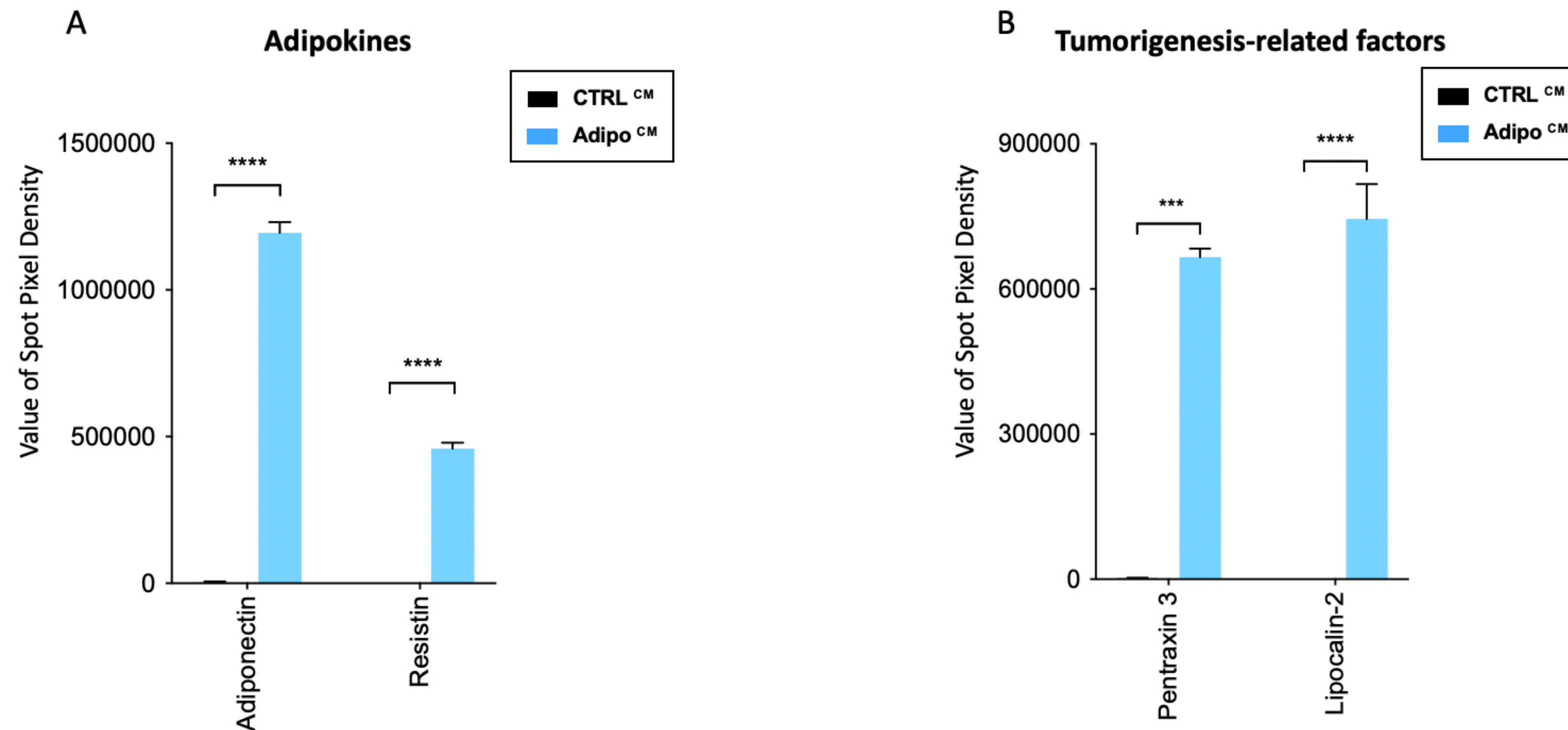


Figure. 31. Mature adipocytes conditioned medium expressed high levels of adipokines and tumorigenesis-related factors. Two membrane-based cytokine array assays, with capture antibodies for relative expression levels of soluble proteins that are spotted in duplicate on the membrane, were incubated with Adipo ^{CM} or control group (1% standard medium) for 24 hours, before undergoing chemiluminescence detection and analysis. Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ±SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

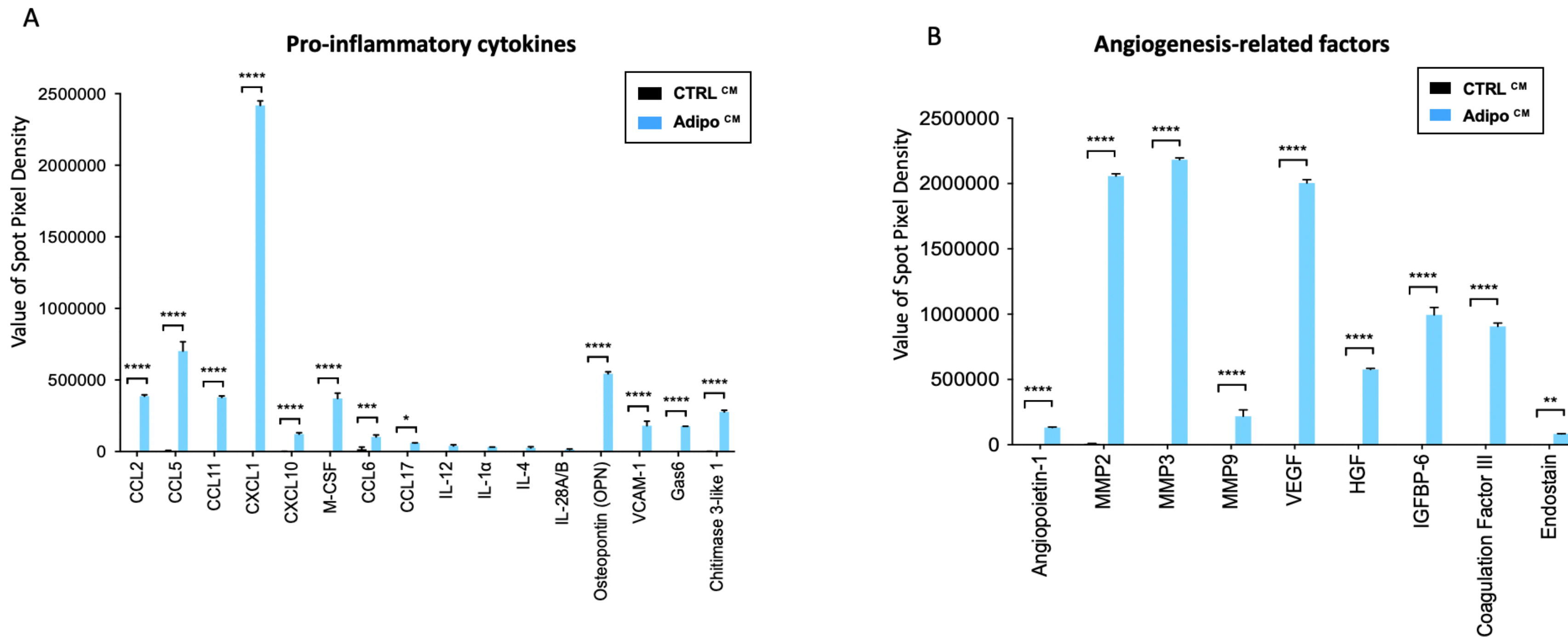


Figure. 32. Mature adipocytes conditioned medium expressed high levels of pro-inflammatory cytokines and angiogenesis-related factors. Two membrane-based cytokine array assays, with capture antibodies for relative expression levels of soluble proteins that are spotted in duplicate on the membrane, were incubated with Adipo ^{CM} or control group (1% standard medium) for 24 hours, before undergoing chemiluminescence detection and analysis. Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ±SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Further detailed evaluation of the role of multiple cytokines, chemokines and soluble proteins in mature mouse 3T3-L1 adipocytes revealed a subset of proteins that are likely to be implicated in the regulation of the adipocyte-induced PCa cell viability that I observed in my models. The effects of adipogenesis on the production of soluble factors was expressed as ratio of control group (CTRL ^{CM}), and adipocyte-derived soluble factors that were considered to be differentially regulated with adipogenesis were shown in figure 33. Supplementary table 1 showed the summary of differentially regulated factors produced by mature mouse 3T3-L1 adipocyte based on the raw data from the protein micro-array assay.

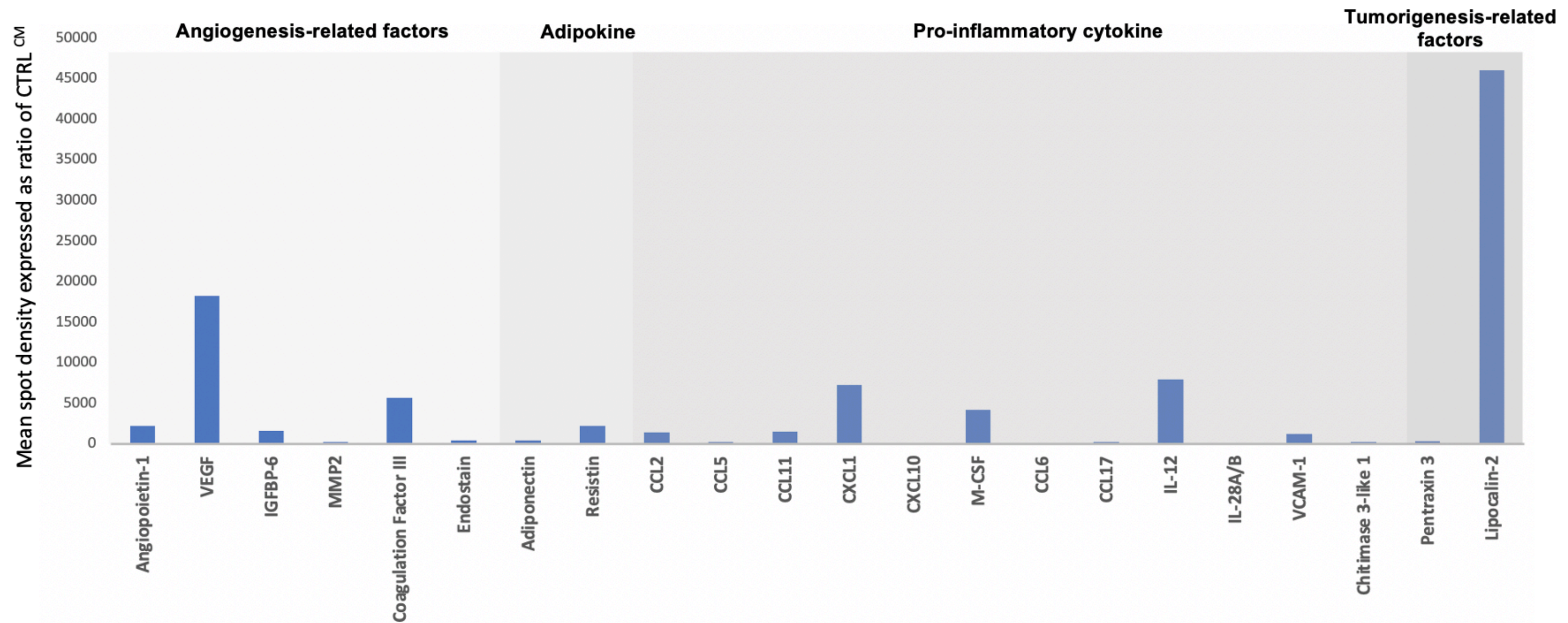


Figure. 33. **Adipogenesis increased pro-inflammatory cytokines and soluble proteins expression levels.** Microarray analysis of soluble factors in conditioned medium from mature mouse 3T3-L1 adipocytes using Proteome Profiler ™ Mouse XL Cytokine Array Kit (R&D systems).

4.5. Discussion

Obesity and adipogenesis have many implications for human diseases, in particular cancer (Divella et al., 2016). Thus, the study of adipocyte development and their role in the regulation of growth and metastatic behaviour of cancer cells have become an area of intense research in recent years. Studies have shown that tumour growth is enhanced in obese individuals and animals, and adipocyte-derived factors enhance the growth of PCa cells in culture (Narita et al., 2019).

In this chapter, I developed an *in vitro* model to study the *in vitro* interactions between adipocyte and PCa cells, and to attempt to examine the mechanism(s) by which adipocytes influence the behaviour of PCa cells with different growth and metastatic abilities. Using my model, I successfully generated mature adipocytes *in vitro*, and I observed their presence in cultures of the mouse pre-adipocytes 3T3-L1 as early as 7 days. Differentiation of pre-adipocytes was initiated by adipogenic medium containing 1µg/ml insulin, 0.5mM IBMX and 1µM DEXA. In my model, insulin was essential for the generation of mature adipocytes. Insulin is a potent adipogenic hormone that plays a key role in regulating the late stages of adipocyte differentiation by inducing glucose and free fatty acid uptake, inhibiting lipolysis and stimulating fatty acid synthesis in adipocytes (Cignarelli et al., 2019). At the molecular level, insulin induces the expression of key regulators of adipocyte differentiation, particularly PPARγ and GLUT4 (Moseti et al., 2016, Jackson et al., 2017), and it triggers the regulation of various transcription factors that include SREBP-1c, p21 and Rho-A (Draznin et al., 2001, Klemm et al., 2001, Laviola et al., 2006).

In agreement with previous published methods, the quantification and follow up analysis of mature adipocytes in my model showed that DEX and IBMX increased and accelerated the differentiation rate of mature adipocytes. This effect was likely due to their ability to enhance the expression of adipocyte-specific gene, in particular PPARγ and C-EBPβ, as previously suggested (Siersbæk et al., 2010). PPARγ agonists are known to enhance adipocyte

differentiation and accelerate their maturation (Akinyeke and Stewart, 2011). In my model, however, I had chosen to refrain from using the PPARγ agonist troglitazone, as previously suggested (Akinyeke and Stewart, 2011). My preliminary work and previous unpublished studies from our group (Kubar, MSc Dissertation, and Idris and AL-Jeffery. personal communication) have noted that troglitazone enhanced the total number of cells in culture to the level that resulted in detachment of the cell monolayer. Furthermore, previous studies by other investigators have shown that troglitazone decreased the growth of human PCa cells *in vitro* and *in vivo* (Kubota et al., 1998, Elisabetta et al., 2000). This is important as it was proposed to expose PCa cells to conditioned medium from mature adipocytes in my model. The protocol used in my model had helped with successfully maintaining mature adipocytes in culture for an extra 5 days, without detachment. As predicted, I observed significant increase in the number of mature adipocytes in culture that was accompanied by marked accumulation of lipid droplets after 10 and 12 days of culture.

There is a growing body of evidence indicating that obesity increases mortality in patients of various cancers, including PCa (Vidal et al., 2017). The interaction between adipocytes and PCa cells plays an important role in the prostatic growth tumour cells and a number of *in vitro* studies have shown that the proliferation of PCa cells (highly metastatic human PC3 and mouse RM1 cells) is significantly increased when cocultured with adipocytes, implying that factors produced by adipocyte enhance the growth of PCa cells (Tokuda et al., 2003, Moreira et al., 2015). My results in the present chapter confirmed these findings by showing that conditioned medium from mature 10-day-old 3T3-L1 adipocytes significantly enhanced the growth of a panel of human hormone-dependent and castration-insensitive human and mouse PCa cells, regardless of their growth and metastatic capabilities. These findings are in broad agreement with previous that have shown that adipocyte-derived factors enhance PCa cell growth (Onuma et al., 2003, Tokuda et al., 2003, Moreira et al., 2015, Gao et al., 2015). In my model, however, adipocyte-derived factors enhanced the growth of the human LNCaP at an earlier time point when compared to castration-insensitive PC3, implying that hormone-

dependent, less metastatic PCa cells are relatively sensitive to the pro-growth effect of adipocyte-derived factors.

PCa patients with bone metastasis experience skeleton related events (Nørgaard et al., 2010). In elderly individuals, adipose tissue constitutes a major component of the bone marrow and visceral organ (Bani Hassan et al., 2018, Mancuso and Bouchard, 2019). Hence, studies that examine the effects of adipose tissue on the growth and spread of highly metastatic PCa cell might be useful for identifying treatment strategies for PCa with bone metastasis. In preclinical study, PCa cell proliferation and motility of the androgen-insensitive and bone-seeking RM1 PCa cells is significantly increased in co-culture with pre-adipocytes and adipocytes conditioned medium (Moreira et al., 2015). My experiment confirmed these findings and showed that the castration-insensitive mouse RM1 PCa cells were indeed more sensitive to the pro-growth effects of adipocyte-derived factors than the hormone-dependent LNCaP and its relatively more metastatic clone C4-2. It is important to note here that all three hormone-insensitive PCa cell lines used in the present study are known to readily metastasise to and grow in distant organs such as the brain, lungs and the skeleton and to cause osteolytic bone damage (Stone et al., 1978, Kaighn et al., 1979, Power et al., 2009). Thus, these effects can be attributed in part to the plethora of systemic growth factors and hormones such as epidermal growth factor (EGF), VEGF and TGF-α or -β that promote the growth of PCa cells (Hellawell and Brewster, 2002).

Obesity is a complex process and it often attributed to altered serum levels of systemic hormones such as estrogen, insulin, IGF-1 or adipokines and various pro-inflammatory cytokines and chemokines (Coppack, 2001, Xu et al., 2003, Weisberg et al., 2003, Freedland and Aronson, 2004, G Paz-Filho 2011, Donohoe et al., 2011). However, little is known about the underlying mechanism by which adipocyte-derived factors affect the behaviour of aggressive and highly metastatic PCa cell remains poorly understood. In this chapter, I utilized protein microarray analysis to measure and identify the adipocyte-derived factors present in the conditioned medium that enhanced the *in vitro* growth of a panel of PCa cells. First, the

present microarray analysis confirmed that conditioned medium from mature adipocytes contains high level of adiponectin and detected significant levels of resistin, two important regulators of adipogenesis and obesity (Fu et al., 2005, Ikeda et al., 2013). The present microarray analysis has also detected significant levels of pro-tumorigenic and pro-angiogenesis factors that include VEGF and various MMPs and chemokines in conditioned medium collected from mature adipocytes when compared to control cultures. This finding is in agreement with previous studies that reported that VEGF enhance the migration of the metastatic DU145 and PC3 cells (Maeda et al., 1997), and implicates provide a mechanism by which adipocyte may enhance the *in vitro* motility of metastatic PCa cells. Furthermore, the fact that VEGF plays an important role in the regulation of angiogenesis and is known to induce tumour vascularization (Carmeliet, 2005) is likely to play a role in the enhanced tumour growth and metastasis that previously reported *in vivo* and in obese individuals (Armanini et al., 1993, Weidner et al., 1993, Gasparini et al., 1997), however further research is needed. My studies have also detected significant increase in the levels of a number of pro-inflammatory cytokines and chemokines in conditioned medium from mature adipocytes, thereby implicating inflammation in the regulation of adipocyte – PCa cell interactions in my models. Factors such as lipocalin 2 and pentraxin 3, detected by the present protein microarray analysis, predict poor prognosis in patients with PCa (Stallone et al., 2014, Ulusoy et al., 2021).

In summary, the results of this chapter showed that mature adipocytes secrete a complex array of adipocyte-specific pro-inflammatory mediators, pro-migratory tumour-associated factors, chemokines and adipokines that enhance the *in vitro* growth of hormone-dependent and castration-insensitive PCa cells. This implies that strategies that disrupt adipocyte-PCa cell crosstalk can be of value in the treatment of PCa in obese patients. In next chapter, I tested the hypothesis that exposure of adipocytes to pro-inflammatory mediators enhances their ability to regulate the motility of highly aggressive and metastatic PCa cell.

CHAPTER 5

THE EFFECTS OF THE PRO-INFLAMMATORY CYTOKINE TNFα ON THE ABILITY OF ADIPOCYTES TO INFLUENCE PROSTATE CANCER CELL METASTATIC BEHAVIOUR *IN VITRO*

5.1. Summary

Obesity is associated with chronic inflammation and a number of studies have detected high levels of circulating pro-inflammatory NFκB-activating mediators such as TNFα and IL-6 in obese patients. Chronic inflammation also contributes to PCa development, and levels of cytokines and chemokines are elevated in obese cancer patients. My studies in chapter 4 have shown that adipocyte-derived factors enhance the growth of PCa, and protein microarray analysis of conditioned medium from mature adipocytes detected elevated levels of a complex array of factors that include pro-inflammatory mediators. Together, these findings implicate inflammation in the regulation of adipocyte – PCa cell interactions in the model described.

TNFα is one of the most important mediators of inflammation. In this chapter, I used TNFα to evaluate the role of inflammation on the ability of mature adipocytes to affect the metastatic behaviour of PCa cell *in vitro*. My experiments showed that conditioned medium obtained from mature adipocytes primed with TNFα had no effects on the *in vitro* growth of a panel of human and mouse hormone-dependent and castration-insensitive PCa cells, when compared to unstimulated control group. In contrast, TNFα enhanced the ability of mature adipocytes to increase the *in vitro* invasion of castration-insensitive human PC3 and mouse RM1, but not hormone-dependent LNCaP PCa cells. Protein microarray analysis of pro-inflammatory factors in conditioned medium from mature adipocytes primed with TNFα showed that these effects were associated with enhanced levels of NFκB-mediated pro-inflammatory mediators, pro-migratory factors, chemokines and adipokines.

Collectively, the results of this chapter indicated that pro-inflammatory response, induced by TNFα, enhanced the ability of mature adipocytes to increase the motility of highly metastatic PCa cells, by a mechanism dependent, at least in part, on increased expression of a complex network of pro-inflammatory mediators, tumour-associated factors, chemokines and adipokines.

5.2. Introduction

Obesity is associated with chronic inflammation and a previous study has detected that high levels of circulating pro-inflammatory mediator TNFα and IL-6 in obese patients (Tzanavari et al., 2010, Eder et al., 2009). Furthermore, TNFα enhances obesity-related insulin resistance in animal models and humans (Hotamisligil et al., 1993, Hotamisligil et al., 1995). Pro-inflammatory cytokines also influence the ability of adipocyte to proliferate, mature and influence other cells in their microenvironment (Coppack, 2001, Xu et al., 2003, Weisberg et al., 2003).

Growing evidence implicates chronic inflammation in the development and progression to advanced metastatic disease of various cancers, including PCa (Baillargeon et al., 2006, Mistry et al., 2007, Taverna et al., 2015). For example, a number of pro-inflammatory NFκB-activating cytokines such as IL-1β, IL-6 or TNFα are upregulated in PCa, and pro-inflammatory cytokines released from adipose tissue influence the development and progression of PCa (Okamoto et al., 1997, Michalaki et al., 2004, Baillargeon, 2006, Mistry et al., 2007). My meta-analysis indicated that IL-1β, IL-6 or TNFα play a role in the interactions between PCa – adipocyte, and in chapter 4, significant levels of a number of pro-inflammatory cytokines and chemokines were detected in conditioned medium from mature adipocytes. This further implicates inflammation in the regulation of adipocyte – PCa cell interactions.

TNFα is a critical mediator of cancer associated chronic inflammation (Szlosarek & Balkwill, 2003). TNFα presents in the tumour microenvironment and various studies have shown that it is a key mediator of chronic inflammation in the tumour microenvironment and adipose tissues (Hotamisligil et al., 1993, Landskron et al., 2014). Increasing evidence indicated that TNFα is tumour-promoting factor, and is linked to all steps of tumorigenesis, including transformation, invasion and metastasis in many cancers (Wang and Lin, 2008). Studies have reported that TNFα facilitates metastasis via driving epithelial-mesenchymal transition (Bates and Mercurio, 2003, Takahashi et al., 2010, Yamauchi et al., 2010), and as a result

contributing to the progression of prostate carcinoma form an androgen sensitive to CPRC (Diaz Jr et al., 2011).

5.3. Aims

Adipocytes regulate the growth of PCa cells but the effects of pro-inflammatory mediators on the ability of adipocyte to enhance PCa metastasis and spread is poorly understood. The aim of this chapter is to evaluate the role of inflammation on the ability of mature adipocytes to affect the in vitro motility and invasiveness of human and mouse PCa cells (Figure 34).

These aims were achieved by examining:

- effects of conditioned medium collected from mature adipocytes primed with TNFα on human and mouse PCa viability, migration and invasion *in vitro*
- the regulation of differentially expression of adipokines and pro-inflammatory cytokines that produced by conditioned medium from the pre-exposure of mature adipocytes to TNFα

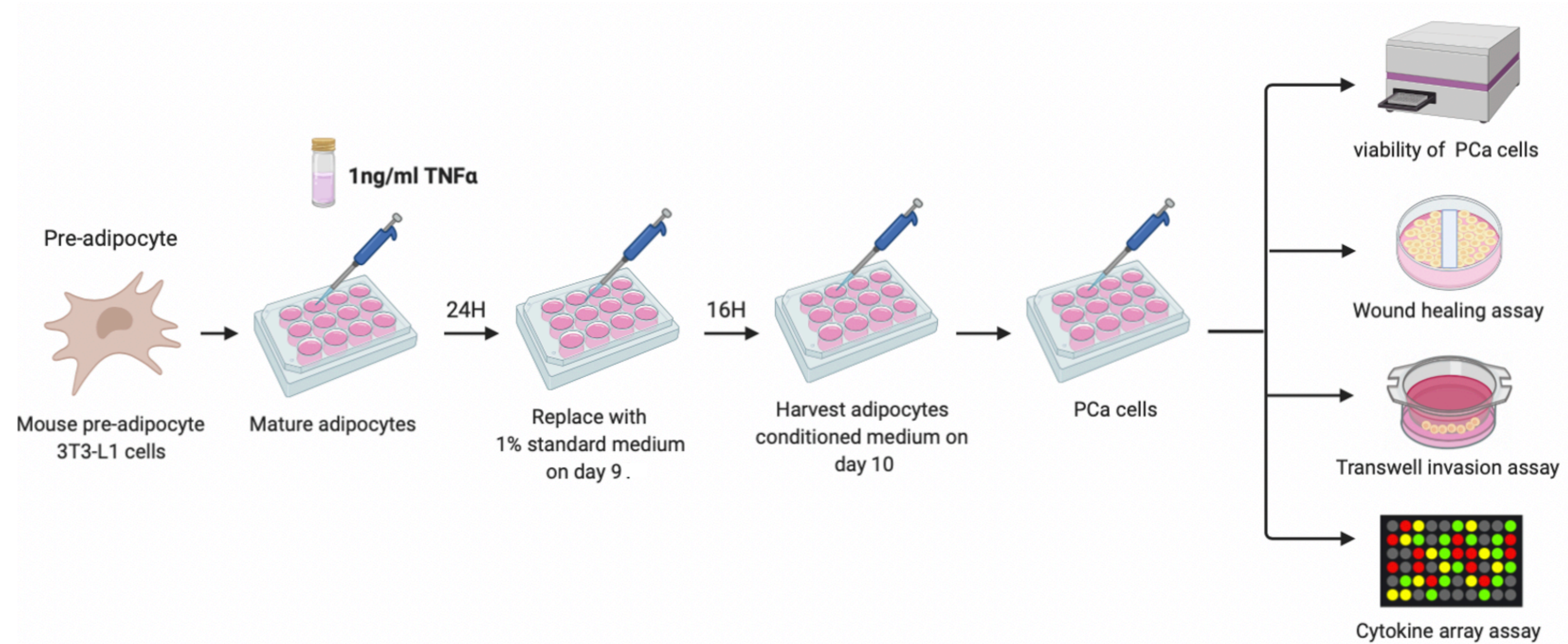


Figure. 34. **Schematic diagram of investigation of factors derived from mature mouse 3T3-L1 adipocytes primed with pro-inflammatory cytokine TNFα.**

5.4. Results

5.4.1. Pre-exposure of mature adipocytes to TNFα failed to enhance their ability to affect the in vitro growth of prostate cancer cells

Among pro-inflammatory cytokines, TNFα, is considered one of the most important mediators of cancer associated chronic inflammation (Szlosarek & Balkwill, 2003). In this chapter, I first examined the effects of conditioned medium from mature 3T3-L1 adipocytes stimulated by 1ng/ml TNFα (Adipo $^{CM/TNF\alpha}$, 20% v/v) on the growth of panel of PCa cells. Cell growth was measured by AlamarBlue assay as described in section 2.2.8. The TNFα concentration of 1ng/ml was chosen on the basis of previous study that have demonstrated that 2-5ng/ml TNFα completely prevented adipogenesis of mouse 3T3-L1 preadipocytes whilst 1ng/ml TNFα was less effective at inhibiting lipid accumulation (Cawthorn et al., 2007).

As shown in figure 35, conditioned medium collected from mature 3T3-L1 adipocytes treated with or without TNFα (1ng/ml) (section 2.2.6.2) were equally effective in enhancing the in *vitro* growth of the hormone-dependant human LNCaP and their metastatic clone C4-2 and castration-insensitive human DU145 and PC3 and mouse bone-seeking RM1 PCa cells.

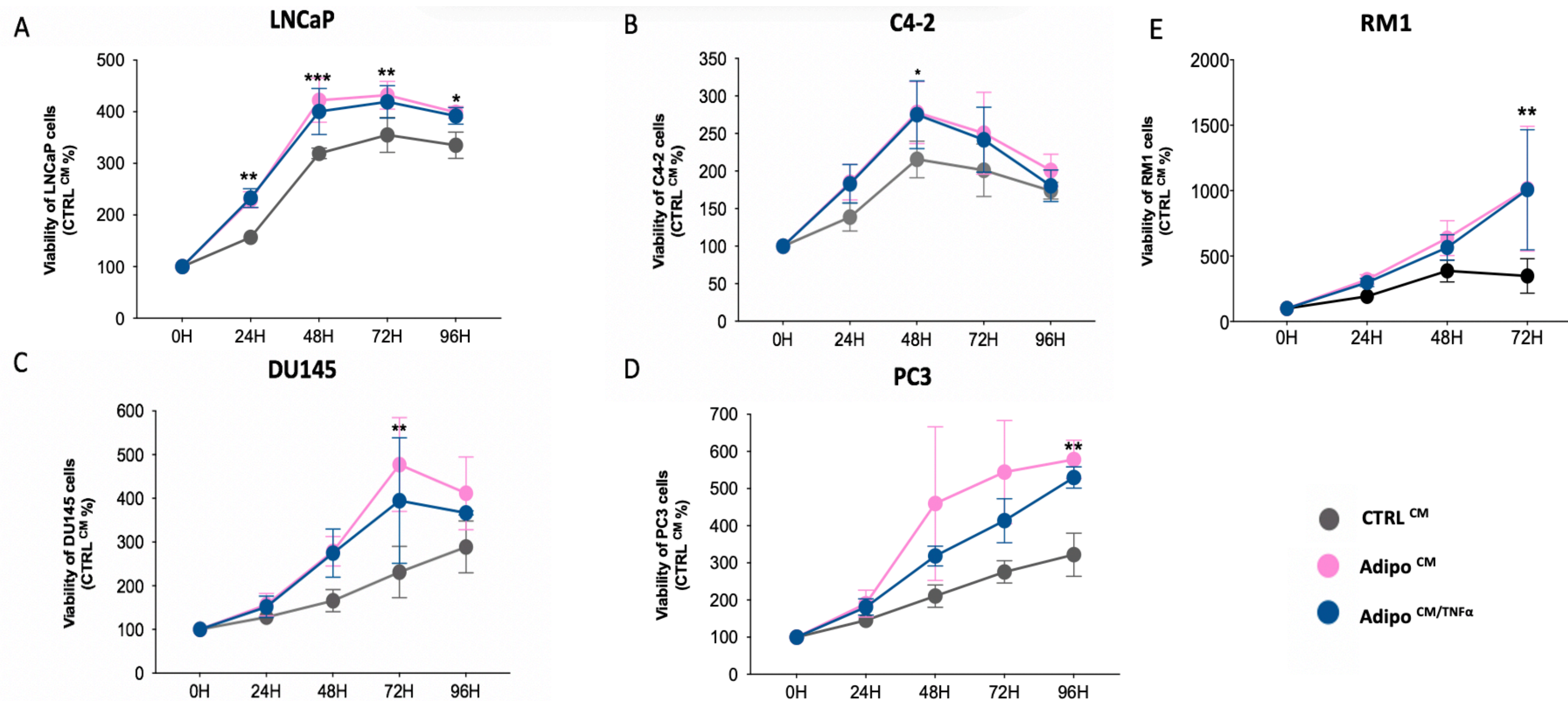


Figure. 35. TNFα failed to enhance the ability of mature adipocytes to increase the growth of PCa cells *in vitro*. Human LNCaP and C4-2, DU145 and PC3 and mouse RM1 PCa cells were plated in 96-well plates. Cell viability was assessed by AlamarBlue. Results are from three independent experiments (N=3). Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (*: Adipo $^{CM / TNF\alpha}$ vs CTRL ^{CM}).

5.4.2. Pre-exposure of mature adipocytes to TNFα enhanced their ability to stimulate the in vitro migration of human prostate cancer cells

Previous studies have reported that TNFα plays a role in promoting PCa motility (Lei et al., 2012). In this chapter, I investigated this further by examining the effects of factors derived from the mature 3T3-L1 adipocytes primed with pro-inflammatory cytokine TNFα on the migration of the hormone-dependent human LNCaP and castration-insensitive PC3 PCa cells. Furthermore, I also tested the effects of TNFα on the migration of hormone-dependent human LNCaP and castration-insensitive PC3 PCa cells in the absence of mature adipocytes or their derived factors. PCa cell migration was measured by wound healing assay as described in section 2.2.9.1.

As shown in figure 36, factors secreted in conditioned medium from mature 3T3-L1 adipocytes that were stimulated by 1ng/ml TNFα (Adipo $^{CM/TNF\alpha}$, 20% v/v) significantly increased the migration of human PC3 cells by 40% after 8 hours, when compared to cultures exposed to adipogenic conditioned from control cultures (Adipo ^{CM}, 20% v/v) ($p < 0.05$).

The exposure of human PC3 PCa cells to 1ng/ml TNFα in the absence of mature adipocytes or their derived factors caused a significant increase in cell migration (26%, 8 hours; and 25%, 12 hours, $p < 0.05$) (Figure 37), when compared with CTRL ^{CM} group. Furthermore, exposure of human PC3 cells to 10ng/ml TNFα for 12 hours caused relatively similar increase in cell migration (27%, 12 hours) (Figure 37).

In contrast, pro-inflammatory cytokine TNFα failed to enhance the ability of mature 3T3-L1 adipocytes to increase the migration of human LNCaP PCa cells, and TNFα had no effect on the migration of hormone-dependent human LNCaP cells directly (supplementary figure 1).

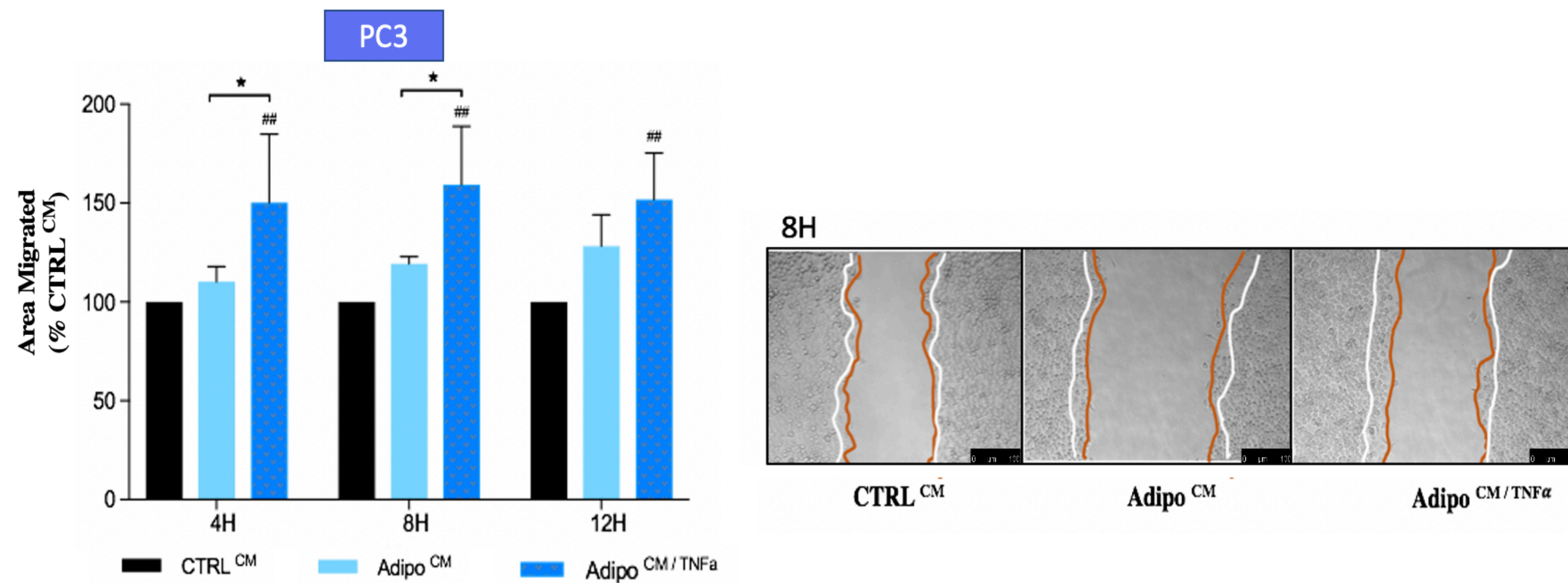


Figure. 36. TNFα enhanced the ability of mature adipocytes to increase the migration of human PC3 PCa cells in vitro. The effects of the derived factors from mature adipocytes stimulated by TNFα on the migration of the metastatic human PCa cells after 1-24 hours was assessed by wound healing assay. Plotted values for the area migrated as the percentage of each time CTRL ^{CM} in 4, 8, and 12 hours. Results are from three independent experiments (N=3). Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (#: Adipo $^{CM/TNF\alpha}$ vs CTRL ^{CM}).

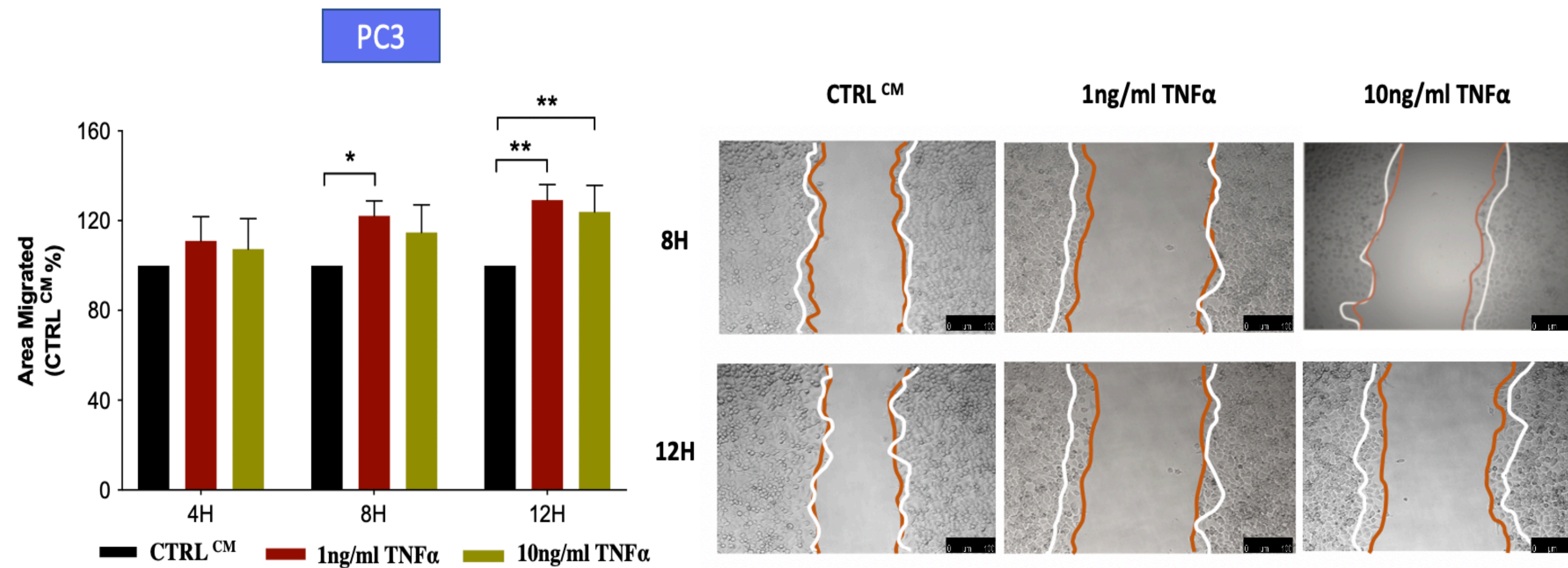


Figure. 37. TNFα enhanced the migration of human PCa cells PC3 *in vitro*. The effects of 1ng/ml and 10ng/ml TNFα on the migration of the highly metastatic human PCa cells PC3 after 1-24 hours was assessed by wound healing assay. Plotted values for the area migrated as the percentage of each time CTRL ^{CM} in 4, 8, and 12 hours. Results are from three independent experiments (N=3). Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

5.4.3. Pre-exposure of mature adipocytes to TNFα enhanced their ability to stimulate the in vitro invasion of human prostate cancer cells

Next, I investigated the ability of inflammation to influence PCa cell invasiveness by testing the effects of the pro-inflammatory cytokine TNFα on the *in vitro* invasiveness capabilities of hormone-dependent human LNCaP and castration-insensitive human PC3 and mouse RM1 PCa cells. Cell invasion was assessed by Transwell invasion assay as described in section 2.2.9.2.

As shown in figure 38 (panel A), factors derived from mature adipocytes stimulated by TNFα (Adipo $^{CM/TNF\alpha}$, 20% v/v) enhanced the invasion of human PC3 cells after 72 hours by 32% and 43%, when compared to cultures exposed to Adipo ^{CM} (20% v/v) and CTRL ^{CM} (20% v/v), respectively ($p < 0.05$). Similarly, treatment of these cultures with 1ng/ml TNFα and 10ng/ml TNFα in the absence of mature adipocytes or their derived factors caused a significant increase in the invasiveness ability of human PC3 PCa cells after 72 hours (30%, 1ng/ml TNFα; 25%, 10ng/ml TNFα, $p<0.05$), when compared with control group (Figure 38, panel B).

The castration-insensitive mouse RM1 PCa cells are known for their aggressive nature and metastatic abilities to organ such as brain and skeleton (Power et al., 2009). As shown in Figure 39 (panel A), the pro-inflammatory cytokine TNFα (Adipo $^{CM/TNF\alpha}$, 20% v/v) enhanced the ability of mature adipocytes to increase mouse RM1 cell invasion by 30% after 48 hours when compared with Adipo ^{CM} – treated group (20% v/v). Similarly, invasion of mouse RM1 cells was increased after exposure to TNFα (10ng/ml) alone for 48 hours (40%, $p < 0.05$) (Figure 39, panel B).

In contrast, pro-inflammatory cytokine TNFα failed to affect the ability of mature 3T3-L1 adipocytes to alter the invasiveness ability of human LNCaP PCa cells. Similarly, the cultures of TNFα without mature adipocyte-derived factors had no effects on the invasion of human LNCaP cells (Supplementary figure 2).

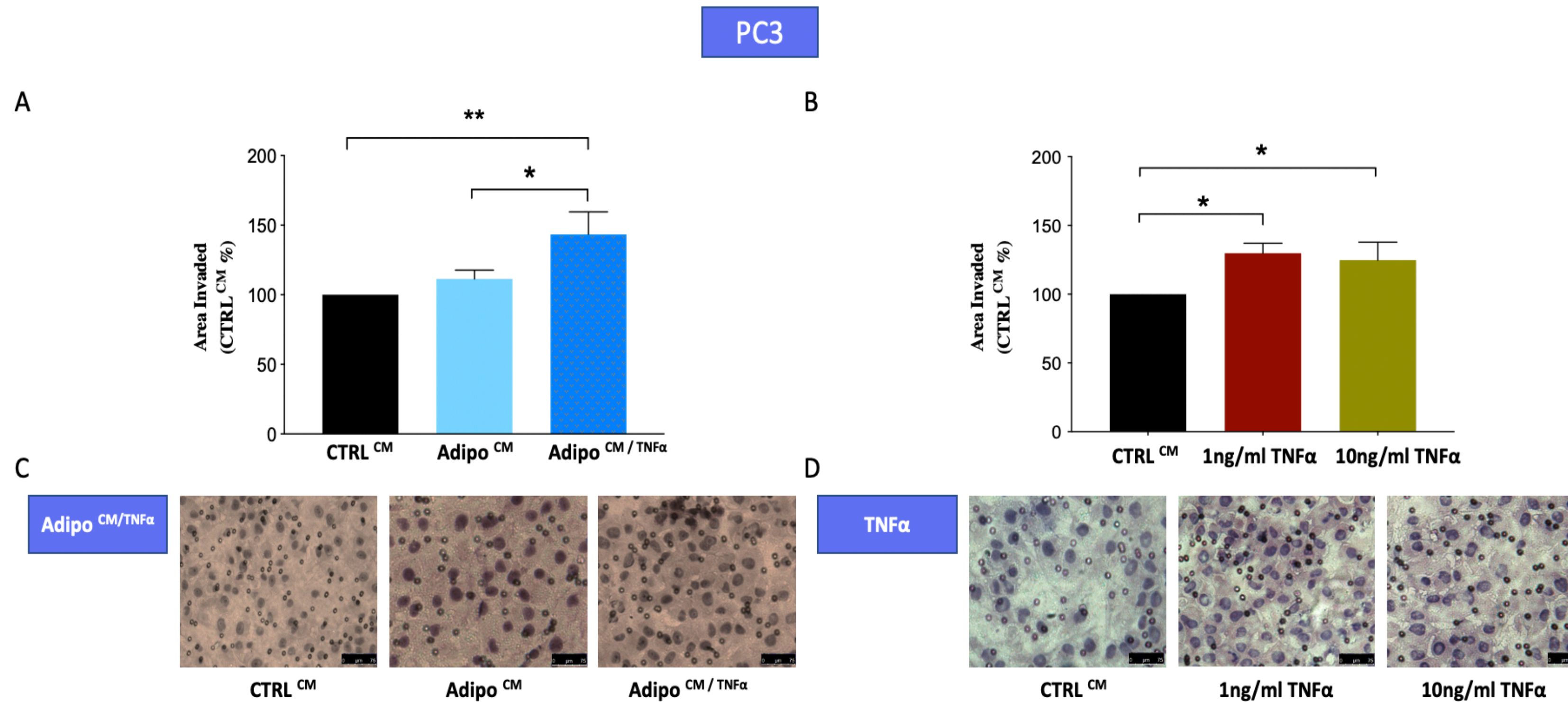


Figure. 38. TNFα enhanced the invasion of human PC3 PCa cells *in vitro*. The effects of the factors derived from TNFα stimulated mature adipocytes (A) or the pro-inflammatory cytokine TNFα (B) on the invasion of the highly metastatic PC3 human PCa cells after 72 hours were assessed by Transwell® invasion. Representative images of PC3 cell invasion (C and D). Results are from three independent experiments (N=3). Data were analysed using one-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

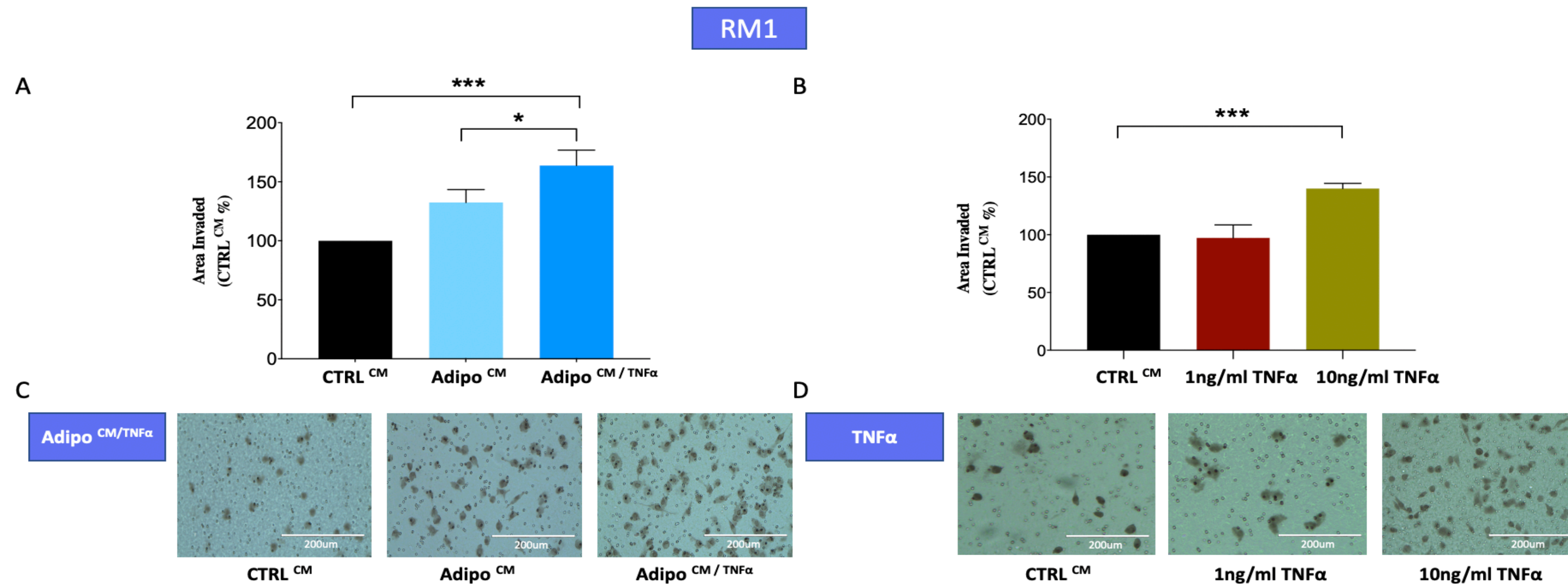


***Figure. 39.* TNFα enhanced the invasion of mouse RM1 PCa cells *in vitro*.** The effects of the factors derived from TNFα stimulated mature adipocytes (A) or the pro-inflammatory cytokine TNFα (B) on the invasion of the highly metastatic RM1 mouse PCa cells after 48 hours were assessed by Transwell® invasion. Results are from three independent experiments (N=3). Data were analyzed using one-way ANOVA test followed by Tukey's multiple comparisons test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (#: Adipo ^{CM} vs Adipo $^{CM/TNF\alpha}$).

5.4.4 Pre-exposure of mature adipocytes to TNFα enhanced the expression levels of adipocyte-derived pro-inflammatory factors

My data in this chapter thus far showed that exposure of mature adipocytes to the pro-inflammatory cytokine TNF increases both migration and invasive capabilities of the human castration-insensitive PCa cells, PC3. In order to examine the mechanism(s) by which TNFα exerts these effects in my model, I utilized the Mouse XL Cytokine Array Assay to measure the levels of 111 pro-inflammatory cytokines, chemokines and adipokines that are known to regulate PCa cell and adipocyte behaviour under inflammatory conditions.

As shown in figure 40 (panel A), detailed analysis of the levels of obesity-related factors revealed that exposure of mature 3T3-L1 adipocytes to TNFα (1ng/ml) significantly enhanced the levels of adiponectin (1.2-fold increase, $p<0.05$) and resistin (1.3-fold increase, $p<0.05$), when compared to conditioned medium collected from un-primed mature adipocytes.

My analysis of cancer-related factors showed that exposure of mature 3T3-L1 adipocytes to TNFα (1ng/ml) significantly enhanced the levels of pentraxin-3 (2.25-fold increase, $p<0.05$), but not Lipocalin 2, when compared to conditioned medium collected from un-primed mature adipocytes (Figure 40, panel B).

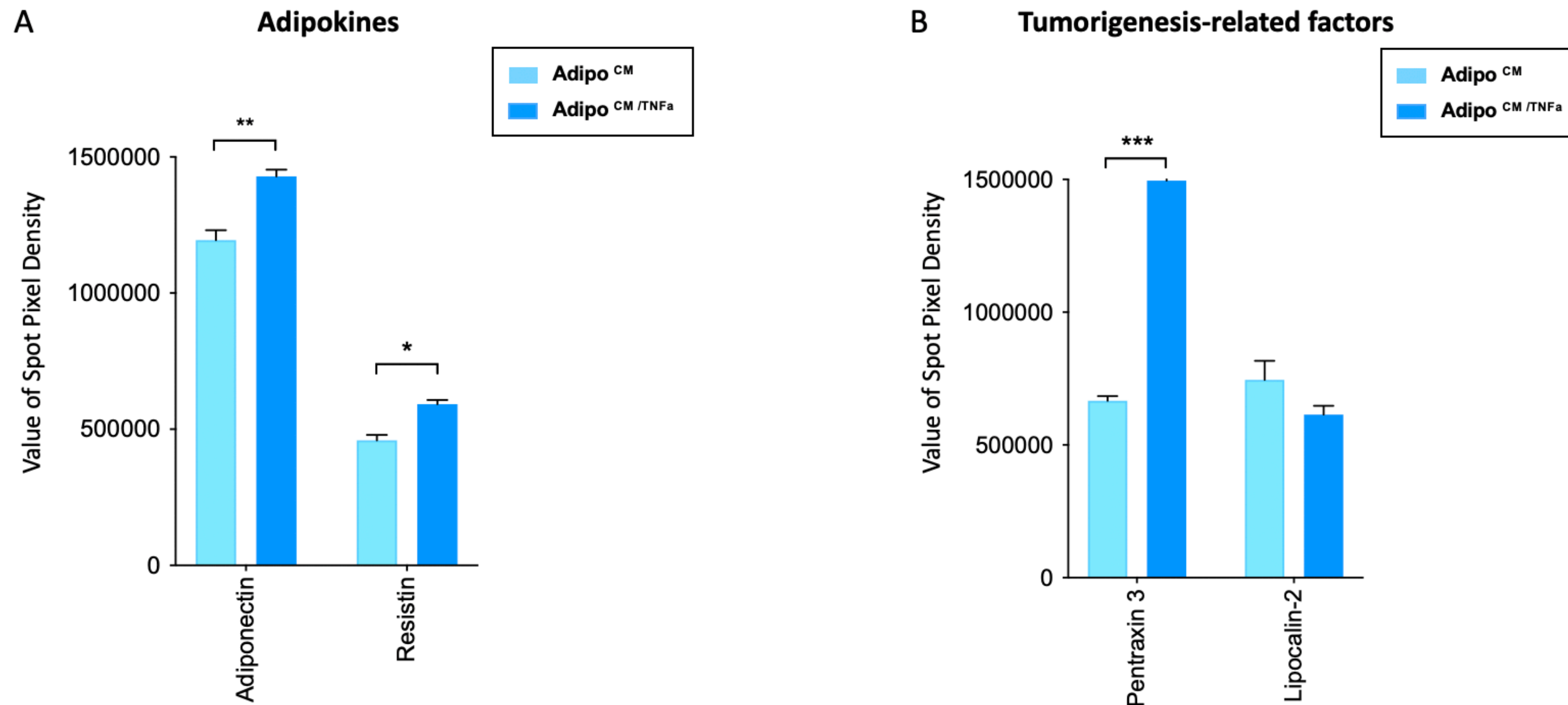


Figure. 40. TNFα increased the secretion level of adipokines and tumorigenesis-related factors in mature 3T3-L1 adipocytes. Two membrane-based cytokine array assays, with capture antibodies for relative expression levels of soluble proteins that are spotted in duplicate on the membrane, were incubated with Adipo $^{CM / TNF\alpha}$ or Adipo ^{CM} for 24 hours, before undergoing chemiluminescence detection and analysis. Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Additionally, I evaluated the levels of pro-inflammatory cytokines and angiogenesis-related factors in conditioned medium from mature mouse 3T3-L1 adipocytes primed with TNFα (1ng/ml). As shown in figure 41 (panel A), priming mature adipocytes with TNFα (1mg/ml) induced the expression levels of adipocyte-derived pro-inflammatory cytokines and chemokines that include CCL2 (3.6-fold increase, $p<0.0001$), CCL5 (2.1-fold increase, $p<0.00001$) and CCL11 (4.8-fold increase, $p<0.0001$), when compared to Adipo ^{CM} from mature adipocyte unexposed to TNFα.

Additionally, TNFα stimulated the expression levels of adipocyte-derived osteopontin (OPN) and Chitinase-3-like protein 1 (1.6-fold increase, $p<0.001$), when compared to Adipo ^{CM}. TNFα also induced higher expression levels of the pro-angiogenesis factors angiopoietin-1 by 2.0-fold, IGFBP-6 by 1.3-fold and MMP3 by 1.2-fold in mature adipocytes, respectively (Figure 41, panel B) ($p<0.05$). In contrast, pro-inflammatory cytokine TNFα attenuate the expression levels of the pro-angiogenesis factors VEGF, MMP-2 and Coagulation factor III by 10%, 29% and 41% respectively, when compared to conditioned medium from mature adipocyte unexposed to TNFα (Figure 41, panel B) ($p<0.05$).

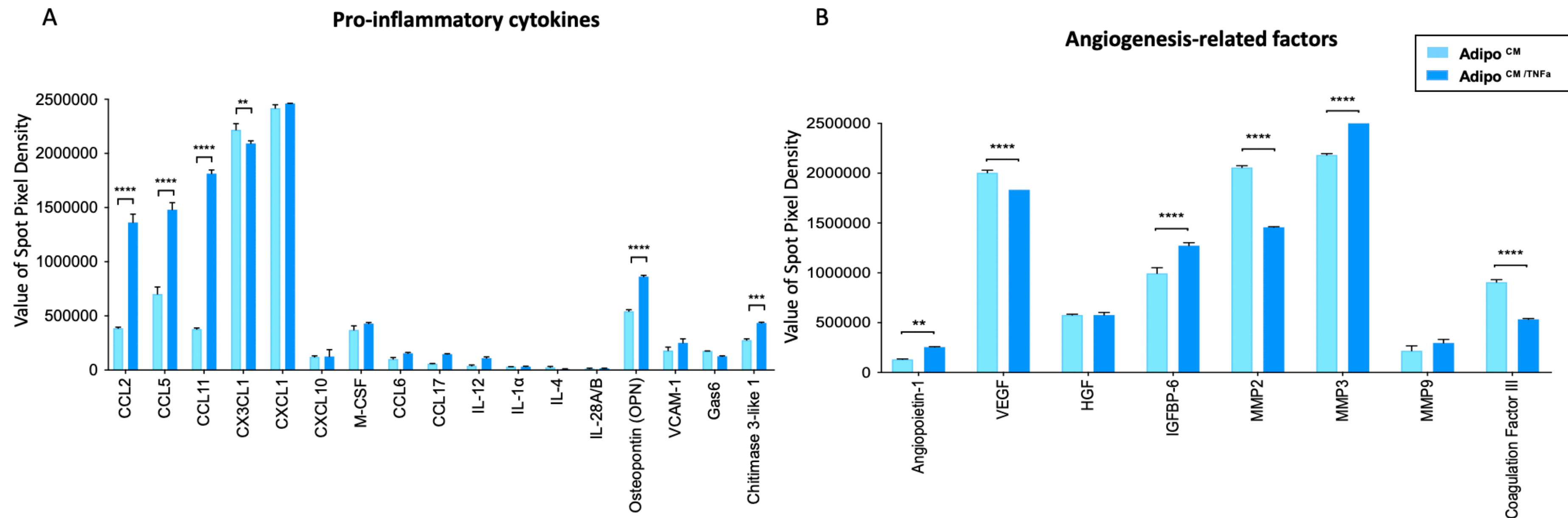


Figure. 41. TNFα increased the secretion level of pro-inflammatory cytokines and angiogenesis-related factors in mature 3T3-L1 adipocytes. Two membrane-based cytokine array assays, with capture antibodies for relative expression levels of soluble proteins that are spotted in duplicate on the membrane, were incubated with Adipo $^{CM / TNF\alpha}$ or Adipo ^{CM} for 24 hours, before undergoing chemiluminescence detection and analysis. Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ±SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Further evaluation of the role of adipocyte-derived soluble factors that implicated in PCa and adipogenesis revealed a subset of pathways and processes that are likely to be implicated in the regulation of the TNFα-driven regulation of the interactions between mature adipocytes and PCa cells that I observed in my models. Figure 42 shows the effects of pro-inflammatory cytokine TNFα stimulated mature adipocytes on the production of soluble factors was expressed as percentage of Adipo ^{CM}. As shown in figure 42, panel A, 15 soluble factors were considered to be differentially regulated with adipogenesis. Figure 42, panel B, shows a Venn diagram of the overlapping role for factors implicated in the regulation of the crosstalk of PCa cells, adipocytes and inflammation cytokine. Supplementary table 2 shows the summary of differentially regulated factors produced by conditioned medium from mature 3T3-L1 adipocytes primed with TNFα based on the raw data from the Mouse XL Cytokine Array Assay used.

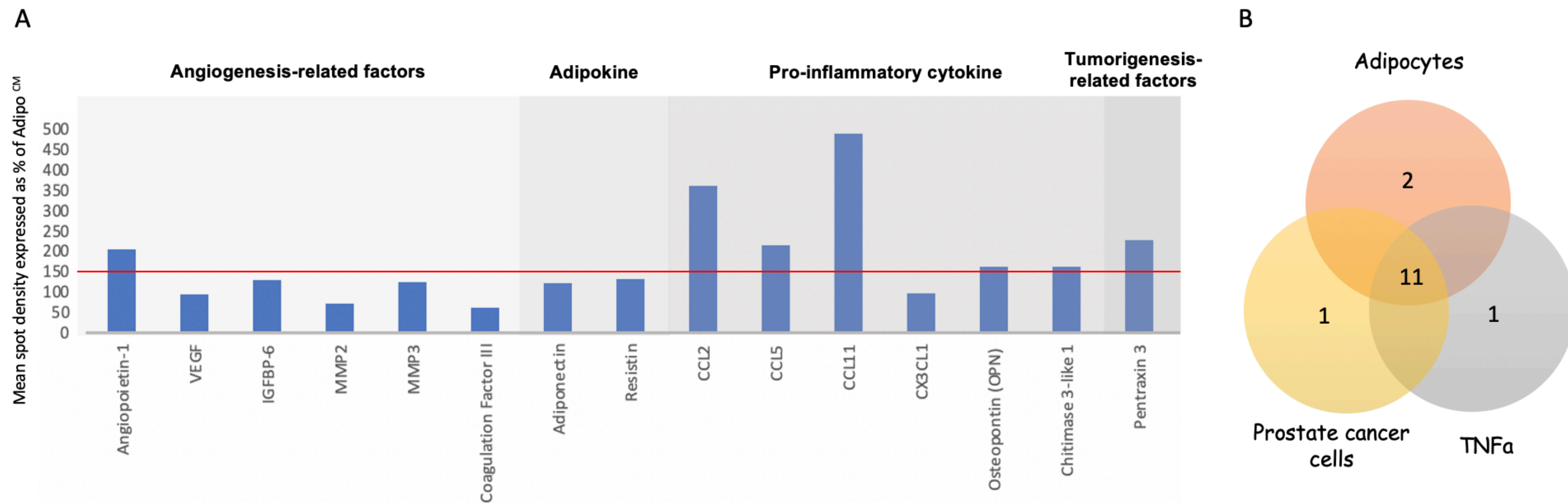


Figure. 42. **Pro-inflammatory cytokine TNFα increased adipocyte-derived pro-inflammatory factors expression level.** A)**.** Microarray analysis of soluble factors derived from mature adipocytes primed with 1ng/ml TNFα, using Proteome Profiler TM Mouse XL Cytokine Array Kit (R&D systems). B)**.** Venn diagram of differentially expressed adipogenesis mediators and pro-inflammatory mediators in conditioned medium from the mature adipocytes that stimulated by 1ng/ml TNFα.

5.5. Discussion

Chronic inflammation in non-cancerous prostate biopsy specimens contribute to the increased development of high-grade prostate tumours. Men with signs of chronic inflammation in prostatic tissue are twice at the risk of developing PCa (Sfanos and De Marzo, 2012, Stark et al., 2015). Furthermore, epidemiological studies have shown that chronic inflammation is associated with increased risk of PCa in clinically obese individuals (Fujita et al., 2019), and previous and current studies in this project have demonstrated that adipocytes regulate the growth of PCa cells. However, the effects of pro-inflammatory mediators on the ability of adipocyte to affect the metastatic abilities of PCa cell during advance stages of the disease is poorly understood. In this chapter, I used the pro-inflammatory factor TNFα as a model pro-inflammatory cytokine to evaluate the role of inflammation on the ability of mature adipocytes to affect the *in vitro* migration and invasion of human and mouse castration-insensitive PCa cells.

TNFα is a classical mediator of inflammation and it is routinely used in high and low concentrations to study the initiation and progression of acute and chronic inflammation *in vitro* and *in vivo* (Tzanavari et al., 2010, Page et al., 2018, Zhao et al., 2020). A number of studies have shown that expression level of pro-inflammatory NFκB-activating mediator TNFα is elevated in states of obesity and insulin resistance (Borst, 2004, Nieto-Vazquez et al., 2008) and PCa (Srinivasan et al., 2010). My investigation in this chapter showed that priming mature adipocytes with the pro-inflammatory factor TNFα had no effects on the *in vitro* growth of both the hormone-dependent and castration-insensitive human and mouse PCa cells tested. TNFα has been implicated in the progression of metastatic PCa and resistance to chemotherapeutic agents (Dennis et al., 2002). This finding led me to hypothesise that exposure of PCa cells to either TNF alone or derived factors from mature adipocytes primed with TNFα influences the motility of PCa cells. To investigate this hypothesis, I examined the effects of conditioned

medium from mature adipocytes treated with TNFα on the migration and invasion of the metastatic, castration-insensitive human PC3 and mouse RM1 PCa cells.

My results confirmed this hypothesis and showed that derived factors from mature adipocytes stimulated by TNFα enhanced invasion of both human PC3 and mouse RM1 cells. My studies also showed that exposure of either PCa cells or adipocytes to TNFα enhanced the migratory and invasive abilities of highly metastatic and castration-insensitive PCa cells. These findings are consistent with previous cancer study that reported that TNFα facilitates the degradation of the ECM and as a result increases the migratory and invasiveness capacity of PCa cells (Ganguly et al., 2014). Furthermore, high expression of TNFα is associated with enhanced PCa progression (Brian et al., 2012), and in broad agreement with studies that have shown that inflammation enhances the metastatic abilities of PCa cells and factors secreted by adipocytes are implicated in the metastatic behaviour of PCa cells (Kaneko et al., 2010, Stark et al., 2015, Duong et al. 2017). Collectively, these findings broadly confirm that crosstalk between adipocytes and cancer cells is mediated by exogeneous pro-inflammatory and other mediators generated and secreted by mature adipocytes in response to exposure to pro-inflammatory factors such as TNFα (Khandekar et al., 2011).

Protein microarray analysis of the expression level of 111 cytokines, chemokines and adipokines in conditioned medium from TNFα stimulated mature adipocytes and their non-stimulated controls revealed that TNFα enhanced the expression levels of a complex network of adipocyte-derived NFκB-mediated pro-inflammatory mediators, pro-migratory factors, chemokines and adipokines. For example, exposure to TNFα significantly enhanced the level of adipocyte-derived pentraxin 3, a recently identified biomarker for predicting prostatic inflammation in PCa patients. This finding is consistent with research that showed that serum level of pentraxin 3 is higher in patients with PCa than in BPH patients (Stallone et al., 2014). Furthermore, a number of pro-inflammatory cytokines including TNFα, LPS and IL-1β enhanced the synthesis of pentraxin 3 (Inforzato et al., 2012). My analysis also detected an

increase in the levels of the chemokine CCL2 and CCL11 (Eotaxin-1). CCL2 is a positive regulator of PCa motility (Loberg et al., 2006, Mizutani et al., 2009, Hao, 2020), whereas CCL11 (Eotaxin-1) has recently been identified as a new diagnostic serum marker for PCa (Agarwal et al., 2013).

PCa is known to metastasise to the skeleton (Gingrich et al., 1996, Nørgaard et al., 2010). Interestingly, my analysis has shown that TNFα stimulated the expression levels of adipocyte-derived OPN, non-collagenous bone matrix protein, that has been implicated in the regulation of PCa bone metastasis (Khodavirdi et al., 2006, Anborgh et al., 2010). These findings imply a role for bone-derived factors in the interactions between mature adipocyte and the castration-insensitive human PC3 and mouse RM1 PCa cells used in the model described. However, further *in vivo* investigation is needed to establish if anti-inflammatory agents such as NFκB inhibitors affect PCa bone metastasis in obese animals.

Another possible mechanism of TNFα-induced motility of PCa cells is the activation of NFκB and related pro-inflammatory signalling pathways such as the mitogen-activated protein kinases (MAPKs) including ERK and p38 which has been reported to regulate the PCa cells migration (Hoesel and Schmid, 2013, Sabio and Davis, 2014). TNFα induced NFκB activation has been shown to regulate cancer cell invasion via up-regulating MMPs expression (Hagemann et al., 2005). According to research on altered MMP-9 expression, the highly metastatic PCa cells showed increased expression of MMP-9 as compared to LNCaP cells (Aalinkeel et al., 2004). Additionally, the 5-α-reductase inhibitors (finasteride and dutasteride) have been used as potential chemo-preventive agents for PCa (Thompson et al., 2003). One research study showed that finasteride may attenuate invasion of PCa cells through the downregulation of MMP-9 (Moroz et al., 2013). Furthermore, TNF enhanced the invasive capability of tumour cells through inducing MMP-3 (Cheng et al., 2007, Gong et al., 2014). This finding is confirmed by the present cytokine array analysis that detected a moderate increase expression level of MMP-3 in conditioned medium obtained from mature adipocytes

exposed to TNFα, thereby suggesting a mechanism for the pro-migratory and pro-invasion effects of TNFα and conditioned medium from mature adipocytes primed with TNFα in the models described.

My analysis also detected an increase in the expression level of pro-angiogenesis factors angiopoietin-1, IGFBP-6 and other factors such as the Chitinase-3-like protein 1 in the conditioned medium from TNFα stimulated mature adipocytes. Whilst all these factors have been shown to play a role in PCa progression (Luyuanli, 2003, Koike et al., 2005, Libreros et al., 2013), further examination of the role that they play in PCa cell – adipocyte interactions in animal models are needed. Future studies also further examine the role of adipokines such as adiponectin in the role of inflammation in adipocyte – PCa interactions. Studies in this chapter showed that adiponectin level in conditioned medium from mature adipocytes exposed to TNFα was modestly increased when compared to control. This finding is inconsistent with previous studies that have shown that reduced adiponectin expression is associated with insulin resistance and TNFα may cause systemic insulin resistance via inhibiting the production of adiponectin (Ruan et al., 2002, Degawa-Yamauchi et al., 2005). Other studies have also shown that reduced levels of adiponectin increase the risk of high grade PCa, when comparing with individuals with benign prostatic hyperplasia (Goktas et al., 2005).

Collectively, the results of this chapter indicate that pro-inflammatory response, induced by TNFα, enhanced the ability of mature adipocytes to increase the motility of highly metastatic, castration-insensitive PCa cells such as the human PC3 and mouse RM1 PCa cells. Whilst the mechanism(s) by which mature adipocytes exert these effects remain to be determined, the results of this chapter suggest that mature adipocyte exert these effects by a mechanism dependent, at least in part, on increased expression and secretion of a complex network of pro-inflammatory mediators, pro-migratory factors and chemokines.

CHAPTER 6

THE ROLE OF TUMOUR ASSOCIATED MACROPHAGE IN ADIPOCYTE – PROSTATE CANCER CELL CROSSTALK *IN VITRO*

6.1. Summary

Macrophages play a key role in inflammation, obesity and TAMs, in particular M2, are the most abundant types of immune cells in the tumour microenvironment. M2 macrophage are implicated in the regulation of various aspects of cancer including tumour growth, angiogenesis, metastasis and colonisation of distant organs. To further examine the role of inflammation in adipocyte – PCa cell crosstalk, I hypothesised that derived factors from mature adipocytes primed with conditioned medium from TAMs influence the growth, migratory and/or invasive ability of hormone-dependent and castration-insensitive human and mouse PCa cells.

In this chapter, M0, M1 and M2 macrophages were first generated from human monocytic THP-1 cells. Mature adipocytes were exposed to conditioned medium from M0, M1 or M2 for 24 hours, and then conditioned medium from primed mature adipocytes was extracted and used to treat a panel of hormone-dependent and castration-insensitive human and mouse PCa cells. The results of these experiments showed that factors derived from mature adipocytes primed with M0-, M1- or M2 -conditioned medium failed to affect the growth of the hormone-dependent and castration-insensitive human and mouse PCa cells tested. Follow up studies on the ability of the alternatively activated M2 macrophage phenotype affects the influence of adipocyte on PCa motility showed that M2-conditioned medium enhanced the ability of mature adipocytes to stimulate the *in vitro* invasion – but not the 2D migration - of the castration-insensitive human PC3 and mouse RM1 PCa cells. Protein microarray analysis of derived soluble factors from both M2 macrophage and mature adipocytes stimulated with M2-conditioned medium showed that these effects were regulated, at least in part, by a complex array of factors that include NFκB-activating pro-inflammatory mediators, pro-migratory factors and chemokines.

Overall, the results of this chapter indicate that TAMs, in particular the M2 phenotype, and their derived factors are implicated in the regulation of the interactions between mature adipocytes and highly metastatic PCa cells.

6.2. Introduction

TAMs are key players in the regulation of inflammation within the tumour microenvironment and are associated with a poor clinical outcome in solid tumours (Mantovani et al., 2002, Kong et al., 2013, Zhang et al., 2013).

Studies in murine PCa models suggested that TAMs that exhibit the M2 phenotype promote tumour growth and metastasis. M2 interact directly with tumour cells and a growing body of evidence show that TAMs and their derived factors promote tumour growth at the primary site and distant metastasis by facilitating angiogenesis, immunosuppression, and inflammation (Condeelis and Pollard, 2006, Redente et al., 2010, Muthana et al., 2011, Chimal-Ramírez et al., 2013, Ashleigh and Matthias, 2018). Several reports have demonstrated that depletion of TAMs in mouse models decreases tumour growth and spread, as well as restores response to chemotherapeutic agents (Fritz et al., 2014, Patwardhan et al., 2014, Mantovani et al., 2017).

Pro-inflammatory cytokines released from adipose tissue are also known to exert an impact on the development and progression of PCa by influencing the function and lineage commitment of TAMs (Baker et al., 2011, Hyo-Yeoung et al., 2020). HFD has been shown to enhances tumour growth and alters the tumour immune microenvironment by enhancing the ratio of M2/M1 in mice (Incio et al., 2016, Hayashi et al., 2018, Fujita et al., 2019). Moreover, TAMs infiltration of tumours has been found to correlate with PCa T stage, higher serum PSA level and GS in PCa patients (Nonomura et al., 2011).

Collectively, these findings suggest that a link between TAMs, prostate cancer and obesity. However, the effects of TAMs and their derived factors on the ability of adipocytes to influence the behaviour of PCa cells and the role of TAMs-derived pro-inflammatory mediators in this process have not been investigated.

6.3. Aims

The aim of this chapter is to study the role of TAMs in adipocyte – PCa cell interactions *in vitro* (Figure 43).

This aim was achieved by assessing:

- effects of conditioned medium that collected from mature adipocytes primed with M2 conditioned medium on the *in vitro* viability, migration and invasion of a panel of hormone-dependent and castration-insensitive human and mouse PCa cells.
- the expression levels of pro-inflammatory cytokines, chemokines and adipokines in conditioned medium from mature adipocytes primed with M2 conditioned medium.

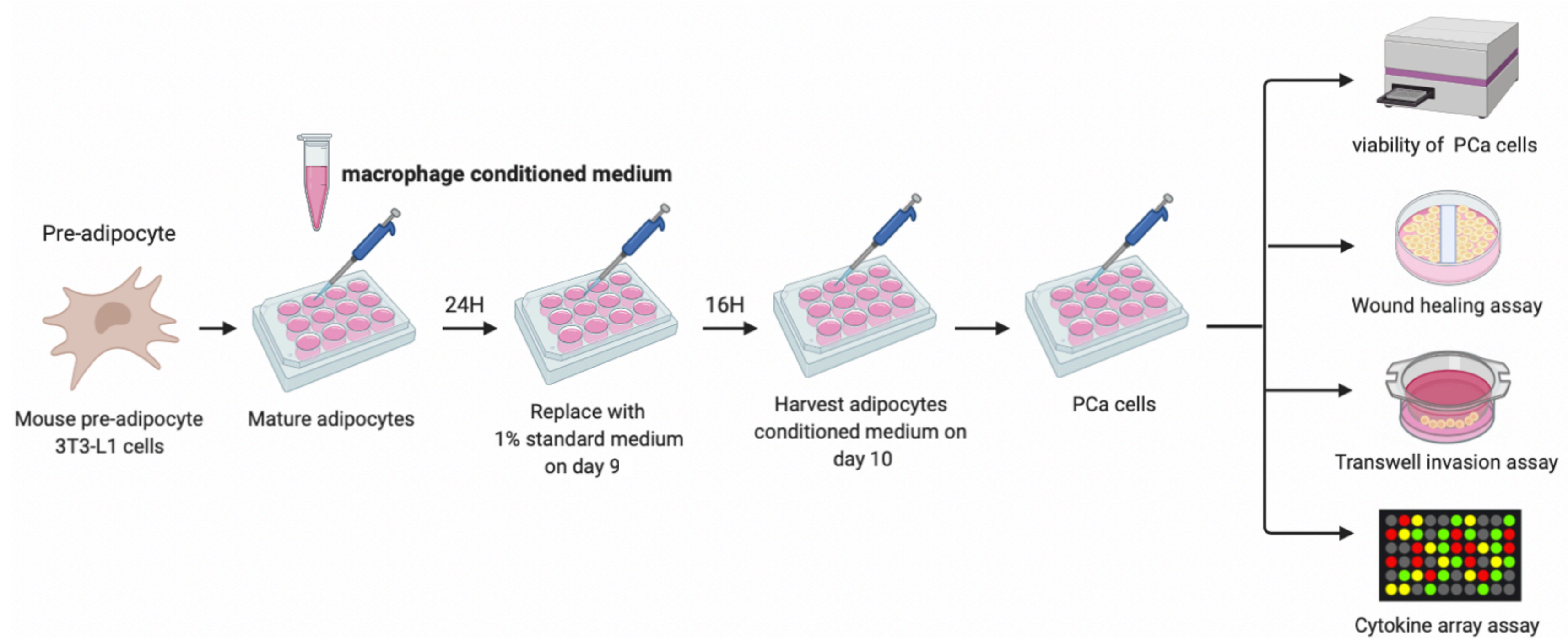


Figure. 43. **Schematic diagram of investigation of factors derived from mature mouse 3T3-L1 adipocytes primed with tumour associated macrophage conditioned medium.**

6.4. Results

6.4.1. Pre-exposure of mature adipocytes to M2 macrophage derived factors failed to influence their ability to affect the in vitro growth of prostate cancer cells

First, TAMs were generated from human monocytic cell THP-1 as described in the Material and Methods (section 2.2.5). Briefly, non-adherent THP-1 were differentiated into adherent M0, M1 and M2 macrophage following an incubation period in PMA, and PTA for M0, IFN-γ and LPS for M1 macrophages and IL-4 and IL-13 for M2 macrophages. Mature 3T3-L1 adipocytes generated as described in the Material and Methods (section 2.2.4) were exposed to TAMs conditioned medium (100% v/v) for 24 hours, and conditioned medium were collected. Next, the effects of conditioned medium collected from mature adipocytes primed with M2 conditioned medium was tested on the *in vitro* growth of a panel of hormone-dependent and castration-insensitive human and mouse PCa cells. PCa cell growth was measured by AlamarBlue assay as described in section 2.2.8.

As shown in figure 44, factors derived from mature adipocytes primed with M0-, M1- or M2-macrophage conditioned medium (Adipo $^{CM/M0}$, Adipo $^{CM/M1}$, Adipo $^{CM/M2}$, 20% v/v) failed to affect the growth of human hormone-dependent LNCaP and castration-insensitive human PC3 and mouse RM1 PCa cells, when compared to cultures exposed to conditioned medium collected from un-primed mature adipocytes (Adipo ^{CM}, 20% v/v).

Further comparative analysis of cell growth across the three PCa cell lines that confirmed that factors derived from mature adipocytes primed with conditioned medium from the pro-tumour M0-, M1-, and M2-macropahges (Adipo $^{CM/M0}$, Adipo $^{CM/M1}$, Adipo $^{CM/M2}$, 20% v/v) was equally ineffective in affecting the growth of hormone-dependent human LNCaP and castration-insensitive human PC3 and mouse RM1 cells (Figure 45).

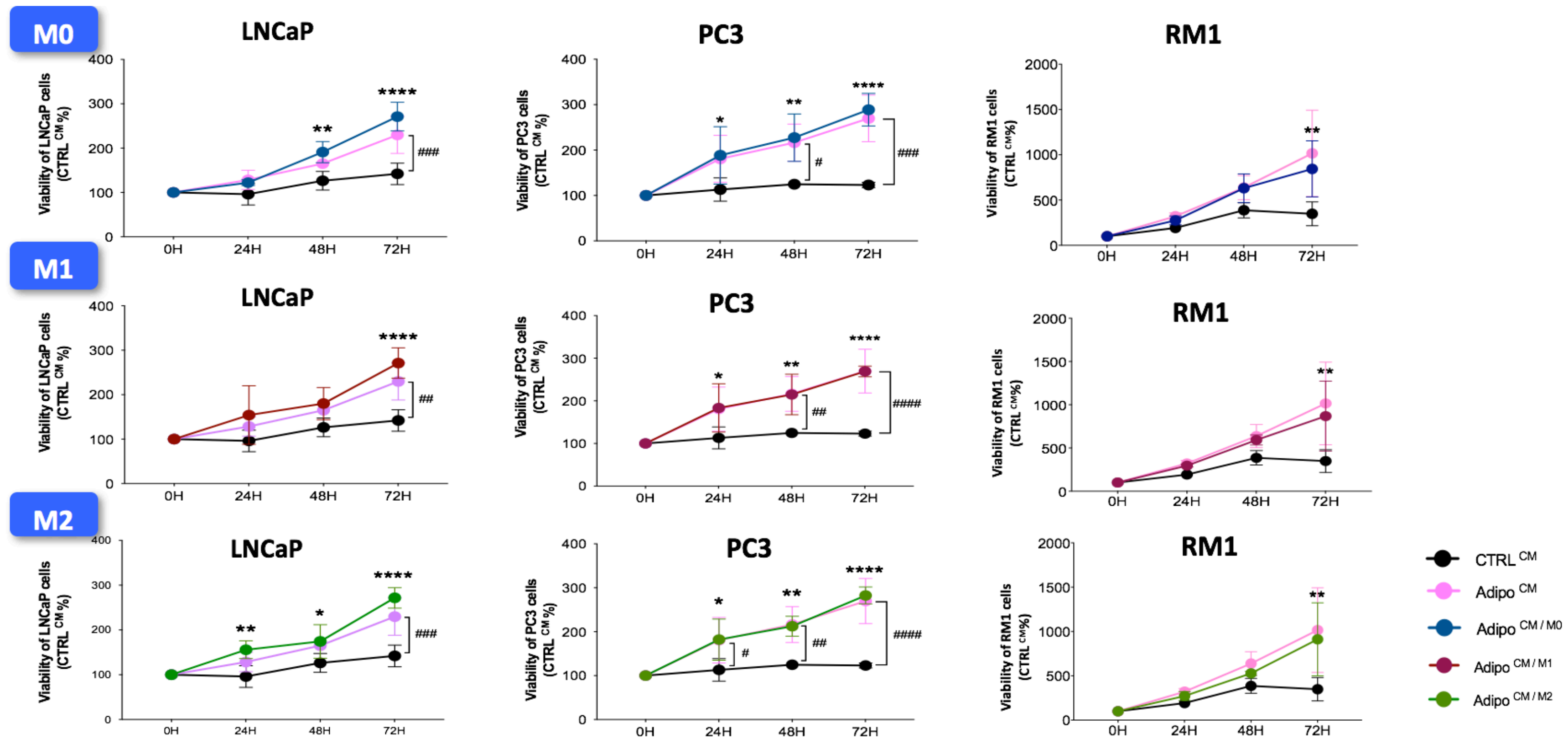


Figure. 44. **TAMs derived factors failed to affect the ability of mature adipocytes to influence the growth of PCa cells *in vitro*.** Human LNCaP and PC3 and mouse RM1 PCa cells were plated in 96-well plates. Cell viability was assessed by AlamarBlue. Results are from three independent experiments (N=3). Data were analyzed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

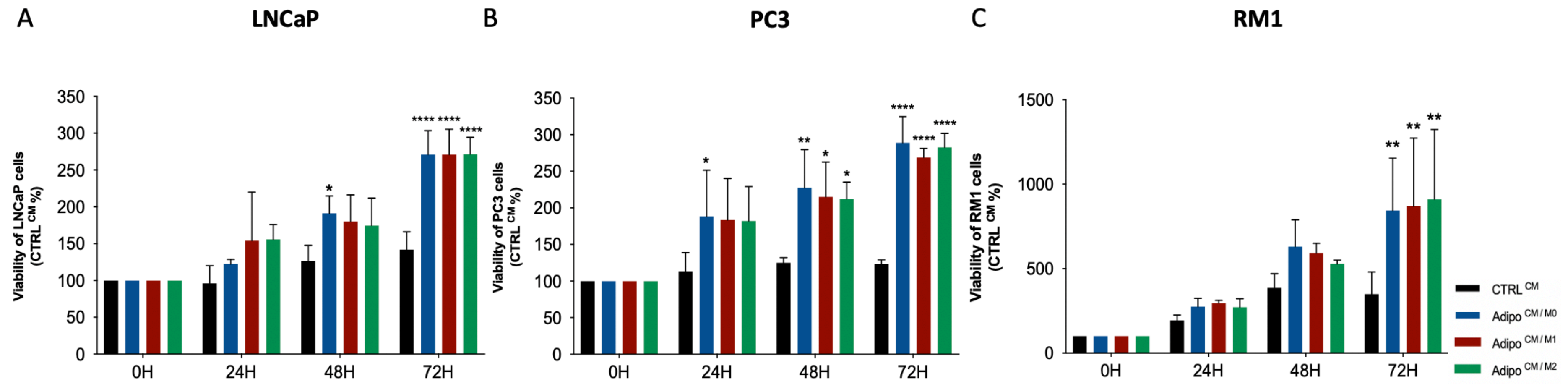


Figure. 45. TAMs derived factors failed to affect the ability of mature adipocytes to influence the growth of PCa cells in vitro. Human LNCaP and PC3 and mouse RM1 PCa cells were plated in 96-well plates. Cell viability was assessed by AlamarBlue. Results are from three independent experiments (N=3). Data were analyzed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

6.4.2. M2 macrophage derived factors enhanced the ability of mature adipocyte to increase the in vitro motility of prostate cancer cells

Previous studies have reported that M2 macrophage phenotype were suggested to have pro-tumour functions that promote tumour metastasis (Chimal-Ramírez et al., 2013, Mills, 2015). Thus, I hypothesised that factors from mature adipocytes primed by M2 macrophages derived factors may affect the motility of PCa cells. In this chapter, I examined the effects of soluble factors that produced by mature 3T3-L1 adipocytes in presence or absence of M2 macrophage conditioned medium on the migratory and invasive capabilities of human PCa cells. Cell migration and invasion were measured by wound healing and Transwell invasion assays as described in sections 2.2.9.1 and 2.2.9.2, respectively.

As shown in figure 46, conditioned medium collected from mature adipocytes primed with M2-macrophage conditioned medium (Adipo $^{CM/M2}$, 20% v/v) failed to affect the *in vitro* migration of hormone-dependent human LNCaP or castration-insensitive human PC3 PCa cells, when compared to cultures exposed to conditioned medium collected from un-primed mature adipocytes (Adipo ^{CM}, 20% v/v).

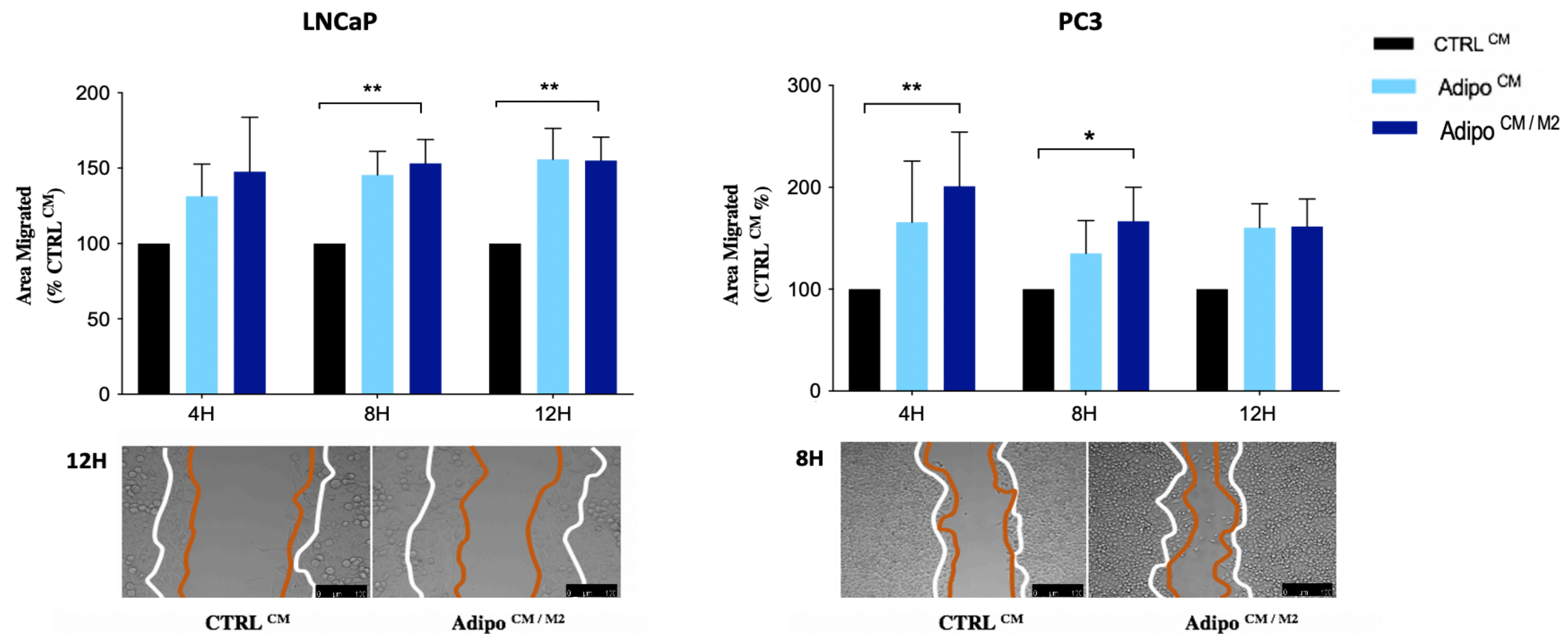


Figure. 46. **M2 macrophage derived factors failed to affect the ability of mature adipocytes to influence the migration of human PCa cells *in vitro*.** *The effects of the derived factors from mature adipocytes stimulated by M2 macrophage on the migration of the metastatic human PCa cells after 1-24 hours was assessed by wound healing assay. Plotted values for the area migrated as the percentage of each time CTRL ^{CM} in 4, 8, and 12 hours. Results are from three independent experiments (N=3). Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (#: Adipo ^{CM} vs CTRL ^{CM}). Magnifications is 10x.*

Next, I tested the effects of factors derived from the mature adipocytes primed with M2 macrophage conditioned medium on the invasion of human LNCaP and PC3 and mouse RM1 PCa cells.

As shown in figure 47, factors derived from mature adipocytes stimulated by M2 macrophage conditioned medium (Adipo $^{CM/M2}$, 20% v/v) enhanced the invasiveness ability of the castration-insensitive human PC3 cells (29% increase, $p < 0.05$, panel A) after 72 hours and mouse RM1 cells (76% increase, $p < 0.05$, panel B) after 48 hours, when compared to cultures exposed to conditioned medium collected from un-primed mature adipocytes (Adipo ^{CM}, 20% v/v).

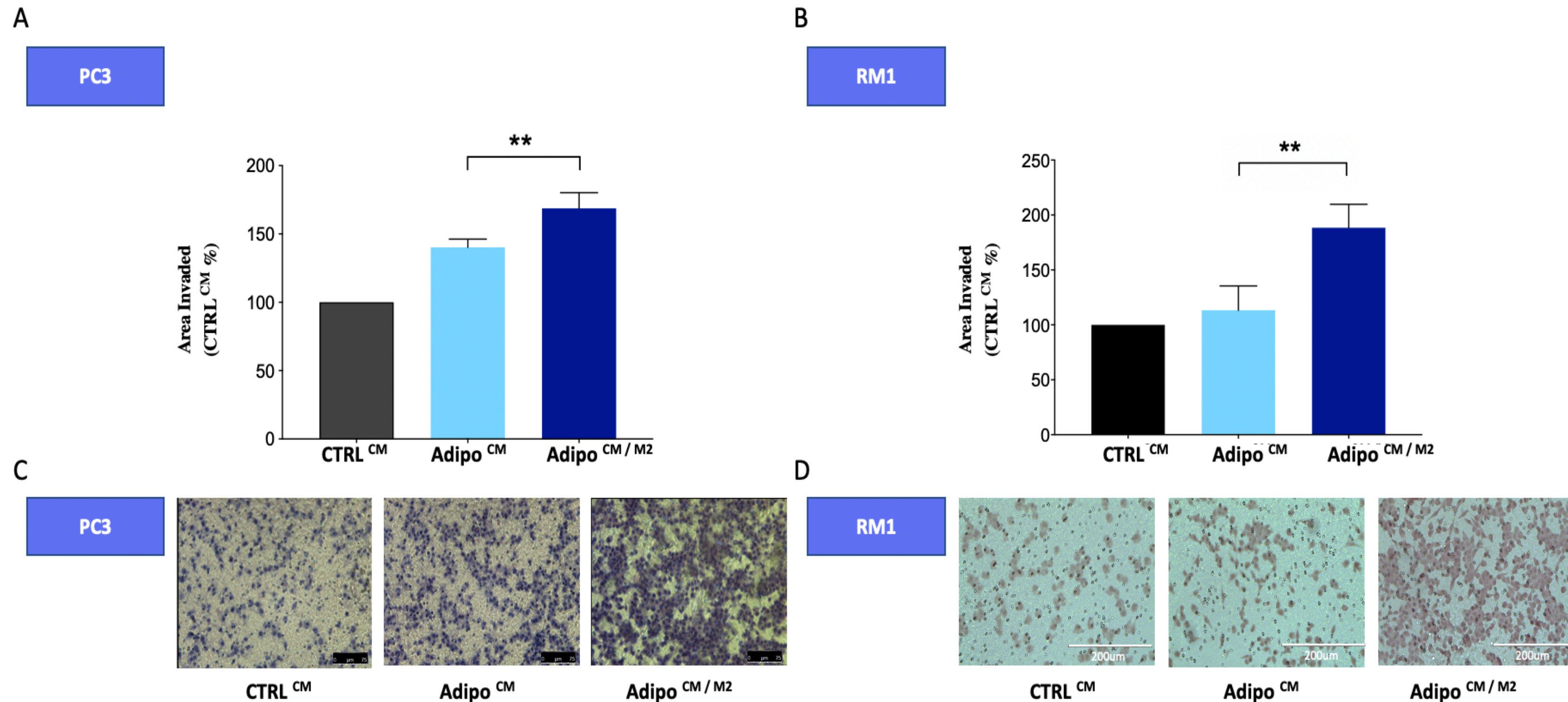


Figure. 47. M2 macrophage derived factors enhanced the ability of mature adipocytes to increase the invasiveness of human PC3 and mouse RM1 PCa cells *in vitro*. The effects of the factors derived from M2 macrophage stimulated mature adipocytes on the invasion of the highly metastatic human PC3 PCa cells (A) after 72 hours or mouse RM1 PCa cells (B) after 48 hours were assessed by Transwell ® invasion. Representative images of PC3 cells (C) and RM1 cells (D) invasion. Results are from three independent experiments (N=3). Data were analysed using one-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

In contrast, M2 macrophage conditioned medium (Adipo $^{CM/M2}$, 20% v/v) failed to exert a statically significant increase in the ability of mature adipocytes to influence the invasiveness capability of the hormone-dependent human LNCaP PCa cells, when compared to cultures exposed to conditioned medium collected from un-primed mature adipocytes (Adipo ^{CM}, 20% v/v) (Figure 48).

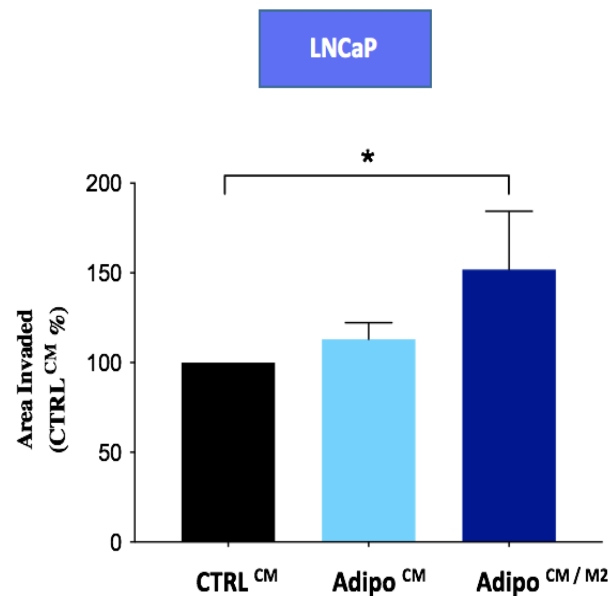


Figure. 48. M2 macrophage derived factors failed to affect the ability of mature adipocytes to increase the invasion of hormone-dependent LNCaP PCa cells. The effects of the factors derived from mature adipocytes primed with M2 macrophage conditioned medium on the invasion of the human prostate cancer cells LNCaP after 72 hours were assessed by Transwell invasion. Results are from three independent experiments (N=3). Data were analysed using one-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

6.4.3. Pre-exposure of mature adipocytes to M2 conditioned medium enhanced the expression levels of adipocyte-derived pro-inflammatory factors

My data in this chapter thus far showed that exposure of mature adipocytes to factors derived from M2 macrophages increased the invasive capability of highly metastatic, castration-insensitive human PC3 and mouse RM1 PCa cells. In order to examine the underlying mechanism by which M2 macrophage exerts these effects in my model, I utilized the Mouse XL Cytokine Array Assay to measure the levels of 111 pro-inflammatory cytokines, chemokines and adipokines in conditioned medium obtained from mature adipocytes primed and un-primed with M2 macrophage derived factors.

As can be seen in figure 49 (panel A), detailed analysis of the level of adipocyte-derived factors in this conditioned medium indicated that exposure of mature adipocytes to M2 macrophage conditioned medium significantly increased the levels of adiponectin (1.4-fold increase, $p<0.01$) and resistin (1.6-fold increase, $p<0.05$), when compared to levels in conditioned medium collected from un-primed mature adipocytes.

Furthermore, my analysis of tumorigenesis-related factors in the same conditioned medium revealed that exposure of mature adipocytes to M2 macrophage conditioned medium significantly enhanced the levels of pentraxin-3 (36% increase, $p<0.05$), when compared to levels in conditioned medium collected from un-primed mature adipocytes (Figure 49, panel B). In contrast, M2 macrophage derived factors reduced the level of Lipocalin 2 by 63% ($p < 0.001$), when compared to levels in conditioned medium collected from un-primed mature adipocytes (Figure 49, panel B).

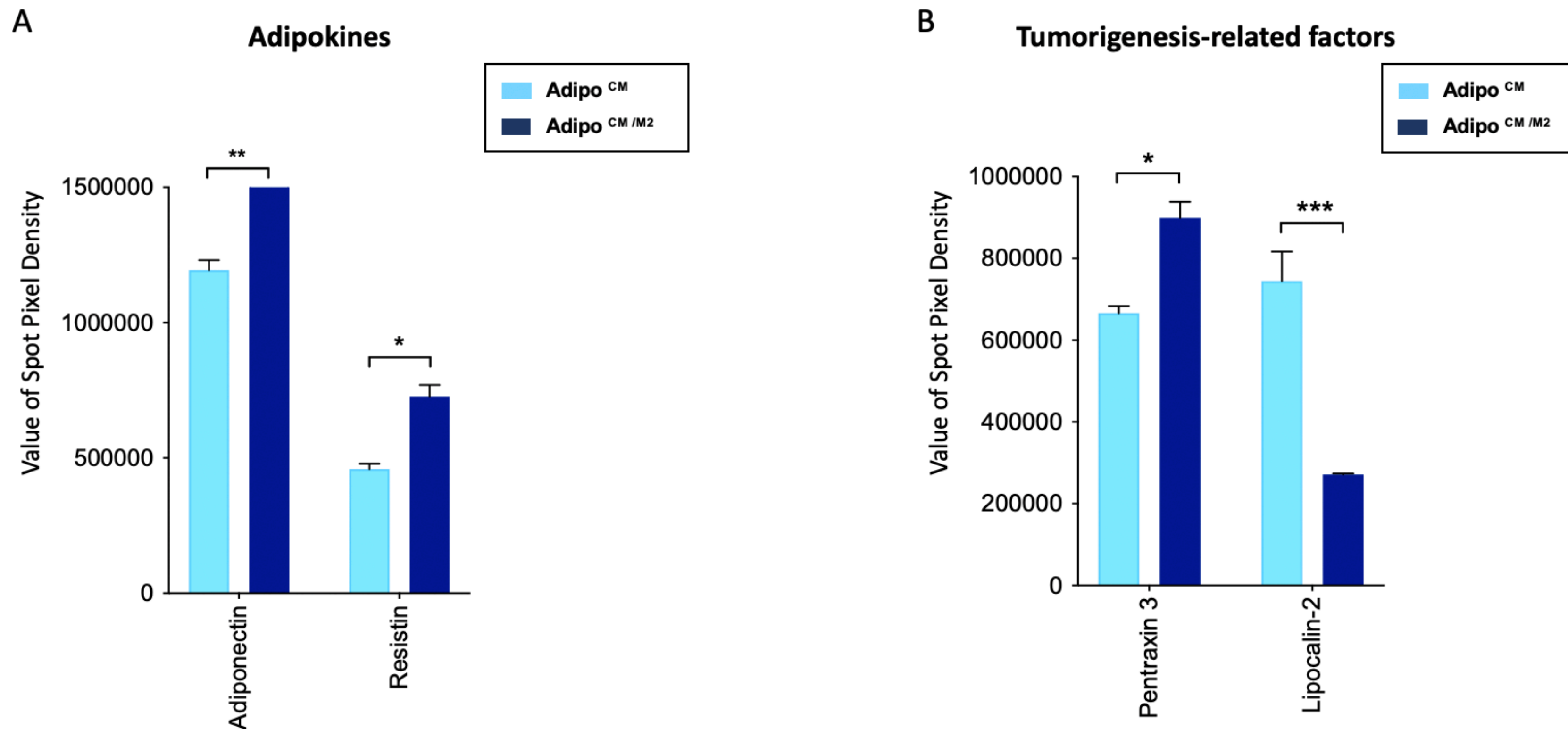


Figure. 49. M2 macrophage derived factors increased the secretion level of adipokines and tumorigenesis related factors in mature 3T3-L1 adipocytes. Two membrane-based cytokine array assays, with capture antibodies for relative expression levels of soluble proteins that are spotted in duplicate on the membrane, were incubated with Adipo $^{CM / M2}$ or Adipo ^{CM} for 24 hours, before undergoing chemiluminescence detection and analysis. Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ±SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Next, I analysed the levels of various pro-inflammatory cytokines and angiogenesis-related factors in conditioned medium from mature adipocytes primed with M2 macrophage conditioned medium. As shown in figure 50 (panel A), M2 macrophage derived factors enhanced the levels of various members of the CCL and CXCL families of chemokines, including CCL2 (4.0-fold, $p<0.0001$), CCL5 (2.6-fold, $p<0.0001$), CCL11 (2.7-fold, $p<0.0001$), CXCL10 (9.4-fold, $p<0.0001$) and CCL6 (6.0-fold, $p<0.0001$), when compared to levels in conditioned medium collected from un-primed mature adipocytes. In addition, TAM conditioned medium stimulated the expression levels of a variety of adipocyte-derived pro-inflammatory cytokines. The list includes M-CSF (1.6-fold), OPN (2.0-fold), VCAM-1 (2.6-fold), Gas6 (2.7-fold) and Chitinase-3-like protein 1 (3.6-fold) ($p<0.0001$).

My analysis of pro-angiogenesis factors showed that exposure of mature adipocytes to TAM conditioned medium significantly enhanced the levels of angiopoietin-1 (4.6-fold, $p<0.0001$), HGF (1.6-fold, $p<0.0001$), IGFB-6 (1.4-fold, $p<0.0001$) and MMP-3 (1.15-fold, $p<0.0001$), when compared to levels in cultures exposed to conditioned medium collected from un-primed mature adipocytes (Figure 50, panel B).

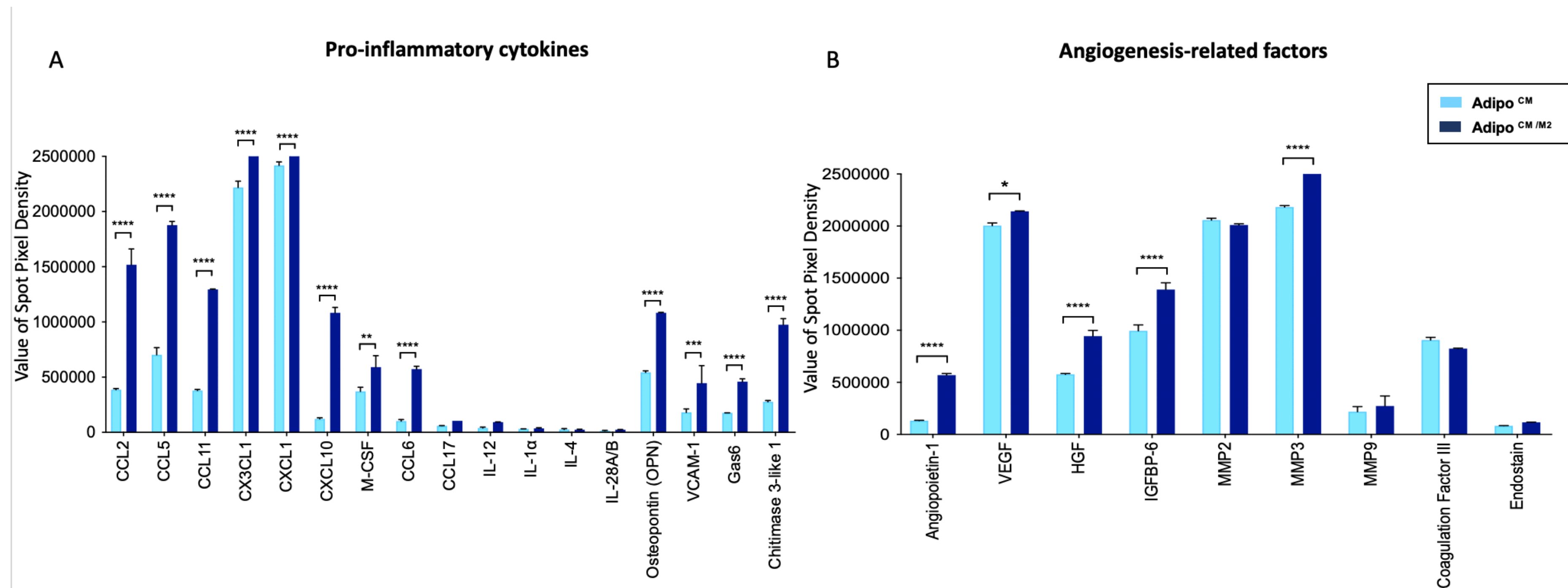


Figure. 50. M2 macrophage derived factors increased the secretion level of pro-inflammatory cytokines and angiogenesis-related factors in mature 3T3-L1 adipocytes. Two membrane-based cytokine array assays, with capture antibodies for relative expression levels of soluble proteins that are spotted in duplicate on the membrane, were incubated with Adipo $^{CM / M2}$ or Adipo ^{CM} for 24 hours, before undergoing chemiluminescence detection and analysis. Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ±SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Further evaluation revealed a subset of pathways and processes that are likely to be implicated in the regulation of the TAM - driven regulation of the crosstalk between mature adipocyte - PCa cell in the described model. The effects of M2 macrophage conditioned medium derived factors stimulated mature adipocytes on the production of soluble factors was expressed as percentage of Adipo ^{CM}. As shown in figure 51 (panel A), 21 soluble adipocyte-derived factors were considered to be differentially regulated with adipogenesis. The figure 51 (panel B) showed that a Venn diagram of the overlapping role for those factors in the regulation of the crosstalk of PCa cells, adipocytes and TAMs. Supplementary table 3 showed the summary of differentially regulated factors produced by conditioned medium from mature 3T3-L1 adipocytes primed with M2 macrophage conditioned medium based on the raw data from cytokine array assay.

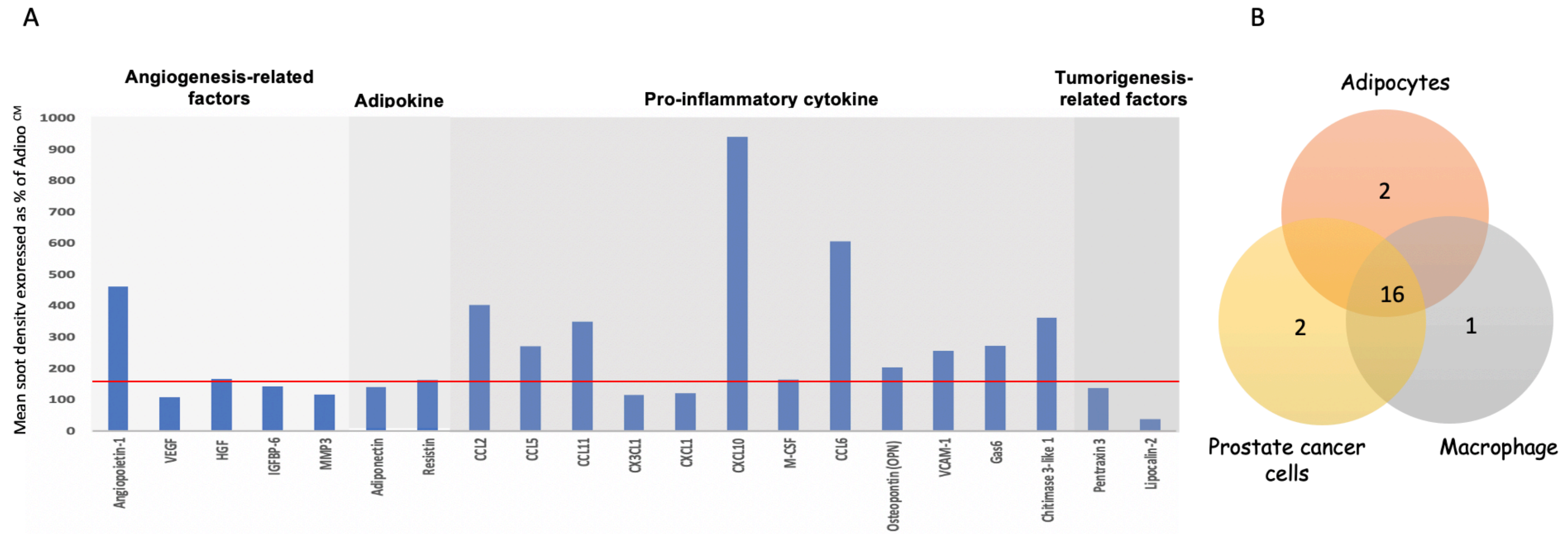


Figure. 51. TAMs derived factors increased the levels of adipocyte-derived pro-inflammatory factors. A). Microarray analysis of soluble factors derived from mature adipocytes conditioned medium treated M2 macrophage conditioned medium, using Proteome Profiler TM Mouse XL Cytokine Array Kit (R&D systems). B). Venn diagram of differentially expressed adipogenesis mediators and pro-inflammatory mediators in conditioned medium from the mature adipocytes that stimulated by M2 conditioned medium.

6.4.4. M2 macrophages secrete high levels of pro-inflammatory, pro-angiogenesis and pro-tumorigenic factors

To explore the mechanism by which the M2 macrophages subtype in the regulation of adipocytes – PCa cells, I utilized protein micro-array analysis to identify and quantify the levels of the soluble adipokines, tumorigenic, pro-angiogenesis and pro-inflammatory mediators in the conditioned medium from M2 macrophages. Using the Human XL Cytokine Array Assay, I analysed the levels of 105 pro-inflammatory cytokines and soluble proteins in conditioned medium obtained from polarized M2 monocytic THP-1 macrophages and their non-polarized control.

First, I detected significant increase in the expression level of the pro-angiogenesis factor MMP-9 ($p<0.01$) in conditioned medium from M2 macrophage when compared with the control group (Figure 52, panel A). My analysis also detected significant increase in the level of the tumorigenic factor uPAR in conditioned medium from M2 macrophage ($p<0.05$), when compared to levels in cultures exposed to conditioned medium collected from non-polarized monocytic THP-1 cells (Figure 52, panel B).

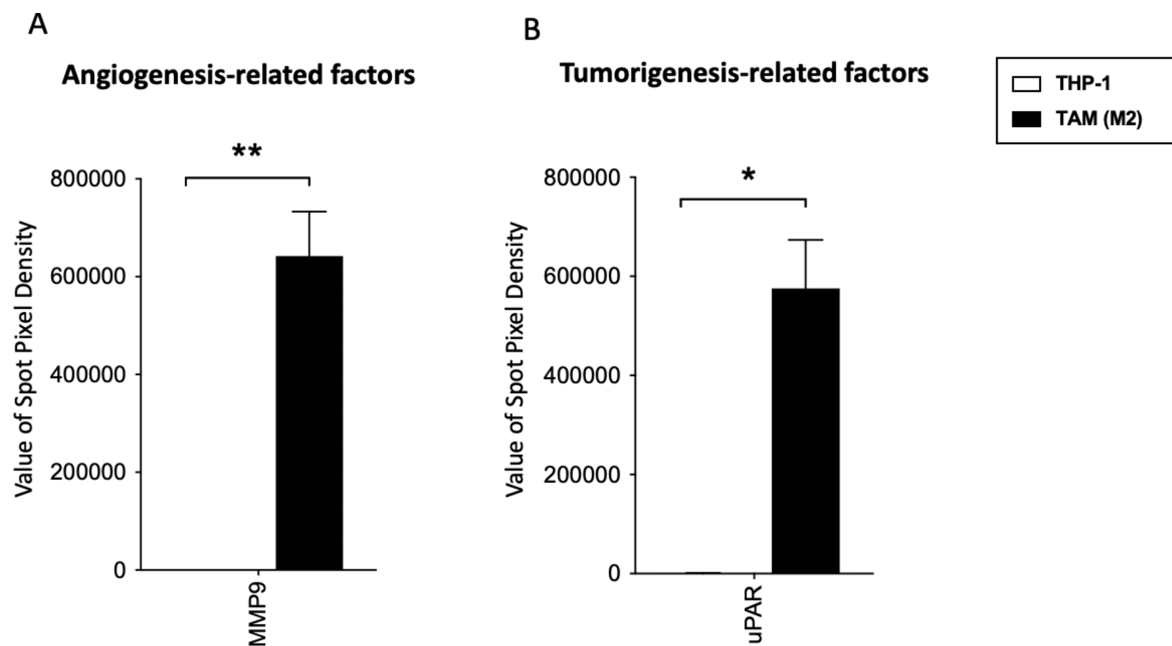


Figure. 52. M2 macrophages secreted high levels of angiogenesis- and tumorigenesis-related factors. Two membrane-based cytokine array assays, with capture antibodies for relative expression levels of soluble proteins that are spotted in duplicate on the membrane, were incubated with conditioned medium from polarised M2 macrophages and their un-polarised THP-1 cells for 24 hours, before undergoing chemiluminescence detection and analysis. Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ±SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

As can be seen in figure 53, polarization of human monocytic cells THP-1 into M2 macrophage subtype is associated with a significant increase in the level of a panel of pro-inflammatory mediators in in conditioned medium from M2 macrophage, when compared to levels in cultures exposed to conditioned medium collected from non-polarized monocytic THP-1 cells ($p<0.05$). The list includes IL-8, MIC-1, Chitinase-3-like protein 1 and various members of the CCL and CXCL families of chemokines, most notably CCL2, CCL3/4 and CXCL10. Supplementary table 4 showed a comprehensive summary of differentially regulated factors identified in M2 macrophage conditioned medium using the protein micro-array assay described.

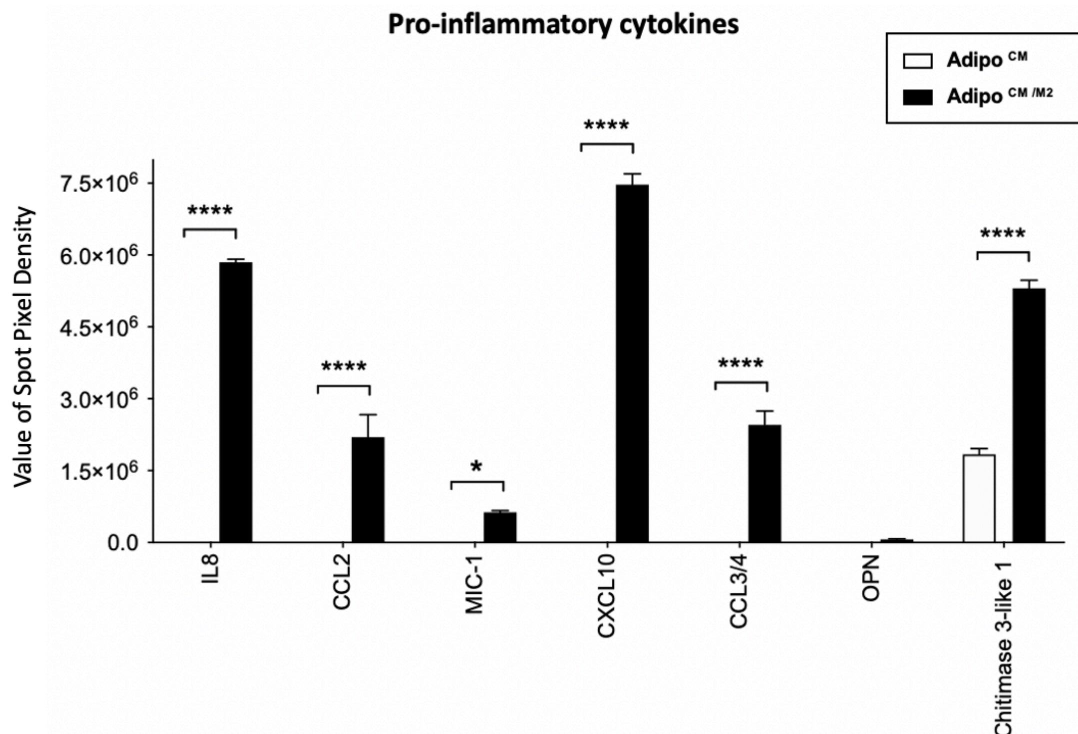


Figure. 53. M2 macrophages secreted high levels of various pro-inflammatory mediators. Two membrane-based cytokine array assays, with capture antibodies for relative expression levels of soluble proteins that are spotted in duplicate on the membrane, were incubated with conditioned medium from M2 macrophage and THP-1 for 24 hours, before undergoing chemiluminescence detection and analysis. Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ±SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

6.5. Discussion

TAMs are the most abundant immune cell populations within the tumour microenvironment. The classically activated M1 macrophage are involved in immunity, cytokine production, microbicidal and tumoricidal activity whereas the alternatively activated macrophage phenotype M2 is implicated in tissue remodelling, immune suppression and tumour progression (reviewed in Biswas et al., 2012). A number of published cancer studies in the literature have implicated TAMs, in particular M2 phenotype, in poor PCa prognosis and accelerated development of the disease (Bingle et al., 2002, Lewis and Pollard, 2006, Lanciotti et al., 2014). Furthermore, other studies have reported the increase of M2/M1 ratio in obese and HFD-fed mice (Weisberg et al., 2003, Hayashi et al., 2018, Fujita et al., 2019). However, little is known if and how different the TAM subtypes and their derived factors affects the ability of mature adipocytes to influence PCa cell motility and metastasis.

My results in this chapter first showed that derived factors from mature adipocytes cultured in presence of conditioned medium obtained from the different TAM subtypes, namely M0-, M1- and M2, had no significant effect on the *in vitro* growth of the hormone-dependent and castration-insensitive human and mouse PCa cells tested. This finding is inconsistent with previous animal studies that shown that M2-type macrophages promote tumour growth *in vivo* (Mantovani and Allavena, 2015, Dalen et al., 2018), a limited number of *in vitro* studies that have shown that the presence of TAMs, in particular M0- and M1-phenotypes, promotes the *in vitro* growth of PCa cells. M2 macrophages are involved in obesity and HFD alter the tumour immune microenvironment to promote cancer progression in experimental animals (Incio et al., 2016, Hayashi et al., 2018, Fujita et al., 2019). These findings led me to hypothesise that exposure of mature adipocytes primed with M2 derived factors influence the motility of PCa cells *in vitro*. In support for this hypothesis, my studies in this chapter confirmed that M2 derived factors enhance the ability of mature adipocytes to increase the invasive capabilities of the highly metastatic and castration-insensitive human PC3 and mouse RM1 PCa cells. This finding is consistent with a recent study published in 2020 that have shown that

conditioned medium from M2 macrophage stimulated the ability of mature adipocytes to increase the invasiveness of PCa cells (Hyo-Yeoung et al., 2020), and in broad agreement with an earlier study that reported that M2 macrophages facilitated the metastasis of PCa (Lewis and Pollard, 2006). Interestingly, the results of this chapter also showed that factors derived from mature adipocytes primed with M2 conditioned medium had no effects on the *in vitro* 2D migration of PCa cells, as assessed by wound healing assay. This finding is inconsistent with the findings above, thereby implying that M2 derived factors enhanced the production of adipocyte-derived factors that enhance cell invasion, but not cell migration and growth.

To investigate the mechanism(s) by which adipocyte and M2 derived factors influence PCa cell motility in my model, I first carried out detailed protein microarray analysis to measure the level of 111 mouse soluble factors in conditioned medium from mature adipocytes primed with M2 macrophage derived factors. My analysis detected a modest increase in the expression level of the angiogenesis-related factor MMP-3 in the conditioned medium from mature adipocytes that stimulated with M2 derived factors. MMPs play an important role in invasion, metastasis and angiogenesis (Kessenbrock et al., 2010), and MMP-3 is known to cause ECM degradation and facilitate tumour cells escape (Chambers et al., 1995, Kessenbrock et al., 2010). A significant increase in adipocyte-derived cytokine and adipokines that are known to contribute to the adipocyte driven PCa cell invasion was detected. For instance, my analysis indicated high level of the CCL and CXCL families of chemokine and adipocyte-derived cytokines that are likely to influence adipocyte cell behaviour in cancer. A significant increase expression of CCL2, CXCL10, OPN, VCAM-1 and Chitinase-3-like protein 1 showed in the conditioned medium from mature adipocytes primed with M2 macrophage derived factors. This is in agreement with the study that illustrated that highly expression of CXCL10 are potential biomarkers for the onset of adipose tissue inflammation during obesity in adipose tissue macrophage, and adipose tissue macrophage exhibits a gene expression profile similar to TAM (Mayi et al., 2012, Kochumon et al., 2020). Furthermore, recent studies have shown

that CXCL10 and its receptor CXCR3 promotes metastasis in several cancer types (Wu et al., 2012, Wightman et al., 2015). Previous studies reported that addition of M2 macrophage derived factors, in particular CCL2, increased the migratory and metastatic ability of PCa cells (Allavena et al., 2007, Lin et al., 2013). Additionally, increased expression of VCAM-1 has been implicated in the adipocyte-macrophage adhesion and PCa cells adherence to the bone forming cells, osteoblasts (Chung et al., 2017, Chang et al., 2018). OPN is expressed predominantly in obese individuals and obese animal models, and previous studies have shown that overexpression of OPN induced a proliferative and invasive advantage to hormone-dependent human LNCaP PCa cells (Khodavirdi et al., 2006, Kiefer et al., 2008). Furthermore, the expression levels of OPN and Chitinase-3-like protein 1 have been first found to increase in visceral adipose tissue from colon cancer patients (Catalán et al., 2011).

My data also showed that M2 derived factors enhanced the secretion of the tumorigenesis-related factors pentraxin 3 and lipocalin-2 by mature adipocytes. This finding is broadly consistent with the previous study that demonstrated that pentraxin 3, a recently identified biomarker, predicts immune escape in PCa (Stallone et al., 2014), and its knockdown suppressed tumour invasive capability by inhibiting the activation of pro-inflammatory NFκB signalling pathway (Ying et al., 2016). The increased level of lipocalin-2 plays a vital role in the regulation of macrophage polarization in obesity-associated inflammation (Catalan et al., 2009, Chakraborty et al., 2012, Guo et al., 2014). These studies implicated lipocalin-2 in adipocyte-macrophage interactions, but further studies are needed to ascertain if these factors play a role in PCa metastasis in obese or HFD animals.

I also carried out detailed microarray analysis that assessed the level of 105 human soluble factors in conditioned medium from polarized M2 monocytic THP-1 macrophages. This analysis detected high level of angiogenesis-related factor MMP-9, pro-tumour factor uPAR and various pro-inflammatory cytokines that are likely to contribute to adipocyte – M2 interactions. This is in agreement with the study by Karine et al. (2009) that reported that the polarization of macrophage to M2 phenotype enhance their ability to secrete of MMP-9 and to

regulate cell migration (Karine et al., 2009). Urokinase-type plasminogen activator (uPA) its receptor (uPAR) plays a significant role in the regulation of tumour progression and metastasis by initiating a proteolytic cascade that degrade components of extracellular matrix (Hu et al., 2014, Chen et al., 2019). Additionally, uPAR has been found to promote the ability of cancer cells to influence macrophage lineage commitment into M2 subtype (Hu et al., 2014). Additionally, studies have shown that M2 macrophage induced tumour cell invasion is inhibited by blocking the expression and activity of uPAR and MMP-9 (Chen et al., 2019). Chitinase-3-like protein 1 is another factor that was reportedly expressed in macrophages at the sites of inflammation and was found to contribute to macrophage lineage commitment into M2 subtype (Lee et al., 2011). Whilst these findings are in broad agreement with the present results that implicate M2 macrophage in regulating adipocytes - PCa cells crosstalk, future studies are needed to examine the role of these M2 derived factors in the ability of mature adipocytes to regulate PCa cell invasion and metastasis in animal models.

In conclusion, the results of my *in vitro* investigation thus far showed that factors derived from mature adipocytes promote the growth and motility of human and mouse PCa cell and priming mature adipocytes with the pro-inflammatory cytokine TNFα and M2 derived factors enhanced their ability to increase the invasion – but not 2D migration - of castration-insensitive human and mouse PCa cells. The follow up detailed protein micro-array analysis showed that these effects are mediated at least in part by a complex network of soluble factors that include NFκB-activating pro-inflammatory mediators, pro-tumorigenic factors, pro-angiogenesis, adipokines and chemokines. The findings of the present chapter led us to hypothesise that altered expression of an unknown key component(s) of the pro-inflammatory NFκB signalling pathway is implicated in the regulation of the interactions between mature adipocyte, immune cells and PCa cells.

CHAPTER 7

ALTERED CANONICAL NFκB SIGNALLING IS ASSOCIATED WITH OBESITY AND PROSTATE CANCER

7.1. Summary

In previous chapters I demonstrated that exogeneous pro-inflammatory mediators, particularly TNFα and M2 derived factors, play a role in the *in vitro* interactions between mature adipocytes and PCa cells. Protein microarray analysis of adipocytes and M2 derived soluble factors identified a number of factors that activate and/or are activated by NFκB signalling. NFκB is highly dysregulated in PCa, and a number of studies have shown that inhibition of the canonical NFκB pathway reduce the metastatic behaviour of PCa cells *in vitro*, *ex vivo* and *in vivo*. Thus, in this chapter, I carried out a detailed retrospective bioinformatic analysis of a number of publicly-available datasets in an attempt to identify the key component(s) of the NFκB signalling pathway implicated in PCa and obesity.

First, my analysis confirmed the involvement of IκBα - an essential regulator of canonical NFκB signalling - in the regulation of various processes associated with PCa and obesity. In PCa, amplification of the gene encodes IκBα (NFKBIA) occupied the majority proportion of the copy number variants in PCa patients. Additionally, my analysis also verified the positive correlation between NFKBIA expression and level of the standard markers of adipogenesis PPARγ and C-EBPβ, and showed that NFKBIA expression is associated with increased risk for obesity. Furthermore, the expression of PSMB5, a key component of the ubiquitin-proteasome pathway and IκBα degradation, is positively associated with reduced overall survival in metastatic PCa patients. In contract, PSMB5 expression was found to be negatively associated with the gene expression of PPARγ and C-EBPβ.

Overall, the results of this chapter indicate that two key components of canonical NFκB signalling pathway, namely IκBα and proteasome, are implicated in the regulation of PCa and adipogenesis. Thus, inhibition of NFκB by targeting of the canonical signalling pathway may be of potential therapeutic value in the treatment of PCa in obese patients.

7.2. Introduction

Inflammation is implicated in PCa and obesity (De Marzo et al., 2007, Dandona et al., 2004). NFκB transcription factors are essential mediators of the inflammatory and immune response (Ting et al., 2017). In the present study, TNFα and a number of pro-inflammatory mediators secreted by M2 have been found to play a role in the regulation of adipocyte - PCa cell interaction. Protein microarray analysis of adipocyte and M2 derived soluble factors identified a number of factors that activate and/or are activated by NFκB signalling. A number of studies have shown that canonical and non-canonical NFκB signalling and the level of pro-inflammatory NFκB-activating cytokines such as IL-1β, IL-6 or TNFα are upregulated PCa cells and adipocytes (Chen & Sawyers, 2002, Ghanim et al., 2004, Shoelson et al., 2007, Karin, 2009). Thus, implicating the pro-inflammatory NFκB signalling pathway in PCa and obesity.

A variety of stimuli, proteins and kinases are involved in the regulation of canonical and non-canonical NFκB signalling (Oeckinghaus and Ghosh, 2009). Canonical NFκB signalling involves the phosphorylation of IκB by the action of IKK complex, which consists of two catalytically active kinases, IKKα and IKKβ, and the adaptor protein NEMO. Once phosphorylated, IκB is targeted for ubiquitination and proteasomal degradation, thereby releasing the p65 (RELA) / p50 (NFKB1) dimers to translocate to the nucleus (Andrea et al., 2011). In non-canonical NFκB signalling, activation of NIK triggers the formation of IKKα dimers, that triggers the phosphorylation and processing of p100. The partial processing of p100 frees the p52 / RelB subunit to translocate to the nucleus (Andrea et al., 2011).

Dysregulation of NFκB signalling is implicated in a variety of inflammatory disorders, metabolic diseases and cancer (Courtois and Gilmore, 2006, Baker et al., 2011). Thus, examining the abnormalities and dysregulation in gene and protein expression of key component of NFκB signalling pathway would aid with the development of new treatments of PCa in obese patients.

7.3. Aim

The aim of this chapter is to employ a bioinformatic approach to establish which component(s) of canonical or non-canonical NFκB signalling is implicated in the regulation of PCa and obesity.

This aim was achieved by examining data from publicly available databases to assess:

- protein interactions of core components of the NFκB signalling pathway
- genetic changes in components of the NFκB signalling pathway in normal and malignant tissue from PCa patients
- DNA copy number variants in components of the NFκB signalling pathway in patients with primary prostate adenocarcinoma and metastatic PCa
- the correlation between expression of key components of NFκB signalling pathway and standard markers of adipogenesis in PCa patients

7.4. Results

7.4.1. Alteration of gene expression of components of the NFκB signalling pathway in prostate cancer and obesity

7.4.1.1. Altered expression level of NFKBIA is associated with increased risk for obesity and related complications

Obesity and inflammation are implicated in all aspects of PCa (De Marzo et al., 2007, Dandona et al., 2004). Insulin resistance and aberrant lipid metabolism are major risk factors in the progression of castration-insensitive PCa (Calle et al., 2003, Deep and Schlaepfer, 2016). The pro-inflammatory NFκB signalling pathway is constitutively upregulated in PCa cells and in obese individuals (Sweeney et al., 2004; Carlsen et al., 2009). Furthermore, my results in previous chapter have shown that mature adipocytes influence the growth of PCa cells via secreting various pro-inflammatory mediators (chapter 4).

To further examine which components of the canonical and non-canonical NFκB signalling pathway is associated with obesity, a bioinformatic analysis of a variety of publicly available resources was carried out using data obtained from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) from STRING database.

As shown in figure 54 (panel A), the analysis confirmed that expression of NFKBIA, CHUK, IKBKB and RELA are involved in adipocytokine signalling. Furthermore, the expression of NFKBIA, IKBKB and RELA have been found to play a role in insulin resistance (Figure 54, panel B). It was also found that NFKBIA contribute to lipid storage (Figure 54, panel C).

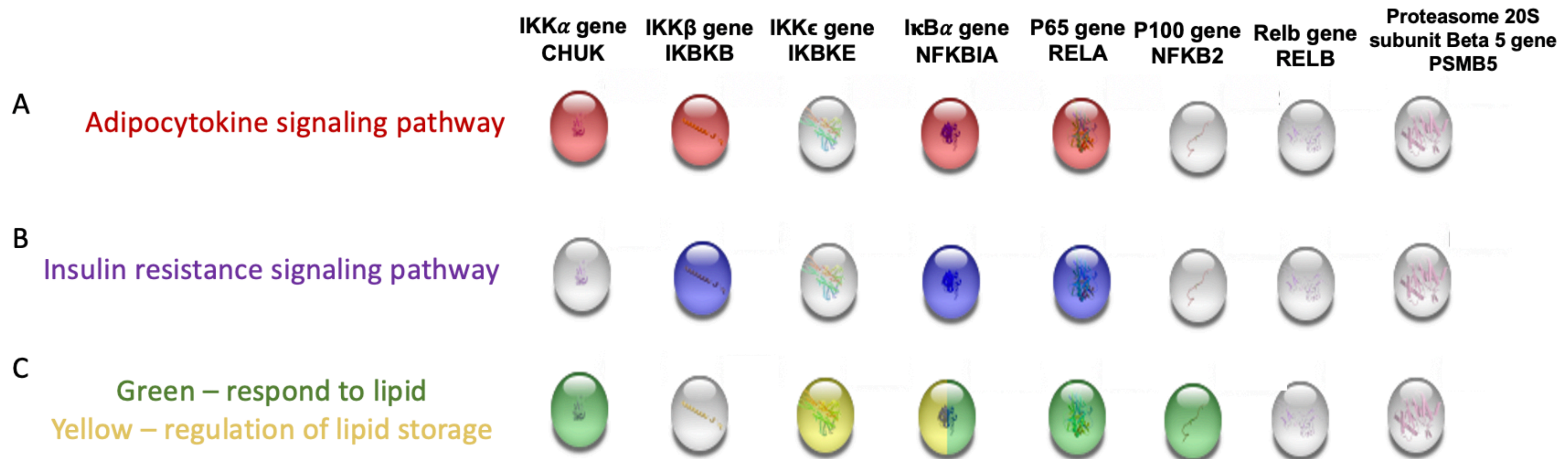


Figure. 54. **Relevant biological functions of NFκB proteins in obesity reported in STRING database.** (A) and (B) Role of NFκB proteins in KEGG pathways. The red-colored nodes link their indicated gene encoding NFκB components to adipocytokine signalling pathway. The purple-colored nodes link their indicated gene encoding NFκB components to insulin resistance signalling pathway. (C) Involvement of NFκB proteins established in biological process. The green-colored nodes link their indicated gene encoding NFκB components to lipid response process. The yellow-colored nodes link their indicated gene encoding NFκB components to lipid storage regulation process. The non-colored nodes have no link to activities addressed.

7.4.1.2. Altered expression level of NFKBIA is associated with inflammatory and immunity response to prostate cancer

Chronic inflammation in benign prostate tissue doubles the risk of developing PCa (Soni et al., 2017). Immune response is an essential component of tumorigenesis in PCa and my results in previous chapters have shown that pro-inflammatory cytokines TNFα and M2 derived factors play a role in adipocyte-driven PCa cell motility (Chapters 5 and 6). The pro-inflammatory cytokines TNFα regulate the immune response via activation of various pathways, particularly NFκB (Ting et al., 2017). TLRs play a critical role in the innate immune system by NFκB activation (Zhao et al., 2014).

To further examine which components of the canonical and non-canonical NFκB signalling pathway is associated with the immune response to PCa, a bioinformatic analysis of a variety of publicly available resources was carried out using data obtained from Gene Ontology and KEGG from STRING database.

My analysis confirmed that the expression of NFKBIA and RELA are implicated in chronic inflammation (Figure 55, panel A). Furthermore, it was found that NFKBIA, CHUK and IKBKB are involved in the regulation of cytokine-stimulated signalling associated with inflammatory and immune responses (Figure 55, panel B).

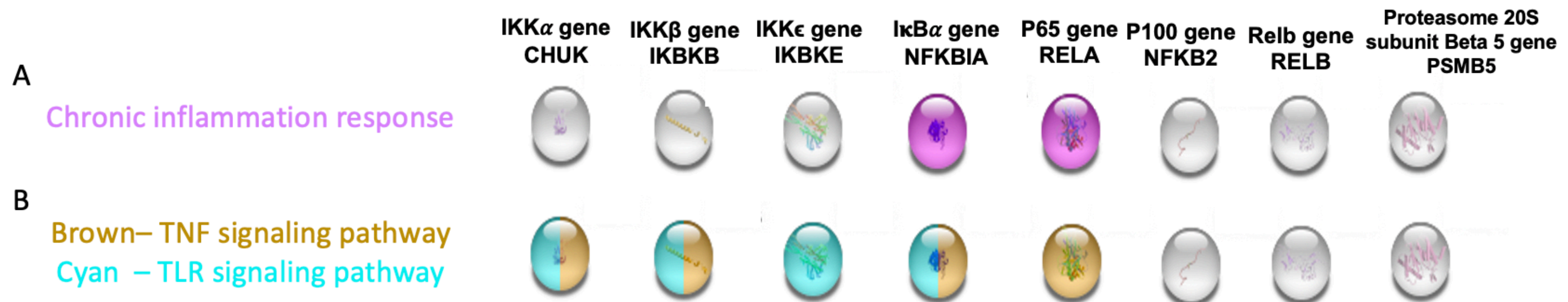


Figure. 55. Relevant biological functions of NFκB proteins in inflammatory and immune response of PCa reported in STRING database. (A) Association of NFκB proteins to biological functions related to inflammation in gene ontology datasets. The light purple-colored nodes link their indicated gene encoding NFκB components to chronic inflammation response. (B) Role of NFκB proteins in KEGG pathways. The brown-colored nodes link their indicated gene encoding NFκB components to TNF signalling pathway. The cyan-colored nodes link their indicated gene encoding NFκB components to TLR signalling pathway. The non-colored nodes have no link to activities addressed.

7.4.1.3. Altered expression level of NFKBIA and PSMB5 is associated with ubiquitin-proteasome signalling

The ubiquitin-proteasome system plays an important role in the activation of canonical NFκB signalling by facilitating the degradation of the inhibitory protein, IκB (Cusack et al., 1999, Yamamoto and Gaynor, 2001). Ubiquitination contributes to the development of PCa (Chen and Zhao, 2013), and obesity-dependent insulin resistance (Gökhan, 2006).

To further examine which components of the canonical and non-canonical NFκB signalling pathway is associated with IκBα ubiquitination, a bioinformatic analysis of a variety of publicly available resources was carried out using data obtained from the KEGG from STRING database.

My analysis confirmed the involvement of NFKBIA in the ubiquitin proteasome pathway and identified the proteasome subunit beta type-5 (PSMB5) as another important regulator in this pathway (Figure 56).

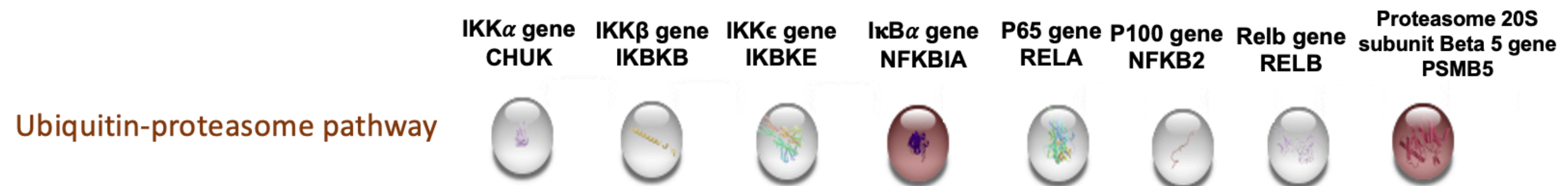


Figure. 56. Relevant metabolism process of NFκB proteins in ubiquitin-proteasome system reported in STRING database. Role of gene expression encoding IκBα in degradation induced NFκB activation. The brownness-colored nodes link their indicated gene encoding NFκB components to ubiquitin-proteasome signalling pathway. The non-colored nodes have no link to activities addressed.

The proteins interaction analysis by KEGG Pathway and Gene Ontology from STRING database are summarized in table 3. The analysis confirmed that NFKBIA, unlike other genes related to proteins in the NFκB signalling pathway, is implicated in all parameter studies. The list includes signaling pathways for adipocytokine, NFκB and ubiquitin-proteasome system.

***Table 3.* Protein interaction analysis of NFκB related genes**

	TNF Pathway	TLR Pathway	Ubiquitin-Proteasome pathway	Chronic Inflammation Response	Adiponectin Pathway	Insulin Resistance Pathway	Lipid Storage
IKKα gene CHUK	√	√			√		
IKKβ gene IKBKB	√	√			√	√	
IKKϵ gene IKBKE		√					√
IκBα gene NFKBIA	√	√	√	√	√	√	√
P65 gene RELA	√			√	√	√	
P100 gene NFKB2							
Relb gene RELB							
Proteasome subunit gene PSMB5			√				

7.4.2. NFKBIA is highly amplified in metastatic prostate cancer

NFKBIA, the gene encoding IκBα, plays an important role in the regulation of canonical NFκB signalling. Depletion of IκBα in PCa cells enhanced apoptosis (Carter et al., 2016) and pharmacological inhibition of IκBα phosphorylation reduces the ability of PCa to metastasise to bone and to cause bone damage (Marino et al., 2019). Thus, IκBα may be of value as a new therapeutic target for the treatment of advanced PCa.

In the present study, I used the online tool STRING to construct interactive maps of genes of NFκB pathway. As shown in figure 57, most interactions between NFκB proteins have been confirmed with curated databases. IκBα interacts with all key components of NFκB signalling pathway, except the non-canonical NFκB signalling protein, IKKε.

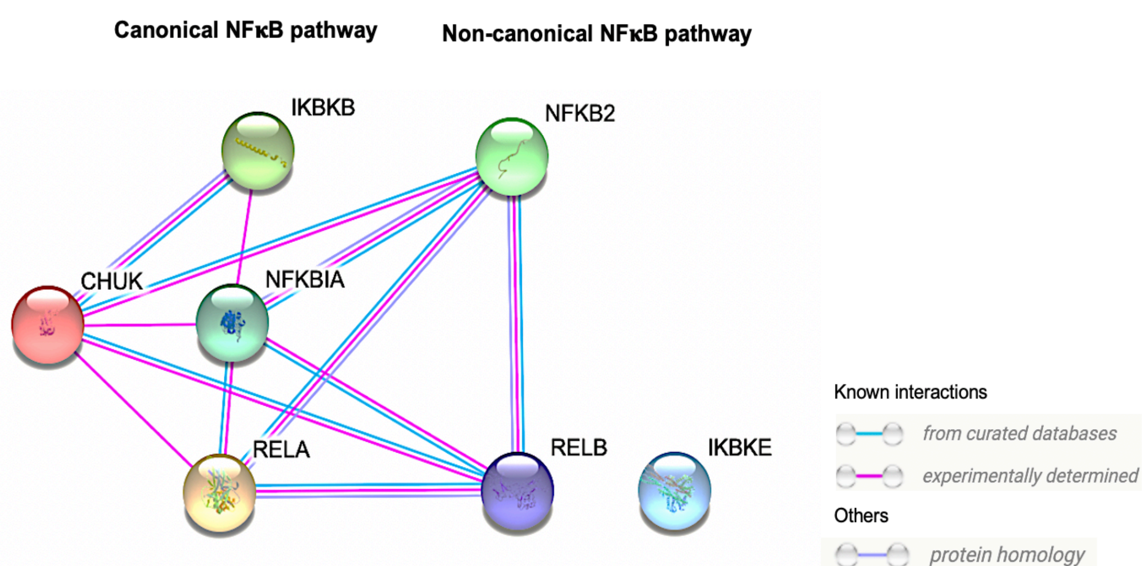


Figure. 57. *Reported interaction of NFκB proteins found in STRING database.* *Canonical and non-canonical* NFκB *proteins predicted and experimentally confirmed interactions between each other. Purple line indicates protein homology, blue line indicates information from curated databases and pink indicates experimentally determined interactions.*

Copy number variants (CNVs), including amplification, mutation and deletion, are important types of somatic aberration (Girirajan et al., 2011). CNVs are known to generate variations in disease phenotype and are an established measure for PCa risk factors (Francesca et al., 2012).

With this in mind, I first carried out a box plots analysis on the mRNA expression of NFκB in normal and cancerous prostatic tissue using the web-based tool GEPIA. As can be seen in figure 58, the analysis showed no evidence for involvement of proteins from the canonical and non-canonical NFκB signalling pathway in PCa in patients (n=492 cases) when compared to prostatic tissue from health control (n=52 cases).

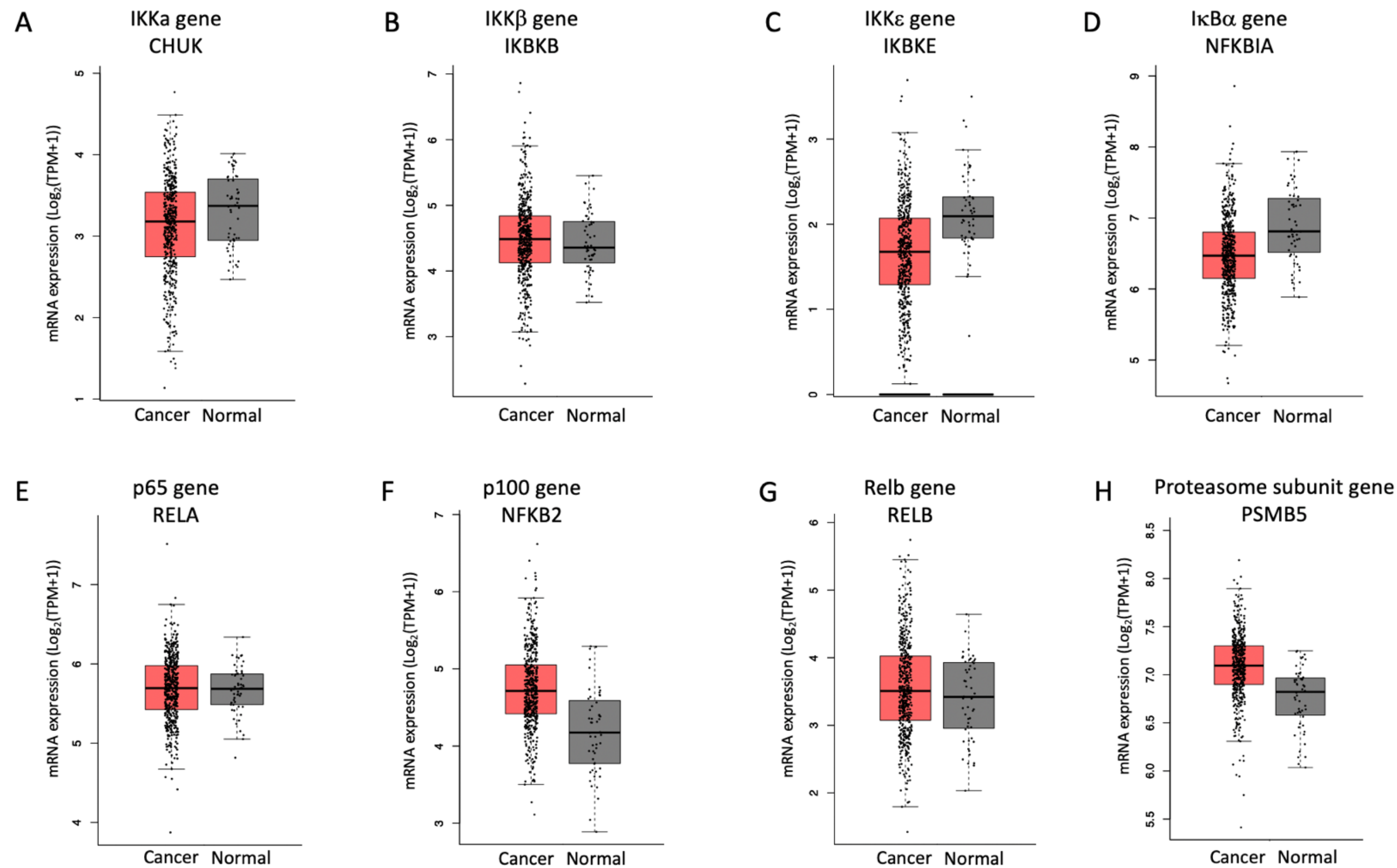


Figure. 58. **Gene expression of NFκB proteins acquired from GEPIA datasets.** (A-H) Expression of key components of NFκB in TCGA prostate cancer samples (n=492) compared to TCGA normal prostate tissue (n=52). Each dot means one tissue sample. Black lines indicate mean ± SD. Red dot plots represent tumour tissue, and grey dot plots represent normal tissue.

Next, I carried out a retrospective analysis of amplifications of genes of key components of the canonical and non-canonical NFκB signalling pathway in a large, combined cohort of PCa patients using cBioPortal online tool. Briefly, I used the PROSTATE (Eur Urol 2017; MSKCC/DFCI 2018; TCGA PanCancer Atlas 2018) database to identify CNVs in prostate adenocarcinoma patient samples, and data from a cohort of patients from the Metastatic Prostate Cancer Project (Provisional, Nov 2019; SU2C 2019) to identify CNVs in patients with advanced PCa.

This analysis revealed that NFKBIA was the most amplified gene from the canonical and non-canonical NFκB signalling pathway in metastatic PCa, where it was amplified in 11.85% of the patients. In comparison, RELA, CHUK and IKBKB copy numbers were amplified in 7.05%, 5.18% and 5.41% of patients (n=474), respectively (Figure 59, panel A). In addition, the analysis showed that the expression of IKBKB and NFKBIA was amplified at in 9% and 4.5% of patients (n=1572) with primary prostate tumours, respectively (Figure 59, panel A).

Further analysis of the combined cohorts of primary and metastatic PCa patients (n=2046) revealed that NFKBIA was amplified in 16.27% of PCa patients; 1.97% exhibited deep deletion and 0.88% exhibited mutations of NFKBIA, and a relatively small percentage (0.2%) exhibited fusion in the NFKBIA locus (Figure 59, panel B).

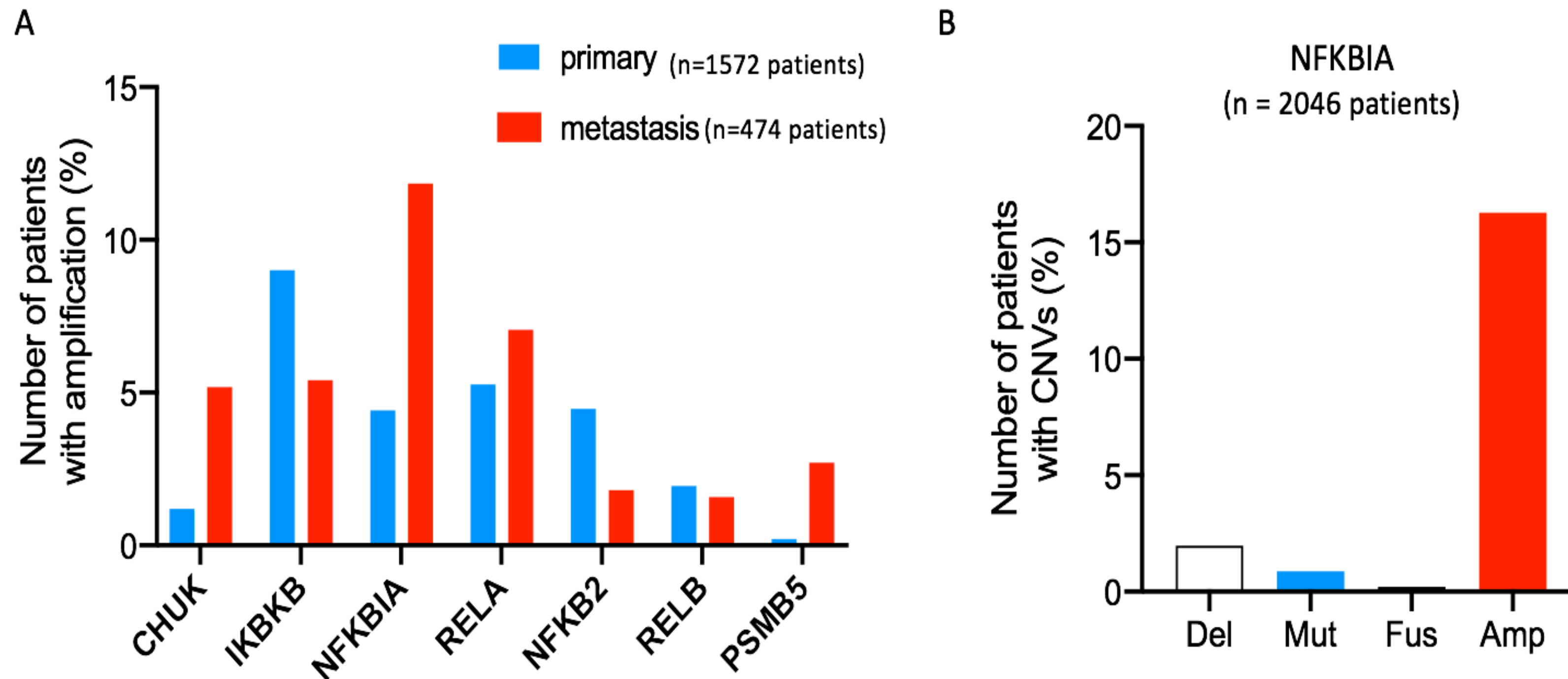


Figure. 59. **Copy number variants of NFκB proteins in patients with prostate cancer using online tool cBioPortal.** (A) Percentage of NFκB genes amplification in prostate adenocarcinoma patients obtained from the PROSTATE database (Eur Urol 2017; MSKCC / DFCI 2018; TCGA PanCancer Atlas 2018) (n=1572 cases) (blue bar), and in metastatic prostate cancer patients obtained from The Metastatic Prostate Cancer Project database (Provisional, Nov 2019; SU2C 2019) (n=474 cases) (red bar). (B) Percentage of gene encoding IkBa alterations involving deletion (Del), mutation (Mut), fusion (Fus) and amplification (Amp) found in primary and metastatic prostate cancer patients.

7.4.3. Altered expression of PSMB5 is associated with reduced overall survival in metastatic prostate cancer patients

NFκB overexpression has been detected in CRPC patients and it is associated with PCa progression and poor prognosis (Jin et al., 2014, Jin et al., 2015). I therefore examined association with alteration in the expression of genes in the canonical and non-canonical NFκB signalling pathway and survival rate in PCa patients by generating overall survival curves separating in primary and metastatic groups using the online database cBioPortal.

As can be seen the Kaplan-Meier curves in figure 60 (panel B), the overall survival in metastatic PCa patients is improved with altered expression of RELB ($p<0.05$). These data also revealed that metastatic PCa patients with overexpression of the proteasome β subunits (PSMB) – key component of the ubiquitin-proteasome pathway - exhibited a median overall survival of 10 months compared to a median overall survival 28 months in patients without alteration (18 months shorter overall survival, $p<0.0001$) (Figure 60, panel B). Interestingly, expression of PSMB5 had no effect on overall survival in the cohort of primary prostate adenocarcinoma patients used in this study (Figure 60, panel A).

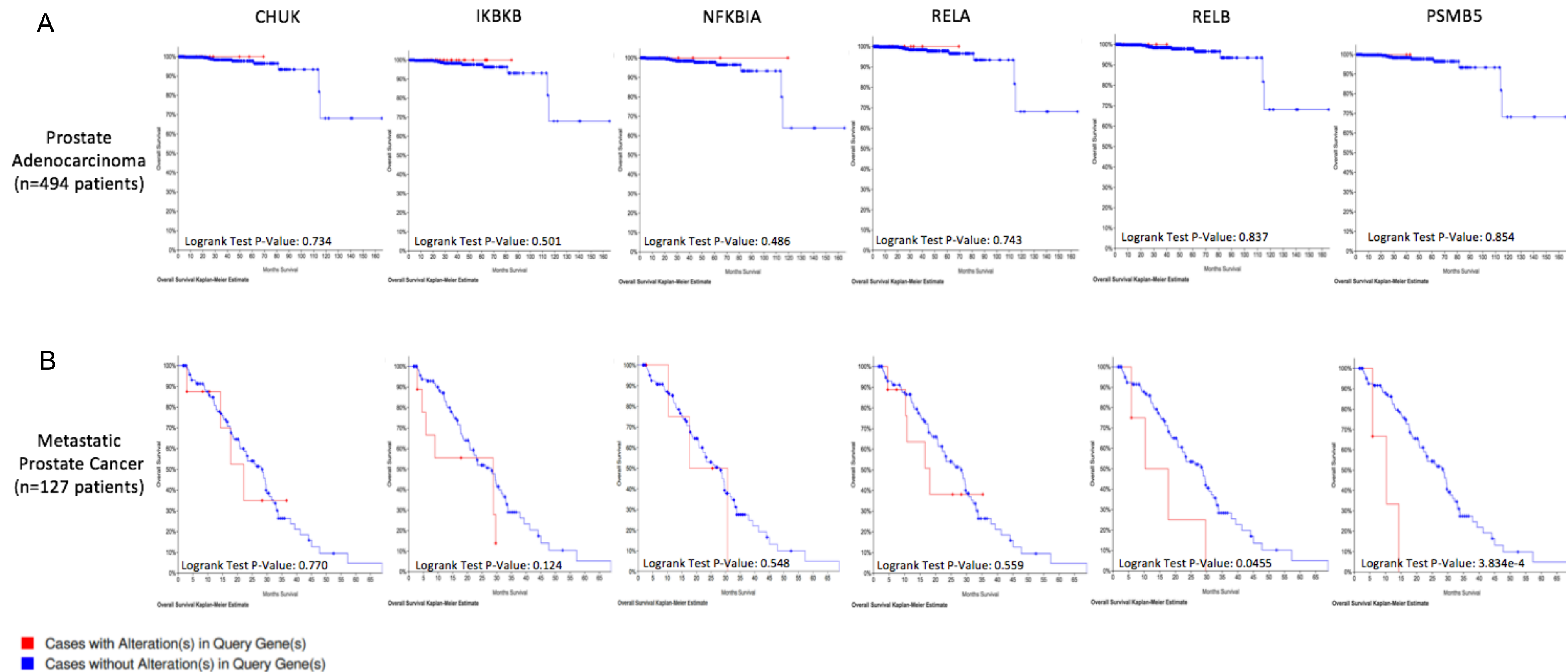


Figure. 60. Kaplan-Meier curves of overall survival of primary and metastatic prostate cancer in altered expression of NFκB proteins using online tool cBioPortal. (A) Kaplan-Meier overall survival plots in prostate adenocarcinoma patients of the PROSTATE (Eur Urol 2017; MSKCC / DFCI 2018; TCGA PanCancer Atlas 2018) studies (n=494 cases) with gene with alteration (red line) and gene without alteration (blue line) of NFκB proteins. (B) Kaplan-Meier overall survival plots in metastatic prostate cancer patients of the PROSTATE (SU2C 2019) and The Metastatic Prostate Cancer Project (provisional, 2019) studies (n=127 cases) with gene with alteration (red line) and gene without alteration (blue line) of NFκB proteins. P-values were calculated with the online tool from log-rank test to compare expression of each NFκB proteins.

7.4.4. Altered expression level of NFKBIA is positively associated with level of markers of adipogenesis

A number of studies have confirmed that obesity is a risk factor in PCa. In patients, obesity increases the incidence, accelerate the progression and contributes to poor clinical outcomes of PCa (Langlais et al., 2019). However, the mechanisms by which obesity exerts these effects are not yet fully understood.

To examine the association between obesity and PCa, Vidal and colleagues conducted a retrospective analysis in 2017 within 4286 radical prostatectomy patients using the Shared Equal Access Regional Cancer Hospital (SEARCH) database. The data showed that overweight (hazard ratio (HR) 1.99, $p = 0.034$) and obesity (HR 1.97, $p=0.048$) are significantly associated with increased PCa mortality. However, obesity and overweight were found not to be associated with disease recurrence ($p \geq 0.189$) (data has not been shown here) (Vidal et al., 2017).

Adipocyte differentiation is controlled by a transcriptional cascade in which PPARγ and members of the C-EBP family are key players (Siersbæk et al., 2010). To investigate if the expression level of key components of the NFκB pathway is associated with standard markers of adipogenesis such as PPARγ and others, I conducted pair-wise gene expression correlation analysis between these parameters in sets of primary tumours and metastatic PCa and visceral adipose tissue using online tool GEPIA. The strength of association was determined by the Pearson's correlation coefficient (R value) (Mukaka, 2012). The analysis revealed that overexpression of NFKBIA in primary and metastatic PCa correlates positively with PPARG (gene encoding PPARγ) (R=0.5) (Figure 61) and CEBPB (gene encoding C-EBPβ) (R=0.73) (Figure 62). In contrast, the gene encoding the proteasome subunit 20S showed an inverse correlation with the expression of PPARγ (R=-0.57) (Figure 61) and C-EBPβ (R=-0.55) (Figure 62). The R values collected from two identified adipogenesis-related markers are summarized in table 4.

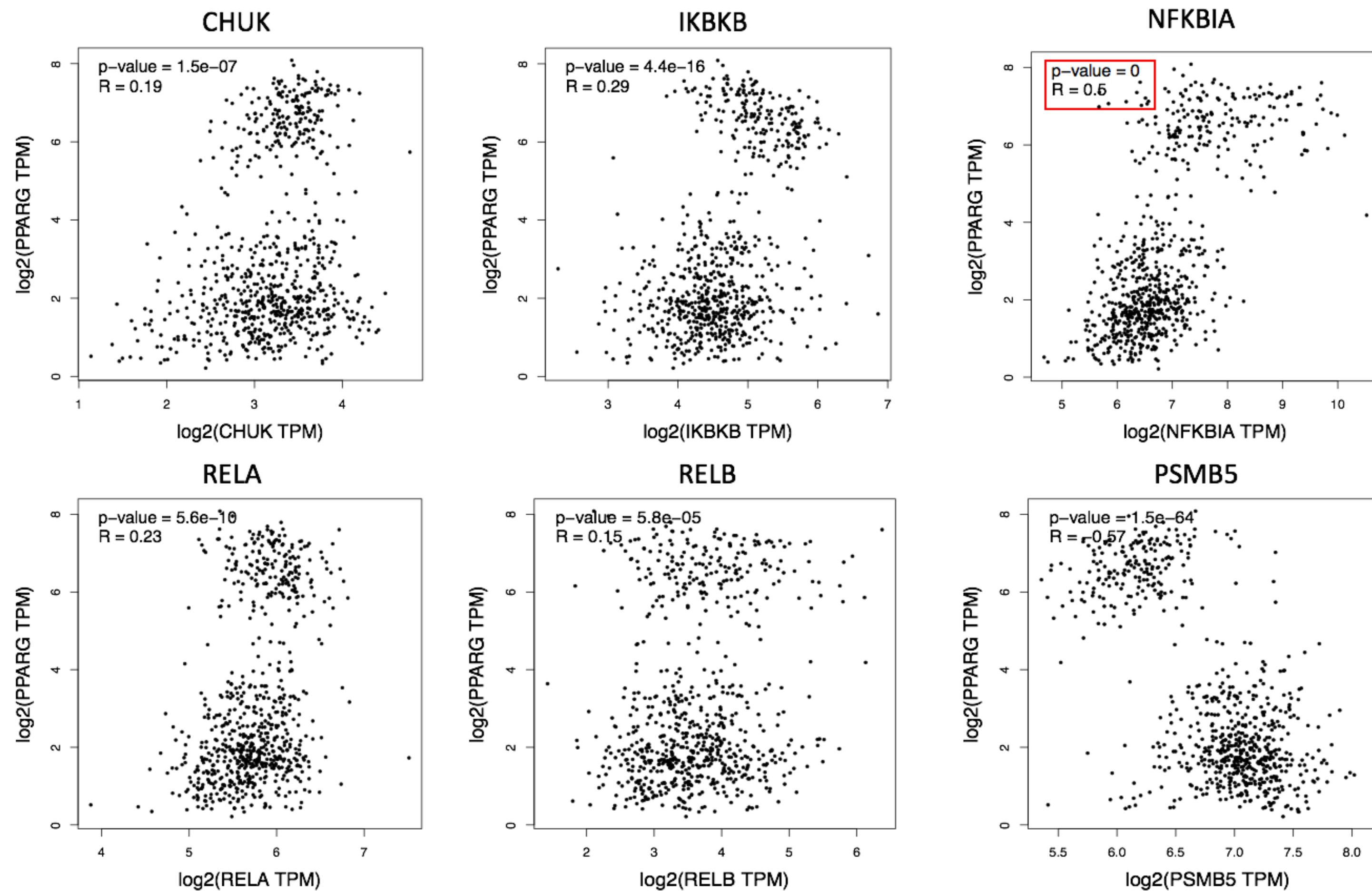


Figure. 61. Correlation of mRNA expression between angiogenesis standard marker PPARγ and NFκB proteins of prostate adenocarcinoma patients using GEPIA. Data collected from TCGA database of prostate adenocarcinoma patients and Genotype Tissue Expression of visceral adipose tissue. The genes of interest can be normalized by other genes. R value is the correlation coefficient indicating the strength of association (R=-1 to 1, -1 means no correlation completely).

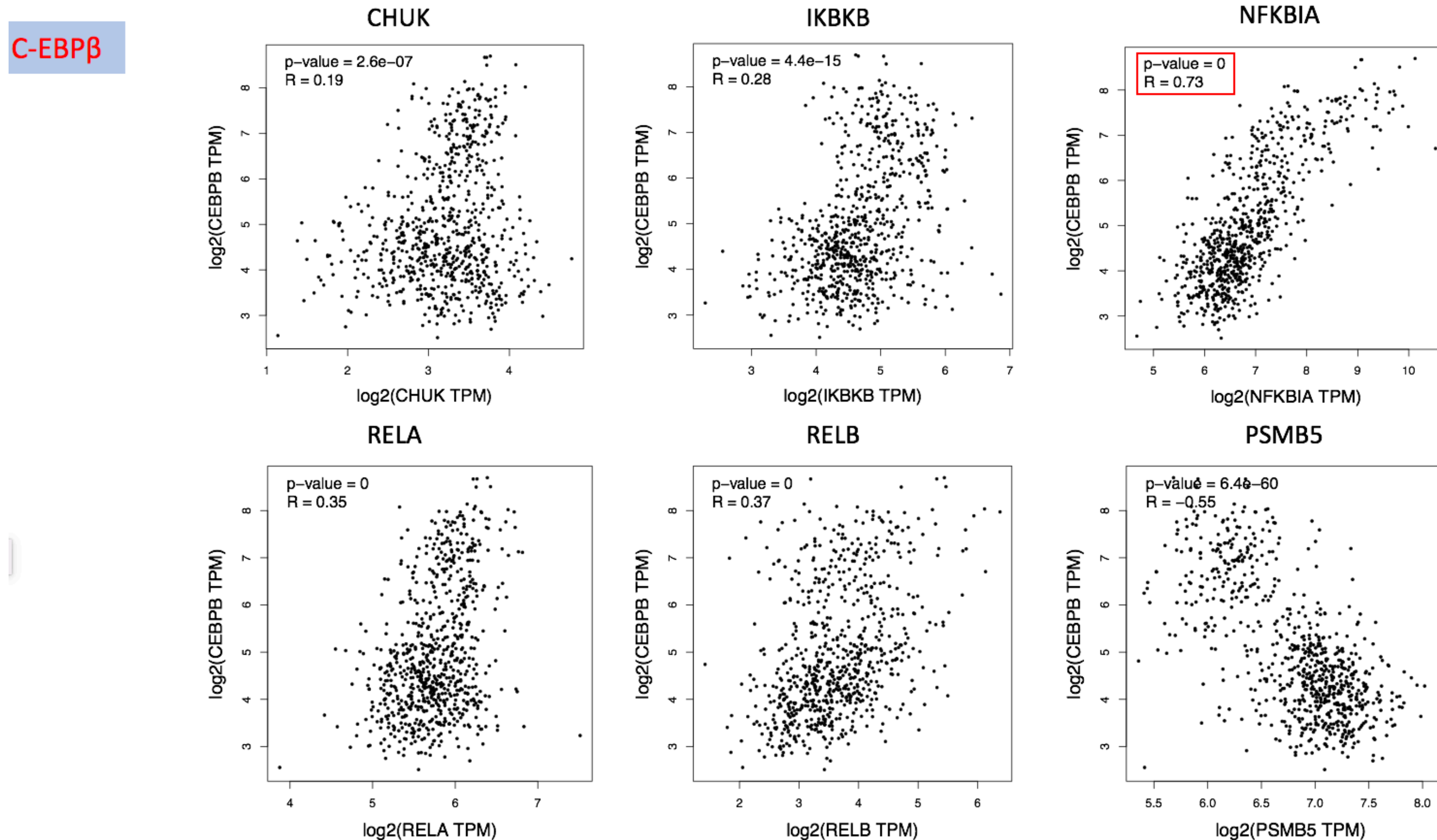


Figure. 62. Correlation of mRNA expression between angiogenesis standard marker C-EBPβ and NFκB proteins of prostate adenocarcinoma patients using GEPIA. Data collected from TCGA database of prostate adenocarcinoma patients and Genotype Tissue Expression of visceral adipose tissue. The genes of interest can be normalized by other genes. R value is the correlation coefficient indicating the strength of association (R=-1 to 1, -1 means no correlation completely).

Table 4. **R values of correlation values of standard markers of adipogenesis cascade with key components of NFκB signalling pathway in PCa patients.**

	PPARγ	**C-EBPβ**
CHUK	0.19	0.19
IKBKB	0.29	0.28
NFKBIA	0.5	0.73
RELA	0.23	0.35
RELB	0.15	0.37
PSMB5	-0.57	-0.55

7.5. Discussion

Obesity and inflammation are major risk factors in PCa (De Marzo et al., 2007, Dandona et al., 2004), and findings from the present research and previous studies indicate a variety of pro-inflammatory mediators of that activate and/or are activated by NFκB play a role in the interaction between PCa cells and adipocytes (Chen & Sawyers, 2002, Ghanim et al., 2004, Shoelson et al., 2007, Karin, 2009). Although the pro-inflammatory NFκB signalling pathway has been implicated in PCa and adipogenesis (Sweeney et al., 2004; Carlsen et al., 2009b), the underlying mechanism(s) by which different components of canonical and non-canonical NFκB signalling pathway regulate PCa cell – adipocyte interaction remains poorly understood.

In this chapter, I utilized data from a number of publicly available databases and resources to investigate the involvement of different components of the canonical and non-canonical NFκB signalling in PCa and obesity. My analysis of protein-protein interaction networks confirmed that the expression of NFKBIA - the gene encoding the key component of canonical NFκB mediator IκBα - plays an important role in the regulation of the pro-inflammatory and adipocytokine signalling pathway tested. Furthermore, NFKBIA has been found to be the most amplified NFκB related gene in metastatic PCa patients when compared to other NFκB related genes that overexpressed in this cohort. Together, these results further confirmed previous findings from a number of studies, including ours, that shown that the expression of key components of the canonical NFκB signalling pathway are implicated in the promotion of PCa progression, metastasis and osteolytic bone damage (Inoue et al., 2007, Jin et al., 2008, Marino et al., 2019). It is interesting, the analysis in this chapter failed to show any association between non-canonical NFκB signalling even though a number of previous studies have shown that key components of this pathway, particularly IKKα are implicated in PCa initiation and progression (Michael, 2006, Mahato et al., 2011, Aljeffery, 2019).

Next, the present gene correlation analysis in this chapter indicated that the gene expression of NFKBIA positively correlates with the expression level of two standard markers of

adipogenesis and obesity, namely PPARγ and C-EBPβ. Previous studies indicated that the adipogenic markers PPARγ is highly expressed in PCa cells (Elisabetta et al., 2000), whilst C-EBPβ levels are elevated in castration-resistant PCa samples (Barakat et al., 2015).

My analysis also confirmed that NFKBIA is involved in the regulation of ubiquitin-proteasome signalling pathway, consistent with a number of previous studies that demonstrated that ubiquitin is the key player in the degradation of the NFκB inhibitor IκBα, IκB kinase activation and nuclear translocation (Chen, 2005, Lichter et al., 2012). However, the results in this chapter showed that the expression of PSMB5 is negatively associated with the gene expression of PPARγ and C-EBPβ. This finding is broadly consistent with previous studies that have shown that obesity is associated with a decrease in plasma ubiquitin level and ubiquitinated proteins accumulation that in turn lead to a reduction in proteasome expression (Ignacio-Souza et al., 2014, Chang et al., 2009). Taken together, these findings indicate that dysregulation of the ubiquitin-proteasome system is an important mechanism involved in the progression of obesity. Thus, targeting the ubiquitin-proteasome-NFκB axis in obese individuals may be of value in the treatment of PCa.

Although the effects of pharmacological inhibition of key components of the TRAF/IKK/NFκB axis on the initiation and progression of PCa cells has been previously investigated (Gasparian et al., 2002, Michael et al., 2002, Michael, 2006, Yemelyanov et al., 2006), the findings in this – and previous - chapters imply selectively targeting the phosphorylation and degradation of IκBα represents a therapeutic strategy that is needed further investigation in pre-clinical models of PCa. In fact, a recent *in vitro* and *ex vivo* studies by our group has demonstrated that treatment of PCa cells with the verified proteosomal and IκB inhibitor Parthenolide reduced their ability to move, influence bone cells and cause osteolysis (Marino et al., 2019). Thus, futher *in vitro* and *in vivo* studies are needed.

CHAPTER 8

PHARMACOLOGICAL INHIBITON OF CANONICAL NFκB SIGNALLING DISRUPTS MACROPHAGE – ADIPOCYTE – PROSTATE CANCER CELL INTERACTIONS *IN VITRO*

8.1. Summary

The canonical NFκB-ubiquitin-proteasome axis plays a key role in inflammation, cancer and obesity. Accumulating evidence from the present *in vitro* studies, meta-analysis and bioinformatic analysis implicates the role of pro-inflammatory cytokine TNFα and a number of M2 and adipocyte derived factors that activate and/or are activated by the NFκB-ubiquitin-proteasome axis in the regulation of adipocyte - PCa cell interaction. In this chapter, I performed a number of mechanistic experiments to examine the activation of canonical IκBα in adipocytes and PCa cells by M2- and adipocyte-derived factors, and test if inhibition of cancer-specific IκBα activation by the verified proteasome inhibitor Bortezomib (BTZ) affects PCa cell – adipocyte - macrophage interactions.

First, Western Blot data showed that the ratio of p-IκBα (active) / total IκBα (inactive) is increased in castration-insensitive human PC3 and mouse RM1 cells by derived factors from mature adipocytes primed with M2 conditioned medium, indicative of canonical NFκB activation. In contrast, priming adipocytes with TNFα failed to enhance their ability to increase p-IκBα/IκBα ratio in human LNCaP and PC3 and mouse RM1 PCa cells. Treatment with BTZ reduced the ability of adipocytes to increase the *in vitro* viability and invasion of PC3 and RM1 cells. In adipocytes, I confirmed that expression level of total IκBα is reduced during mouse adipocyte differentiation and maturation, indicative of enhanced activation of canonical IκB/NFκB signalling pathway. Finally, I showed that p-IκBα/total IκBα ratio is increased in mature adipocytes primed with conditioned medium from M2 macrophages.

Overall, the results of this chapter confirm the role of the canonical IκBα/ubiquitin-proteasome/ NFκB axis in the interactions of M2 macrophages, adipocytes and castration-insensitive PCa cells, and indicate that treatment with the proteasome / IkBa degradation inhibitor BTZ may be of value in the reduction of PCa cell growth and motility in obese individuals. However, further *in vivo* studies are needed to confirm the findings of the present *in vitro* studies.

8.2. Introduction

In previous chapters I showed that the pro-inflammatory TNFα and a number of M2 and adipocyte-derived factors that are known to activate and/or are activated by NFκB signalling enhanced the *in vitro* motility of highly metastatic castration-insensitive PCa cells. Canonical NFκB signalling plays a role in PCa initiation and progression to metastatic disease and it is implicated in obesity (Carlsen et al., 2009a, Vykhovanets et al., 2011, Jin et al., 2015). Over recent years, it has become evident that canonical NFκB is one of the key signalling pathways that provides a link between PCa and obesity. Thus, in this chapter I hypothesise that M2 and adipocyte-derived factors activate canonical NFκB signalling in PCa cells, and pharmacological inhibition of this pathway disrupts the ability of M2 macrophages and/or mature adipocytes to enhance the behaviour of PCa cells.

Activation of canonical NFκB signalling is regulated by the phosphorylation and proteasomal degradation of IκBα. A number of studies have shown that treatment with the verified inhibitor IκBα, parthenolide, inhibits the growth and induces apoptosis in a variety of PCa cancer cells and restores the sensitivity of androgen-insensitive PCa cells to chemotherapeutic agetns including docetaxel and enhances radiosensitivity of PCa cell lines such as DU145 and PC3 via NFκB inhibition (Bork et al., 1997, Hehner et al., 1998, Shanmugam et al., 2006, Kawasaki et al., 2009). A recent *in vitro* and *ex vivo* studies by our laboratory has shown that parthenolide reduced the motility of bone-seeking PCa cells and inhibited their ability to cause bone damage (Marino et al., 2019). The ubiquitin-proteasome system plays a key role in the regulation of canonical NFκB signalling, and the proteasome inhibitor BTZ is known to inhibit the phosphorylation of IκBα, reduce NFκB nuclear translocation and induce apoptosis of castration-insensitive PCa cells (Adams et al., 1999, Williams et al., 2003, Adams, 2004, Zheng et al., 2015).

8.3. Aims

The aim of this chapter is to test the hypothesis that macrophage and adipocyte-derived factors activate the canonical NFκB-ubiquitin-proteasome axis in PCa, and pharmacological inhibition of IκBα expression and phosphorylation status disrupts macrophage – adipocytes – PCa cell crosstalk. The proteasome inhibitor BTZ is approved for the treatment of metastatic cancers such as of relapsed multiple myeloma by the Food and Drug Administration (Robert et al., 2006, Mateos et al., 2006). Thus, I utliized this agent to test the effects of inhibition of canonical NFκB-ubiquitin-proteasome on the ability of M2 macrophages and/or mature adipocytes to enhance PCa viability and invasion *in vitro* (Figure 63).

These aims were achieved by examining:

- the protein expression of IκBα and p-IκBα in mouse mature adipocytes cultured in the presence and absence of the pro-inflammatory TNFα or conditioned medium from M2 macrophages
- the protein expression of IκBα and p-IκBα in PCa cells exposed to conditioned medium from mature adipocyte primed with TNFα or conditioned medium from M2 macrophages
- the effects of BTZ on the *in vitro* viability and invasion of the castration-insensitive human PC3 and mouse RM1 PCa cells

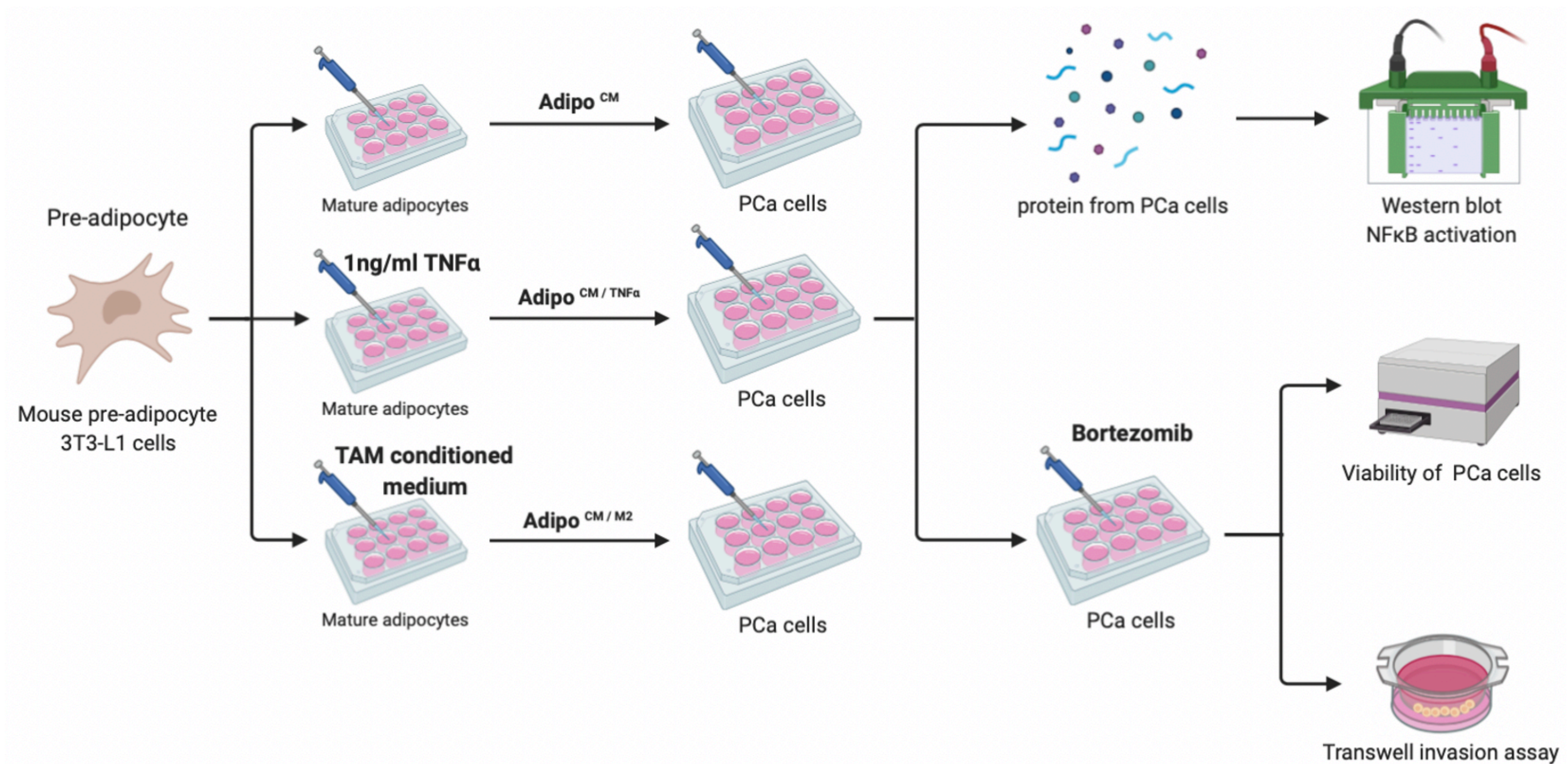


Figure. 63. **Schematic diagram of the investigation of effects of the role of canonical NFκB activation in the regulation of macrophage, mature adipocyte and PCa cell interactions.**

8.4. Results

8.4.1. Canonical NFκB activation in PCa cells is increased by derived factors from mature adipocytes primed with M2 conditioned medium

To investigate the role of canonical NFκB activation in the ability of M2 macrophage to affect adipocyte – PCa crosstalk, I first assessed changes in the ratio of phosphorylated (p-IκBα) to total (Tol-IκBα) IκBα in hormone-dependent and castration-insensitive human and mouse PCa cells cultured in the presence and absence of conditioned medium from mature mouse mature 3T3-L1 adipocyte that were primed with conditioned medium from M2 macrophages. The expression of phosphorylated and total κBα was assessed by Western Blot analysis as described in section 2.3.

As shown in figure 64, exposure of castration-insensitive human PC3 and mouse RM1 PCa cells to factors derived from mature adipocytes primed with M2 conditioned medium (Adipo $^{CM/M2}$, 20% v/v) caused a significant increase in p-IκBα to IκBα ratio (71% increase in human PC3 cells, $p<0.001$) (Figure 64, panel C) and (45% increase in mouse RM1 cells, $p<0.01$) (Figure 64, panel D), when compared to cultures exposed to adipogenic conditioned medium (Adipo ^{CM}, 20% v/v).

In contrast, M2 macrophage derived factors failed to enhance the ability of mature adipocytes to increase in p-IκBα/IκBα ratio in hormone-dependent human LNCaP PCa cells (Figure 64, panel B). The individual expression level of p-IκBα and IκBα in castration-insensitive human PC3 and mouse RM1 PCa cells from the experiments above are shown in supplementary figure 3.

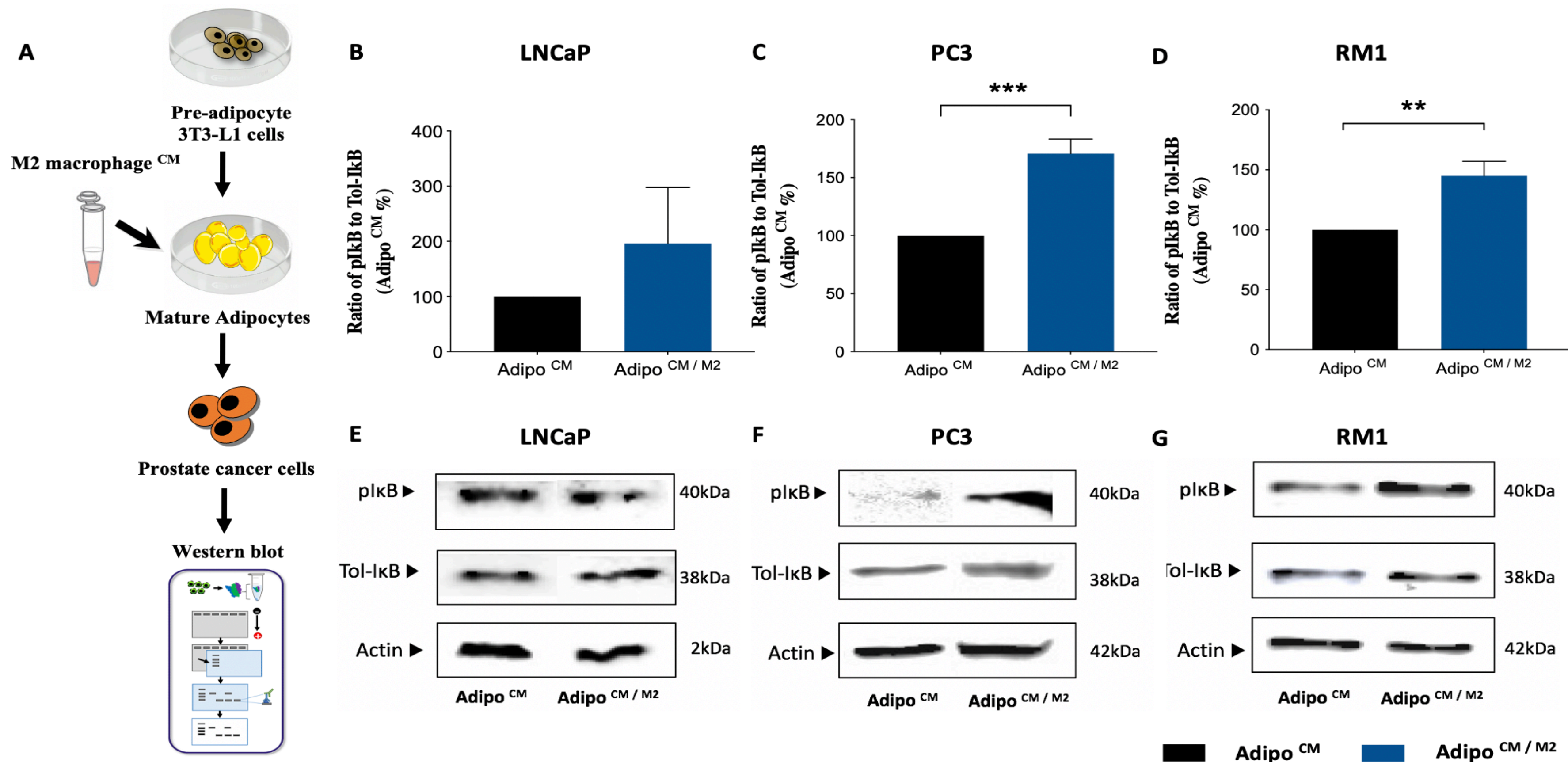


Figure. 64. M2 macrophage derived factors enhanced the ability of mature adipocytes to increase canonical NFκB activation in human and mouse PCa cells. (A) Graphic illustration for this experiment. (B-D) Western blot quantification represents the ratio of p-IκBα to IκBα levels that normalized to Adipo ^{CM} – treated group. (E-G) Total cell lysates were prepared as described in the materials and methods from PCa cells treated with conditioned medium. The blots were analysed for the indicated antibodies and anti-rabbit β-actin was used as the loading control. Results are from three independent experiments (N=3). Data were analysed using unpaired T-test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

8.4.2. Priming mature adipocytes with the pro-inflammatory TNFα failed to enhance their ability to increase canonical NFκB activation in human and mouse prostate cancer cells

To investigate the role of canonical NFκB activation in the ability of exogeneous inflammatory mediators to affect adipocyte – PCa crosstalk, I measured the ratio of p-IκBα to Tol-IκBα in hormone-dependent and castration-insensitive human and mouse PCa cells cultured in the presence and absence of conditioned medium from mature mouse mature 3T3-L1 adipocyte that primed with TNFα.

As shown in figure 65, conditioned medium collected from mature adipocytes (Adipo $^{CM/TNF\alpha}$, 20% v/v) primed with TNFα (1ng/ml) failed to affect p-IκBα/Tol-IκBα ratio in hormone-dependent human LNCaP (figure 65, panel B) or castration-insensitive human PC3 (figure 65, panel C) and mouse RM1 (figure 65, panel D) PCa cells, when compared to cultured exposed to conditioned medium collected from un-primed mature adipocytes (Adipo ^{CM}, 20% v/v). The individual expression level of p-IκBα and IκBα in castration-insensitive human PC3 and mouse RM1 PCa cells from the experiments above are shown in supplementary figure 4.

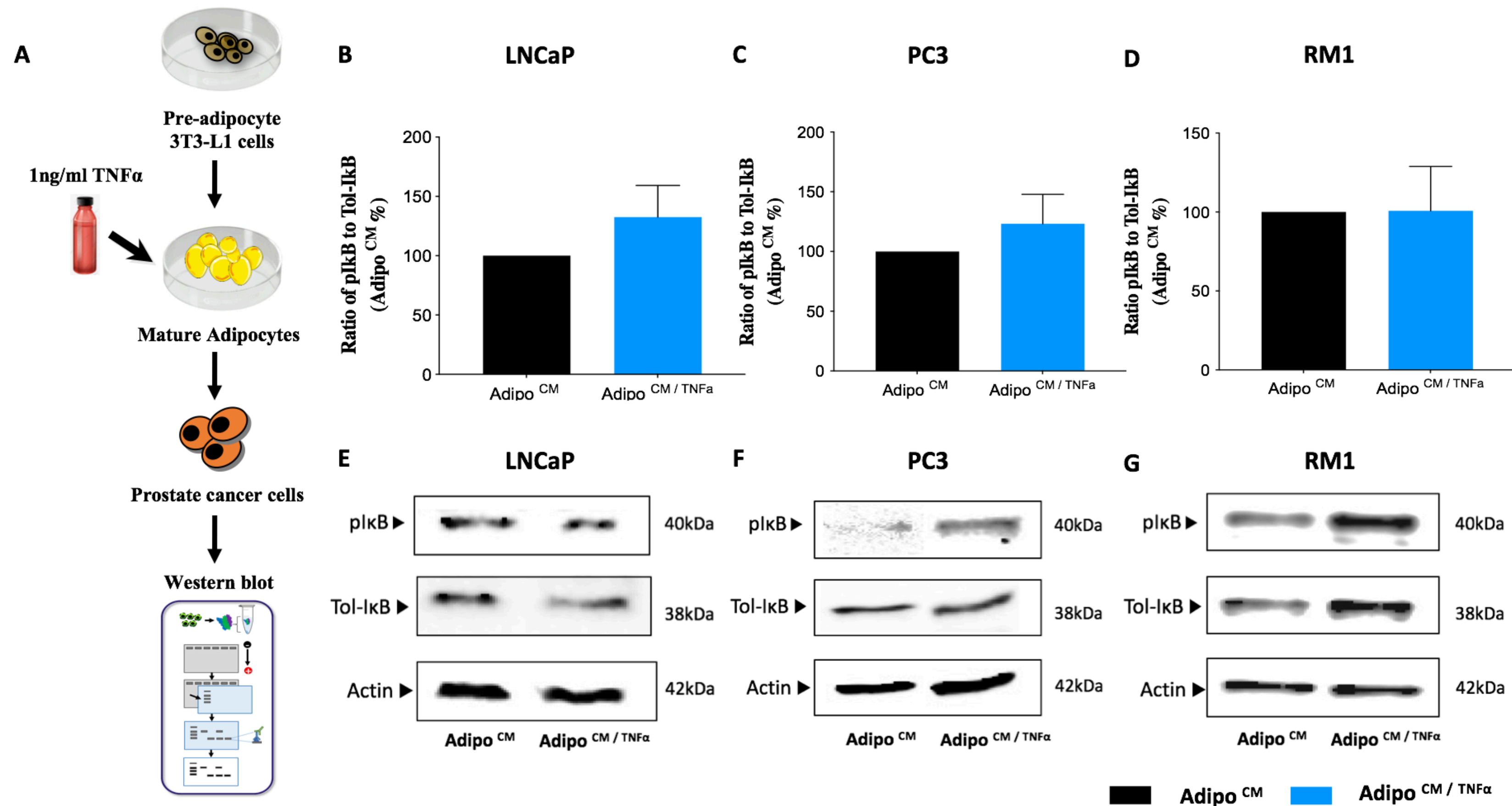


Figure. 65. TNFα failed to affect the ability of mature adipocytes to increase canonical NFκB activation in human and mouse PCa cells. (A) Graphic illustration for this experiment. (B-D) Western blot quantification represents the ratio of p-IκBα to IκBα levels that normalized to Adipo ^{CM} – treated group. (E-G) Total cell lysates were prepared as described in the 'materials and methods' from prostate cancer cells treated with conditioned medium. The blots were analysed for the indicated antibodies and anti-rabbit β-actin was used as the loading control. Results are from three independent experiments (N=3). Data were analysed using unpaired T-test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

8.4.3. Canonical NFκB activation is increased in mature adipocytes primed with conditioned medium from M2 macrophages

To investigate the role of canonical NFκB activation in adipocyte – macrophage crosstalk, I assessed p-IκBα/Tol-IκBα ratio in mature 3T3-L1 adipocytes cultured in the presence and absence of pro-inflammatory cytokine TNFα or factors derived from M2 macrophages. The expression of phosphorylated and total κBα was assessed by Western Blot analysis, as described in section 2.3.

As shown in figure 66, exposure of mature adipocytes to M2 conditioned medium caused a significant 2-fold ($p<0.05$) increase in p-IκBα/IκBα ratio (figure 66, panel A), when compared to controls. In contrast, exposure of mature adipocytes to the pro-inflammatory cytokine TNFα (1ng/ml) failed to affect p-IκBα/IκBα ratio (figure 66, panel D). The individual expression level of p-IκBα and IκBα in castration-insensitive human PC3 and mouse RM1 PCa cells from the experiments above are shown in supplementary figure 5.

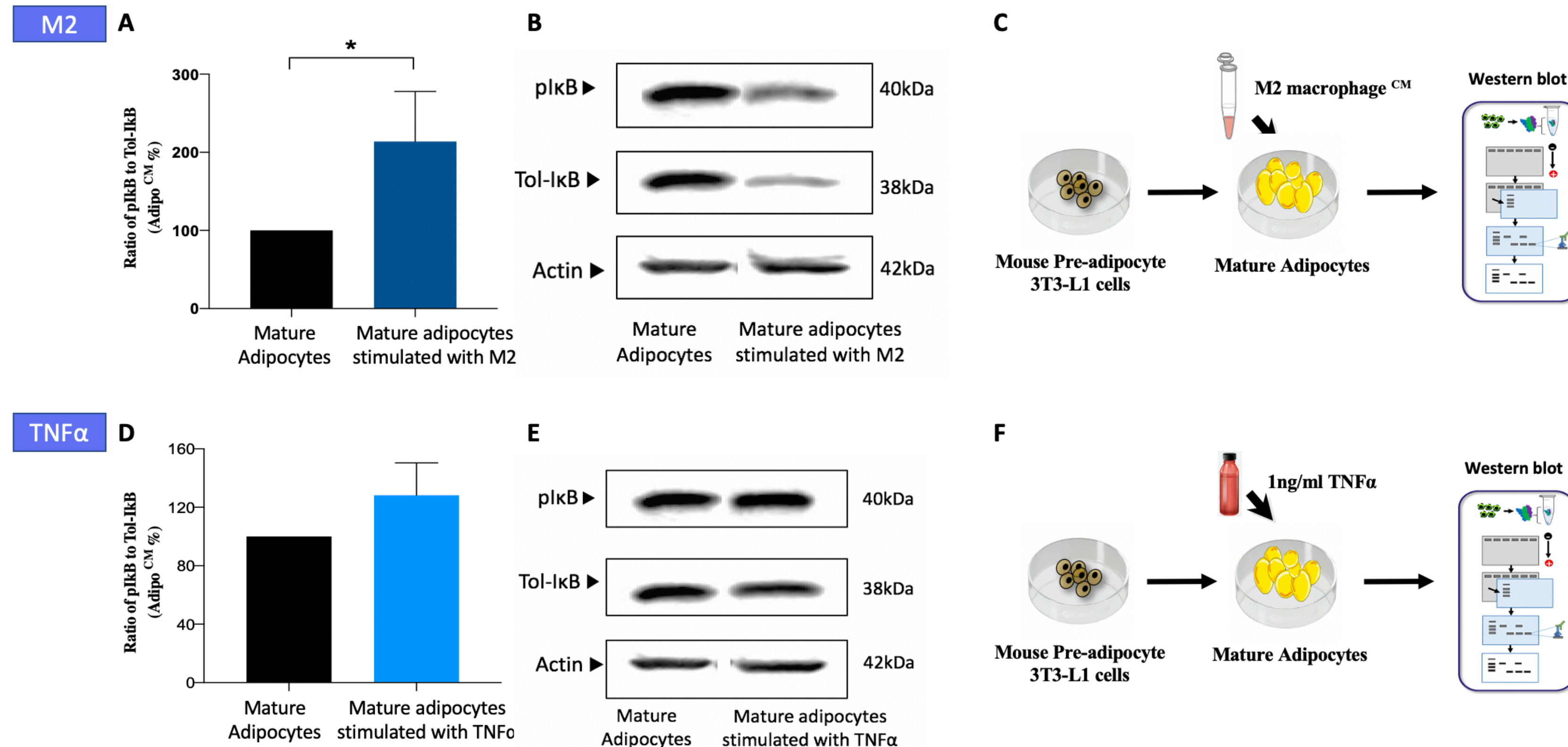


Figure. 66. M2 macrophage derived factors, but not TNFα, increased the canonical NFκB activation in mature adipocytes. (A and D) Western blot quantification represents the p-IκBα / IκBα ratio that normalized to protein expression in mature adipocytes. (B and E) Total cell lysates were prepared as described in the 'materials and methods' from mature adipocytes stimulated by pro-inflammatory cytokine TNFα or by factors derived from M2 macrophage conditioned medium. The blots were analysed for the indicated antibodies and anti-rabbit β-actin was used as the loading control. (E and F) Graphic illustration for the experiments. Results are from three independent experiments (N=3). Data were analysed using unpaired T-test (Prism 7.0). Error bars indicate mean ± SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

8.4.4. Expression level of total IκBα is reduced during mouse adipocyte differentiation and maturation

Finally, to examine canonical NFκB activation during adipocyte differentiation and maturation, I measured the expression of total IκBα (Tol-IκBα) in pre- and mature adipocytes. In this chapter, mature adipocytes were generated from the mouse pre-adipocyte 3T3-L1 as described in the Material and Methods (section 2.2.1.1). Total protein from pre- and mature adipocytes was collected on day 0, 3, 7, 10 and 12 and the expression of Tol-IκBα was assessed by Western Blot analysis, as described in section 2.3.

As shown in figure 67, the expression level of total IκBα was significantly reduced in mouse 3T3-L1 adipocyte after 7, 10 and 12 days, indicative of activation of canonical NFκB signalling and ubiquitin-proteasome system. Expression of IκBα was significantly reduced by 47%, 48% and 72% after 7 ($p < 0.05$), 10 ($p<0.05$) and 12 ($p<0.001$) days, respectively.

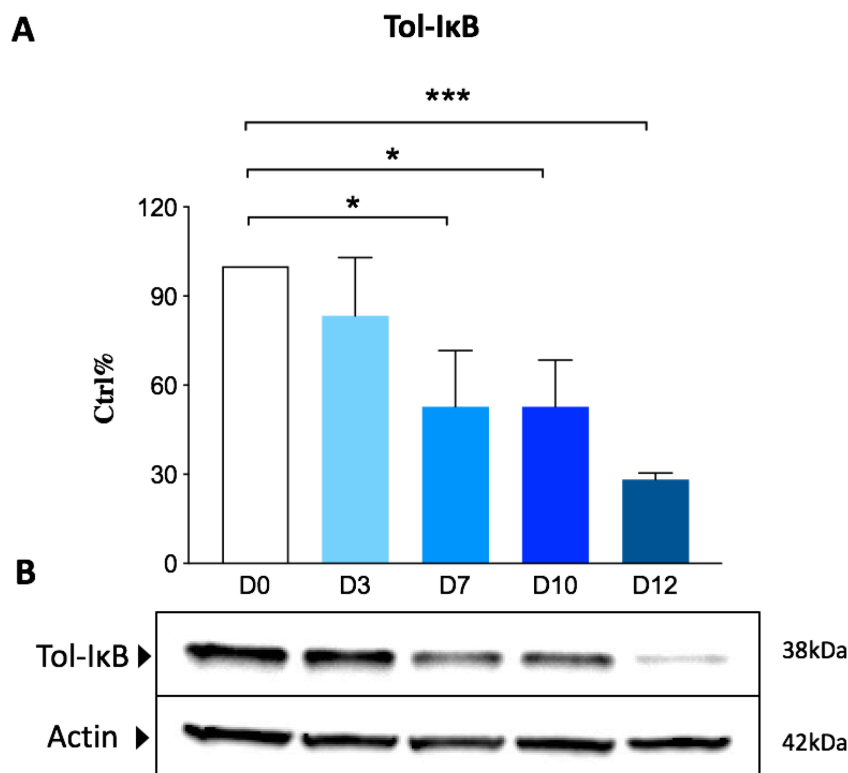


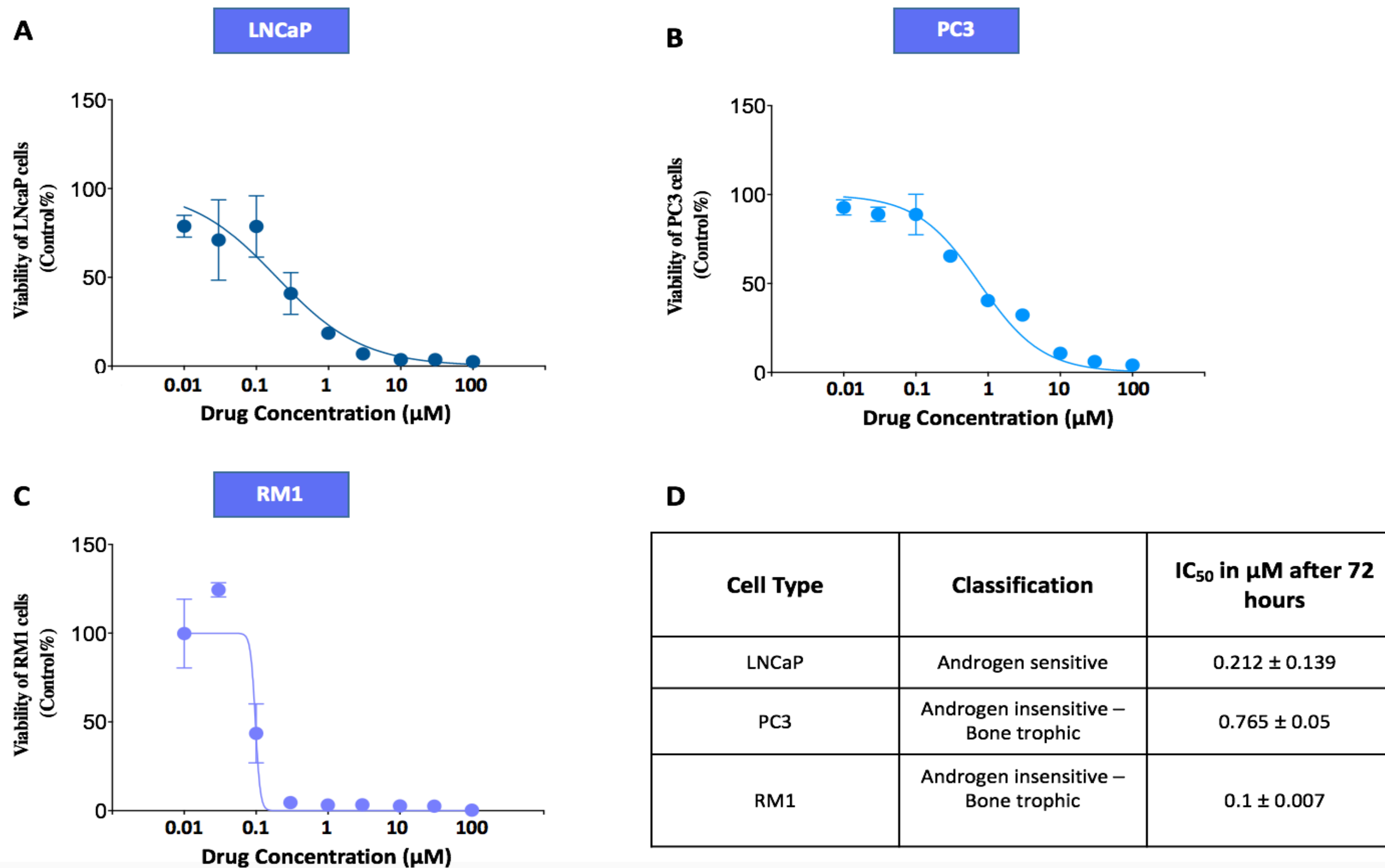
Figure. 67. Protein expression of total IκBα is reduced during mouse adipocyte differentiation and maturation. (A) Western blot quantification represents the changes in the level of key components of canonical NFκB pathway. This quantification of NFκB expression relative to actin and normalized to pre-adipocytes group level (D0). (B) Total cell lysates were prepared as described in the 'materials and methods' from D3, D7, D10 and D12's adipocytes. The blots were analysed for the indicated antibodies and anti-rabbit β-actin was used as the loading control. Results are from three independent experiments (N=3). Data were analysed using one-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

8.4.5. Bortezomib reduced the ability of mature adipocyte to increase the viability and invasiveness of prostate cancer cells in vitro.

My bioinformatic analysis has implicated the role of canonical NFκB-ubiquitin-proteasome in the regulation of PCa and obesity. The proteasome inhibitor BTZ is approved for the treatment of metastatic cancers (Robert et al., 2006, Mateos et al., 2006), and in this section, I utliized this agent to test the effects of inhibition of canonical NFκB signalling on the ability of M2 macrophages and mature adipocytes to affect the *in vitro* viability and invasion of PCa cells.

First, I examined the effects of different concentration of BTZ (0 – 100μM) on the viability of the castration-insensitive human PC3 and mouse RM1 and hormone-dependent human LNCaP cells. Cell growth was measured by AlamarBlue assay, as described in section 2.2.8.

As shown in figure 68, the proteasome inhibitor BTZ reduced the viability of human LNCaP and PC3 and mouse RM1 PCa cells in a concentration-dependent manner after 72 hours. The half maximal inhibitory concentrations (IC_{50}) for BTZ are shown in figure 68, panel D.



Cell Type	Classification	IC_{50} in µM after 72 hours
LNCaP	Androgen sensitive	0.212 ± 0.139
PC3	Androgen insensitive – Bone trophic	0.765 ± 0.05
RM1	Androgen insensitive – Bone trophic	0.1 ± 0.007

Figure. 68. The proteasome inhibitor BTZ reduced the *in vitro* viability of a panel of murine and human PCa cells with different metastatic abilities *in vitro*. Dose-response curves of the proteasome inhibitor bortezomib on the viability of (A) human LNCaP and (B) human PC3 and (C) mouse RM1 prostate cancer cells after 72 hours assessed by Alamar Blue assay. (D) IC_{50} values were obtained as described in methodology section. Data obtained from technical repeat experiments. Error bars indicate mean ± SD.

After determining the tested concentration of BTZ by IC_{50} assay, I examined if BTZ influence the ability of PCa cells to grow in the presence of mature adipocyte-derived factors. As can be seen in figure 69 (panel A), exposure of castration-insensitive human PC3 PCa cells to 0.3μM BTZ significantly attenuated the increase of cell growth by mature adipocyte-derived factors (Adipo ^{CM} (0.3 μM BTZ), 20% v/v) by 2.4-fold after 48 hours ($p<0.05$) and 3.6-fold after 72 hours ($p<0.001$), when compared to control cultures (Adipo ^{CM} (Vehicle), 20% v/v).

Similar inhibitory effects were also observed in cultures of castration-insensitive mouse RM1 PCa cells pre-treated with 0.03μM BTZ and then exposed to mature adipocyte-derived factors (Adipo ^{CM} (0.03 μM BTZ), 20% v/v) for 24 (1.3-fold reduction, $p<0.05$), 48 (2.5-fold, $p<0.0001$) and 72 (3.1-fold, $p<0.0001$) hours, when compared to control cultures (Adipo ^{CM} (Vehicle), 20% v/v) (Figure 69, panel B).

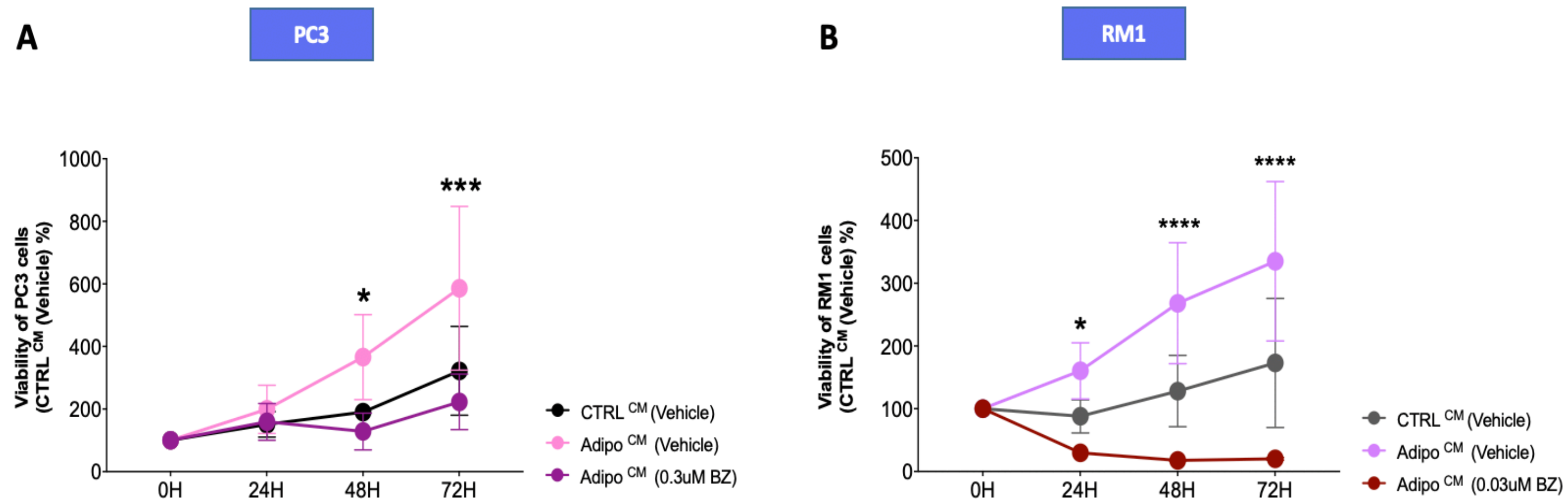


Figure. 69. BTZ attenuated the ability of mature adipocytes to increase the viability of human and mouse PCa cells *in vitro*. The effects of the proteasome inhibitor bortezomib on the viability of human LNCaP (A) and PC3 (B) and mouse RM1 (C) prostate cancer cells after 72 hours as assessed by Alamar Blue assay. Results are from three independent experiments (N=3). Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ (*: Adipo ^{CM} (Vehicle) vs Adipo ^{CM} (BTZ)).

My previous *in vitro* study showed that pro-inflammatory cytokine TNFα enhanced the ability of mature adipocytes to increase the invasive capability of human PC3 and mouse RM1 PCa cells. To investigate the effects of the proteasome inhibitor BTZ on these effects, I tested the effects of TNFα to influence the ability of mature adipocytes to affect the invasion of the castration-insensitive human PC3 and mouse RM1 cells in the presence and absence of BTZ. Cell invasion was measured by Transwell invasion assay, as described in section 2.2.9.2.

As shown in figure 70, exposure of castration-insensitive human PC3 and mouse RM1 PCa cells to BTZ at the indicated concentration significantly reduced the invasion of human PC3 (35%, $p<0.05$) after 72 hours (Figure 70, panel A) and mouse RM1 (36%, $p<0.01$) after 48 hours (Figure 70, panel B) cultured in the presence of conditioned medium from mature adipocytes primed with TNFα (Adipo $^{CM/TNF\alpha}$ (BTZ), 20% v/v) , when compared to control cultures (Adipo ^{CM} (Vehicle), 20% v/v).

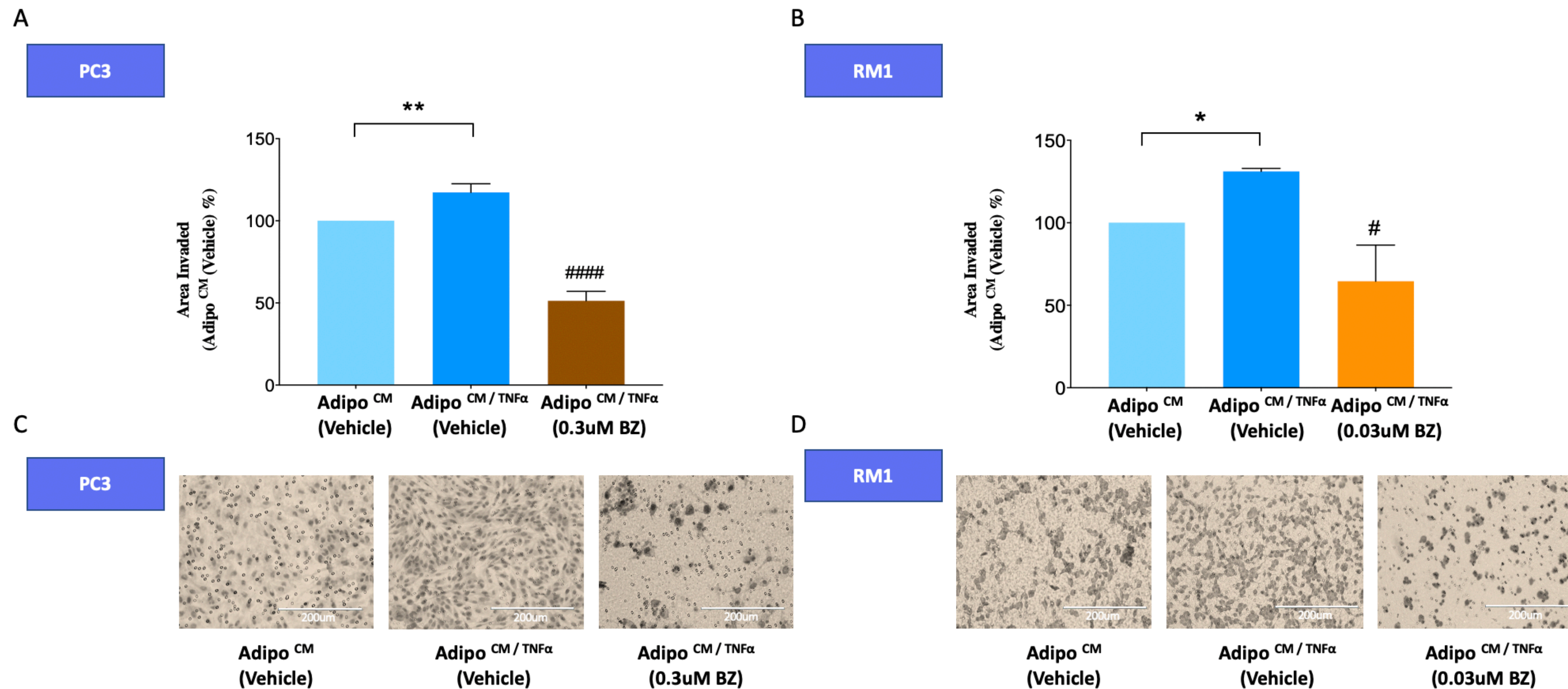


Figure. 70. BTZ attenuated the effect of pro-inflammatory cytokine TNFα on the ability of mature adipocytes to increase human and mouse PCa cell invasion *in vitro.* Human PC3 and mouse RM1 PCa cells were pre-exposure to factors derived from pro-inflammatory cytokine stimulated mature adipocytes. The effects of the proteasome inhibitor on the invasion of human PC3 prostate cancer cells (A) after 72 hours or mouse RM1 prostate cancer cells (B) after 48 hours were assessed by Transwell® invasion. Representative images of PC3 cells (C) and RM1 cells (D) invasion. Results are from three independent experiments (N=3). Data were analysed using one-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (#: Adipo ^{CM} (Vehicle) vs Adipo $^{CM/TNF\alpha}$ (BTZ)).

My previous *in vitro* study also showed that M2 macrophage derived factors enhances the ability of mature adipocytes to increase the invasive capability of human PC3 and mouse RM1 PCa cells. Next, I assessed the effects of the proteasome inhibitor BTZ on the invasion of castration-insensitive human PC3 and mouse RM1 cells exposed to derived factors from mature adipocyte-derived cultured in the presence and absence of M2 conditioned medium. Cell invasion was measured by Transwell invasion assay in section 2.2.9.2.

As shown in figure 71, exposure of castration-insensitive human and mouse PCa cells to BTZ at the indicated concentration significantly reduced the invasion of human PC3 (49%, $p<0.05$) after 72 hours (Figure 71, panel A) and mouse RM1 (42%, $p<0.05$) after 48 hours (Figure 71, panel B) cultured in the presence of mature adipocytes primed with M2 conditioned medium (Adipo $^{CM/M2}$ (BTZ), 20% v/v), when compared to control cultures (Adipo ^{CM} (Vehicle), 20% v/v) ($p<0.05$).

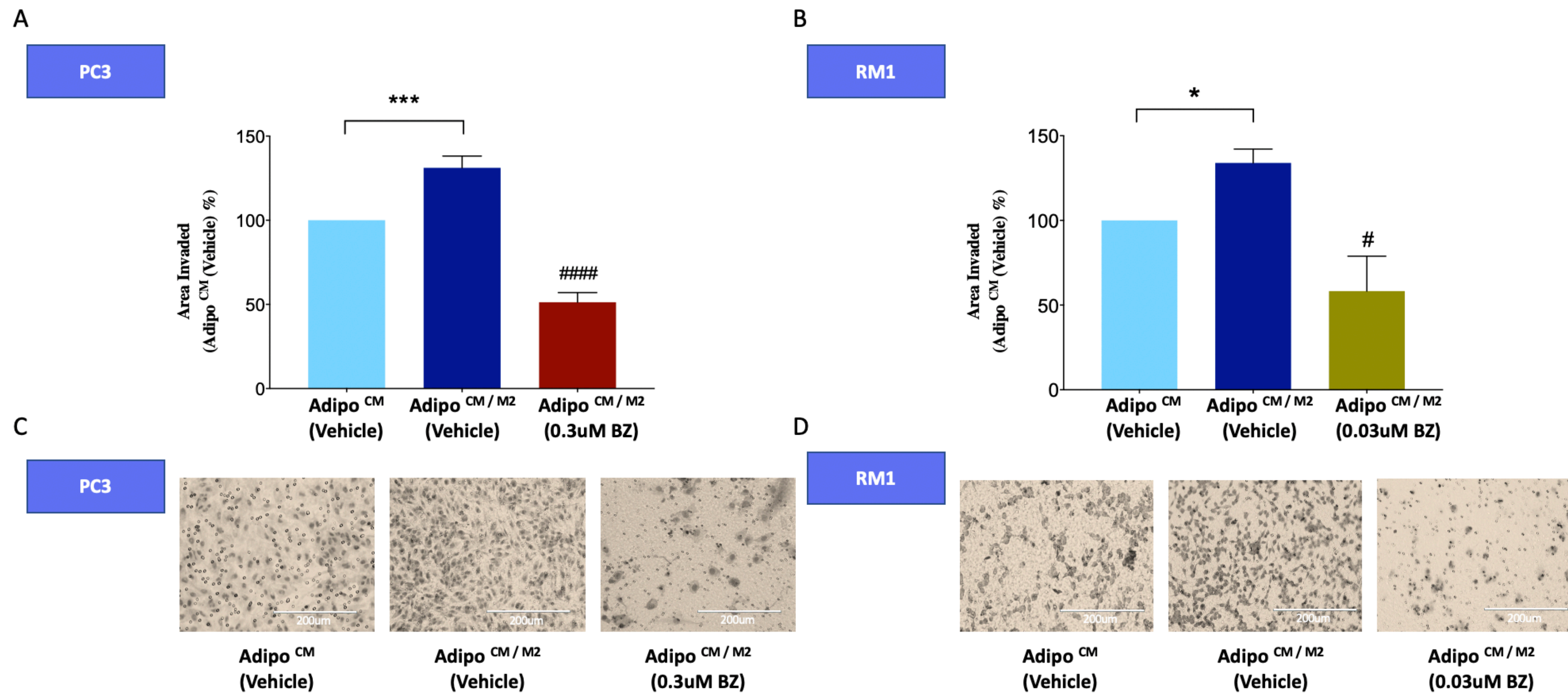


Figure. 71. BTZ attenuated the effect of M2 derived factors to affect the ability of mature adipocytes to increase the invasion of human PC3 and mouse RM1 PCa cells *in vitro*. Human PC3 and mouse RM1 prostate cancer cells were pre-exposed to factors derived from M2 macrophage conditioned medium stimulated mature adipocytes. The effects of the proteasome inhibitor on the invasion of human PC3 prostate cancer cells (A) after 72 hours or mouse RM1 prostate cancer cells (B) after 48 hours were assessed by Transwell® invasion. Representative images of PC3 cells (C) and RM1 cells (D) invasion. Results are from three independent experiments (N=3). Data were analysed using one-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (#: Adipo ^{CM} (Vehicle) vs Adipo $^{CM/M2}$ (BTZ)).

8.5. Discussion

The present bioinformatic analysis suggested that the canonical NFκB-ubiquitin-proteasome axis plays a role in inflammation, PCa and obesity, and the findings from the *in vitro* studies implicated pro-inflammatory factors, namely TNFα and a number of M2 and adipocyte derived factors, that are known to activate and/or are activated by NFκB, in the regulation of macrophage - adipocyte - PCa cell interactions.

IκBα is a critical regulator of the canonical NFκB-ubiquitin-proteasome signalling pathway. In resting cells, IκB is present in the cytoplasm as a latent, inactive signalling protein bound to p65/RELA (Gilmore, 1999). Phosphorylation of IκB by the IKKα/β/γ complex leads to its degradation, which enables the p65/RELA dimers to translocate to the nucleus where it activates various genes implicated in inflammation, cancer and obesity (Claudia et al., 2004, Andrea et al., 2011). Although, several studies have reported that NFκB activation plays an essential role in PCa and adipogenesis (Michael et al, 2002; Inoue et.al. 2007, Baker et al., 2011, Catrysse and van Loo, 2017), few studies tested the effects of selective inhibition of canonical NFκB activation on the ability of adipocytes to influence PCa cell behaviour in the presence of TAMs or their derived factors.

The results in this chapter confirmed that factors secreted by the TAMs sub-type M2 enhanced the ability of mature adipocytes to activate canonical NFκB signalling in highly metastatic PCa cells. The evidence comes from the Western Blot data that showed that the ratio of active p-IκBα over inactive total IκBα was increased in castration-insensitive human PC3 and mouse RM1 exposed to derived factors from mature adipocytes primed with M2 conditioned medium. In contrast, priming adipocytes with TNFα failed to cause the same effect. This suggests that M2-derived factors, other than TNFα, regulate macrophage – mature adipocyte – PCa cell crosstalk in the models described. Further examination of factors in cultures of adipocytes primed with M2 conditioned medium by protein microarray analysis (chapter 6, section 6.4.3.)

failed to detect TNFα, but it revealed that M2 macrophage-derived factors enhanced the secretion of a number of adipocyte-derived factors that have previously been reported to activate canonical NFκB signalling. The list includes CXCL1, CXCL10, VCAM-1 and M-CSF.

The chemokine CXCL1 is highly abundant in PCa tumours (Ferrer et al., 1998), and it has been found to stimulate NFκB activation in PCa cells and to enhance in vivo growth and in vitro motility of castration-insensitive PCa cells (Moore et al., 1999, Kuo et al., 2012). CXCL10 is another factor that has been shown to regulate PCa metastasis. Overexpression of CXCL10 promoted cell motility in highly metastatic DU145 and PC3 cells, but not in the localized LNCaP PCa cells (Nagpal et al., 2006, Wu et al., 2012). The effects CXCL10 on NFκB activation in PCa cells is unknown, but Jin et al. (2017) showed that the level and activity of CXCL10 and its receptor CXCR3 positively correlated with NFκB activation in breast cancer cells (Jin et al., 2017). Previous study has also shown that serum level of M-CSF is significantly increased in advanced PCa patients with bone metastasis when compared to those in non-metastatic patients (Ide et al., 2008), and genetic inactivation of IκBα in mice increased the expression level of M-CSF in prostatic tissue when compared to normal tissue (Jin et al., 2013). A number of studies have also implicated VCAM-1 in PCa cancer, and Wong and colleagues reported increased expression of VCAM-1 and NFκB activation in hormone-dependent LNCaP cells upon exposure to macrophage derived factors (Wong et al., 2009). Collectively, these results suggest that PCa cell-specific activation of canonical NFκB signalling by CXCL1, CXCL10, VCAM-1 and M-CSF is likely to be the mechanisms by which adipocytes influence the behaviour of highly metastatic PCa cells such as the human PC3 and mouse RM1 PCa cells used in this study.

To further test and validate the hypothesis, I assessed the effect of inhibition of canonical NFκB activation on PCa cell motility by treating PC3 and RM1 cells with the verified IκB /proteasome inhibitor BTZ prior to the addition of derived factors from adipocytes primed with M2 or TNFα. Although a number of previous studies showed that castration-insensitive human

DU145 and PC3 cells undergo apoptosis when treated with BTZ (Adams et al., 1999, Zheng et al., 2015), the present experiments are novel because few studies investigated the effects of BTZ on PCa cell behaviour in models adipogenesis. The result of the present experiments confirmed that BTZ inhibited the growth and invasion of PC3 and RM1 cells. This is consistent with my hypothesis and previous studies in the literature that showed that BTZ inhibited NFκB activity and suppressed tumour volume and metastasis in PCa mouse models (Adams et al., 1999, Papandreou and Logothetis, 2004). Whilst these results suggest that IκB and proteasome inhibition by BTZ disrupts adipocyte - PCa cell interactions, it is likely that a number of other mechanisms are involved. For example, BTZ is known to induce apoptosis in cancer cells, thus apoptotic pathways such as caspase-8 and 9 are likely to contribute to the effects observed in the present models (Kubiczkova et al., 2014).

To confirm the role of canonical NFκB activation in adipogenesis in the present models, I assessed canonical NFκB activation during adipocyte differentiation. The results showed that expression level of total IκBα was significantly reduced during mouse 3T3-L1 pre-adipocyte differentiation into mature adipocytes. This further implicates canonical IκBα / NFκB signalling in adipocyte maturation, and it is consistent with a number of previous studies that showed that canonical NFκB activation is enhanced during adipocyte differentiation and in HFD fed mice (Hill et al., 2015, Vykhovanets et al., 2011). Interestingly, the present finding is inconsistent with the work by Berg.et.al (2004) that showed that IκBα expression level was increased in mature adipocytes, suggesting reduced canonical NFκB activation (Berg.et.al. 2004). One likely explanation for this is that enhanced adipocyte differentiation and obesity often lead to insulin resistance that has been shown to be associated with reduced NFκB activation (Arkan et al., 2005, Yekollu et al., 2011, Su et al., 2009). Thus, further studies should examine if enhanced NFκB activation in the adipocyte model described is maintained beyond day 12.

My results in this chapter also showed that M2 derived factors - other than TNFα - enhanced canonical NFκB activation in mature adipocytes, as evidenced by increased p-IκBα/IκBα ratio. Further examination of data from microarray analysis (chapter 6, section 6.4.4.) indicated that M2 conditioned medium contains high levels of the pro-inflammatory mediators IL-8, CCL2 and CXCL10. IL-8 belongs to the CXC family of chemokines, which are secreted by macrophages to promote inflammation, and infiltration of adipose tissue (Remick, 2005, Yamaguchi et al., 2015, Hasan et al., 2019). In obesity, enhanced IL-8 production is associated with NFκB activation caused by free fatty acids (Joshi-Barve et al., 2003). Similarly, NFκB activation is also associated with enhanced expression of CCL2 in tumour microenvironment (Bussard et al., 2016). Together, these results confirm the role of canonical NFκB signalling in the regulation of the ability of M2 to influence mature adipocyte – PCa cell interactions in the *in vitro* model described.

Interestingly, the present data indicate that TNFα does not activate IκB phosphorylation in mature adipocytes neither it plays a role in the ability of M2 macrophage to influence NFκB activation in PCa cells in the presence of mature adipocytes. One plausible explanation for this is that TNFα causes rapid increase in NFκB activation in a time-dependent manner, which is often detected in seconds and minutes (Miyamoto et al., 1994, Dvoriantchikova and Ivanov, 2014). In the mechanistic experiments described in this study, IκBα phosphorylation was measured in adipocytes after hours of exposure to exogeneous TNFα. Thus, further mechanistic studies are needed to examine the effects of TNFα on NFκB activation in pre- and matures adipocyte after shorter exposure periods.

In summary, the findings of the present chapter confirm the role of canonical NFκB activation in the regulation of mature adipocyte - PCa cell interactions in the presence of M2 macrophages, and it also provides pharmacological, in vitro evidence that shows that inhibition of canonical NFκB-ubiquitin-proteasome axis at the level of IκBα by BTZ may be of therapeutic benefits in the reduction for PCa in obese patients. Thus, further *in vivo* investigation of the

effect of pharmacological inhibition of IκBα by agents such as BTZ and Parthenolide (Marino et al., 2019) on PCa metastasis in lean and obese mouse models is needed.

CHAPTER 9

GENERAL DISCUSSION

PCa is the most common non-skin cancer in the UK and the fifth leading cause of cancer death worldwide. Approximately, 90% of men diagnosed with PCa survive the disease for 5 years or more. However, survive rate in men diagnosed with distant metastases such as those in bone drops to 30% (Atlanta, 2003, Kirby, 2009, Key Non Parliamentary Papers Office for National, 2020). Obesity is a common public health problem. It a major contributory factor to PCa recurrence and metastasis, and it has been shown to reduce survival rate in PCa patients (Gong et al., 2007, Agalliu et al., 2015, Zhang et al., 2015). Thus, there is an urgent need to develop and test novel treatments for advanced PCa in obese patients.

Inflammation is a risk factor in both PCa and obesity (Kumar et al., 2004, Baker et al., 2011, Taverna et al., 2015). There is a growing body of evidence that implicates the pro-inflammatory NFκB signalling pathway in various aspects of PCa development and adipogenesis (Berg et al., 2004, Carlsen et al., 2009a, Jin et al., 2015, Staal and Beyaert, 2018). In this project, I present evidence from retrospective meta and bioinformatic analysis, as well as *in vitro* functional and mechanistic studies that implicates inflammation in the regulation of the *in vitro* interactions between adipocytes and PCa cells and shows that M2 derived factors enhance the ability of mature adipocytes to stimulate canonical NFκB activation and motility of highly metastatic PCa cells. I also present pharmacological evidence that shows the verified proteasome / NFκB inhibitor BTZ reduced the ability of mature adipocytes to enhance the *in vitro* motility of the highly metastatic human PC3 and mouse RM1 PCa cells in the presence of exogenous TNFα or endogenous M2 derived factors.

In chapter 3, I first carried out a meta-analysis that examined the association between the expression levels of adipocyte-derived factors and PCa development and progression. This provided preliminary evidence that confirmed that adipokines regulate *in vitro* motility of highly metastatic castration-insensitive PCa cells (Tang and Lu, 2009, Huang et al., 2011), and high levels of NFκB-related factors play a role in HFD-induced obesity in mice (Laurent et al., 2016, Hayashi et al., 2018). The lack of sufficient number of human and pre-clinical studies suggests

that further human, *in vivo* and *in vitro* studies are needed to confirm the role of the pro-inflammatory NFκB signalling pathway - in particular factors that activate or activated by this pathway - in the regulation of obesity driven PCa. Thus, I went on to successfully develop, optimise and utilize the mouse 3T3-L1 adipocyte model to study mature adipocyte – PCa – macrophage crosstalk (Figure 73).

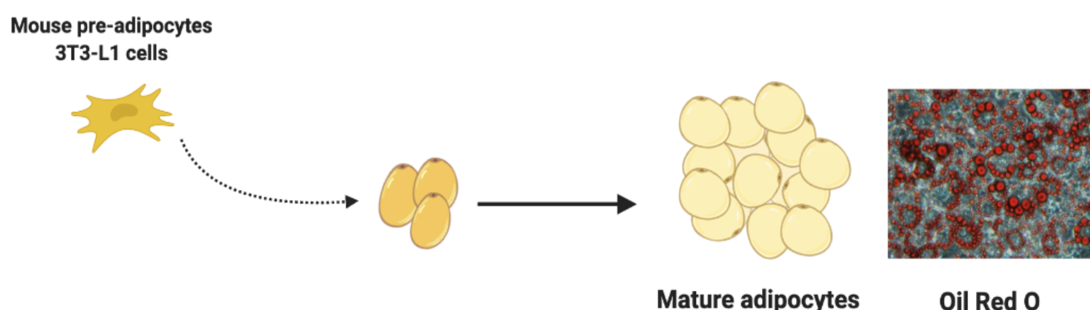


Figure. 73. Schematic summary of differentiation and maturation of adipocytes in the mouse 3T3-L1 pre-adipocyte model described. Refer to text and chapter 4 for details.

In chapter 5 and 6, I used this model (Figure 73) to study the *in vitro* interactions between adipocytes and PCa cells in the presence of exogenous (TNFα) and endogenous (M2-derived) pro-inflammatory mediators, respectively. Consistent with the findings from the meta-analysis (Chapter 3), these *in vitro* studies confirmed that mature adipocytes secrete various factors that enhance the *in vitro* growth of both hormone-dependent and castration-insensitive PCa cells (Figure 74). These findings are also consistent with previous *in vitro* and *in vivo* studies that showed that adipocyte-derived factors affect the growth and metastasis of hormone-dependent and castration-insensitive PCa cells (Tymchuk et al., 2001, Onuma et al., 2003, Tokuda et al., 2003, Moreira et al., 2015, Hu et al., 2018). Using protein microarray analysis, I analysed the levels of adipocyte-derived factors in conditioned medium from day 10 mature adipocytes. This experiment revealed that the aforementioned effects were associated with

significant increase in the levels of an adipocyte-derived array of pro-inflammatory mediators, pro-migratory tumour-associated factors, chemokines and adipokines (Figure 74).

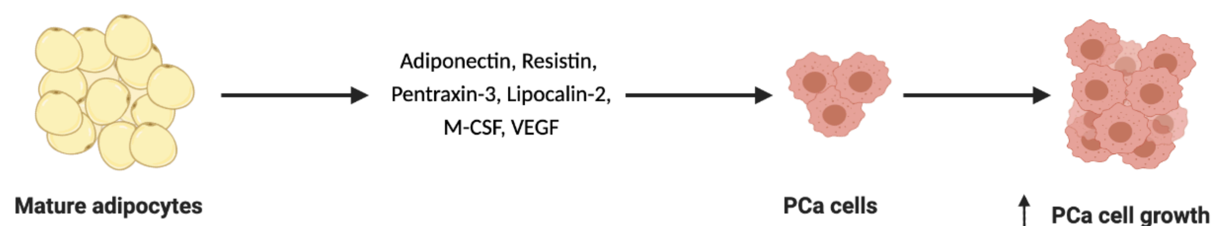


Figure. 74. Schematic summary of regulation of PCa cell growth by mature adipocyte derived factors in the mouse 3T3-L1 pre-adipocyte model described. Refer to text and chapter 4 for details.

Further investigation of the action of these adipocytes derived factors showed that mature adipocytes in my model are likely to increase the growth of highly metastatic PCa cells by mechanisms dependent, at least in part, on increased expression of factors that include adiponectin and resistin, which are known to stimulate adipocyte differentiation and lipid accumulation (Fu et al., 2005, Ikeda et al., 2013). Additionally, the presence high levels of the pro-tumour growth factors Pentraxin-3 and Lipocalin-2 in the adipogenic conditioned medium is also likely to contribute to the described effects. A number of previous *in vitro* studies reported that Lipocalin-2 knockdown reduced cell growth in castration-insensitive PCa cells (Tung et al., 2013, Ding et al., 2016). Although the effects of Pentraxin-3 on PCa cells growth is currently unclear, Stallone and colleagues showed that Pentraxin-3 is a biomarker for predicting PCa progression (Stallone et al., 2014).

Protein microarray analysis also detected high level of adipocyte-derived M-CSF, which has also been found implicated in PCa development. Jingying et al. (2013) showed that inhibition of M-CSF in combination with radiotherapy exerted an inhibitory effect on PCa tumour growth (Jingying et al., 2013). Interestingly, the present analysis detected increased level of the angiogenesis-related factor VEGF in conditioned medium from mature adipocyte. Although no previous studies have reported evidence for a tumour-promoting role for VEGF in *in vitro* models, a number of *in vivo* studies have shown that VEGF promote tumour growth and its

inhibition suppresses the growth and spread of highly metastatic human PCa tumours in mice (Borgström et al., 1998, Melnyk et al., 1999). Furthermore, high levels of matrix metallopeptidases including MMP-2, -3 and -9 have also been detected in adipogenic conditioned medium, and have been implicated in motility and metastasis of PCa cells (reviewed in Gong et al., 2014).

Whilst these results together indicate that the adipocyte-derived factors described play a role in the growth of PCa cells, further characterisation of the role and mechanism(s) by which these factors in the regulation of adipocyte - PCa cell crosstalk is needed. Thus, future *in vitro* studies should examine the effects of adipocyte conditioned medium on the growth of PCa cells lacking the VEGF, Pentraxin-3 or Lipocalin-2 receptors. Anti-angiogenic therapy currently being tested for the treatment of PCa (Melegh and Oltean, 2019), thus future *in vivo* studies should consider the testing the anti-tumour effect of anti-VEGF agents, such as the recombinant humanized monoclonal antibody Bevacizumab, in obese nude mice bearing PCa.

Obesity is associated with chronic inflammation, and a number of studies have detected high levels of circulating pro-inflammatory mediators that activate NFκB including TNFα and IL-1β, and those their expression is enhanced by NFκB activation such as IL-6 in obese patients (Hotamisligil et al., 1995, Lagathu et al., 2006, Eder et al., 2009). The present *in vitro* studies confirmed these findings. In chapter 3, meta-analysis of included *in vitro* studies revealed an association between the levels of PCa cell-derived IL-1β and the presence of adipocytes (Herroon et al., 2019). TNFα, is the master regulator of chronic inflammation (Chen and Sawyers, 2002, Karin, 2009b, Calcinotto et al., 2012), and high levels of TNFα have been detected in obese individuals (Tzanavari et al., 2010), suggesting a role of TNFα in the regulation of adipogenesis and obesity.

In chapter 5, I used exogenous human recombinant TNFα to examine if classical pro-inflammatory mediators regulate the ability of mature adipocytes to affect the behaviour of PCa cells *in vitro*. These studies showed that conditioned medium obtained from mature

adipocytes primed with TNFα enhanced their ability to increase the *in vitro* invasion of castration-insensitive human PC3 and mouse RM1 cells. Interestingly, I found that TNFα had no effect on the ability of adipocyte to affect the motility of the hormone-dependent human LNCaP PCa cells (Figure 75).

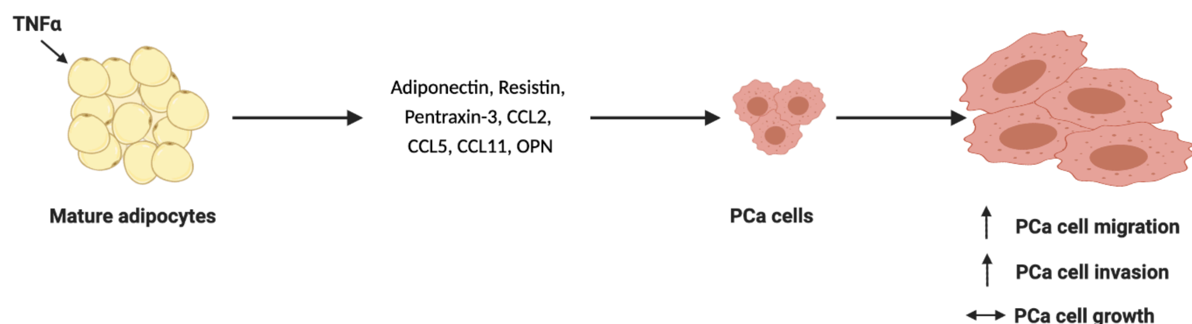


Figure. 75. Schematic summary of regulation of PCa cell motility by factors derived from mature adipocytes primed with exogeneous TNFα. Refer to text and chapter 5 for details.

This finding is consistent with previous studies that shown that TNFα enhanced the migratory capacity of PCa cells (Maolake et al., 2018), and in accordance with previous studies that have shown that inflammation enhance the metastatic abilities of PCa cells (Stark et al., 2015, Duong et al., 2017). These findings are important because they suggest that pro-inflammatory mediators such as TNFα influence the behaviour of highly metastatic PCa cells but not the localized PCa cells, such as the hormone-dependent LNCaP cells described. However, further *in vivo* studies in the preclinical model described in Figure 78 are needed to confirm this hypothesis.

Protein microarray analysis of pro-inflammatory factors in adipocytes conditioned medium further confirmed this, and it also implicated factors other than TNFα in the regulation of adipocyte – PCa cell interactions. It showed that TNFα enhanced the ability of mature adipocytes to increase the motility of highly metastatic PCa cells by a mechanism dependent, at least in part, on increased expression of various mediator that include the adipokines Adiponectin and Resistin. Priming mature adipocytes with TNFα also caused the secretion of

high levels of various adipocyte-derived chemokines and pro-inflammatory mediators (Figure 75). The list includes CCL2, CCL11, CCL5, Chitinase-3-like protein 1 and OPN. It is important to note that factors such as Adiponectin and Resistin as well as others including CCL2, CCL11 and CCL5 are secreted by adipocytes in the absences of TNFα, but their levels have been found to be multiple magnitudes higher when TNFα is present.

Review of current scientific literature confirms the role of the aforementioned factors in the regulation of TNFα signalling, and showed that excess CCL2, OPN and Resistin induced PCa cells to acquire a strong proliferative and invasive potential *in vitro* (Khodavirdi et al., 2006, Kim et al., 2011, Lin et al., 2013,). Furthermore, CCL2 and CCL11 promoted the invasion and migration of highly metastatic PCa cells (Loberg et al., 2006, Zhu et al., 2014), and CCL5 inhibition suppressed the migration of PCa cell to bone (Urata et al., 2018). Clinical study also indicated that adiponectin expression positively correlates with PCa metastasis (Rider et al., 2015).

Collectively, these findings suggest that strategies that successfully disrupt adipocyte - PCa cell crosstalk in the presence and absence of pro-inflammatory mediators, in particular TNFα can be of value in the inhibition of localized hormone-dependent prostatic growth and reduction of castration-insensitive PCa motility and metastasis and growth at distant sites such as the skeleton. Whist the experiments in this project have used a panel of human and mouse representative of hormone-dependent and castration-insensitive PCa cells to validate these findings, it is important to note that the present findings were limited to the mouse 3T3-L1 adipocyte model. Thus, future studies should consider reproducing the present results in cultures of mature adipocytes generated from other models, in particular the human adipocyte model described in (Nieman et al., 2011, Ribeiro et al., 2012). More functional studies that investigate the mechanisms by which adipocytes derived CCL2 (CCR2), CCL5 (CCR5) and CCL11 (CCR3) affect PCa cell motility are also needed. For example, future *in vitro* studies should examine if knockdown or pharmacological inhibitions of CCR2 or CCR3 or CCR5

receptors affect the ability of adipocytes to enhance the *in vitro* motility of highly metastatic PCa cells or their growth and metastatic spread in obese mice.

Macrophages produce various pro-inflammatory mediators that directly or indirectly regulate cancer cell behaviour. TAMs, in particular M2, interact with tumour cells via releasing soluble factors that promote tumour growth and metastasis (Condeelis and Pollard, 2006, Redente et al., 2010, Muthana et al., 2011, Ashleigh and Matthias, 2018). In this project, I used the human THP-1 cell model to successfully generate M2 macrophages and provided evidence to implicate M2 derived factors in the regulation of PCa cell – adipocyte crosstalk.

These *in vitro* studies showed that priming mature adipocytes with M2 conditioned medium enhanced their ability to increase the invasion of highly metastatic PCa cell, without affecting cell growth or 2D migration (Figure 76). These findings are in accordance with a recent study that implicated TAMs in the regulation of ability of adipocytes to enhance the invasion of PCa cells (Hyo-Yeoung et al., 2020). I also showed that factors in conditioned medium from mature adipocytes primed with M2 derived factors increased in the expression levels of the chemokines CCL2 and CXCL10, pro-tumour factors Pentraxin 3 and pro-inflammatory mediators OPN and VCAM-1 (Figure 76). The role of these factors in the regulation of the metastatic behaviour of PCa cells has been previously described in a number of studies (Khodavirdi et al., 2006, Wu et al., 2012, Lin et al., 2013, Ding et al., 2016, Chang et al., 2018), and elevated levels of Pentraxin 3 was detected in PCa patients (Stallone et al., 2014).

On the other hand, analysis of factors in the conditioned medium from polarized M2 monocytic THP-1 macrophage identified the chemokines CCL2 and CXCL10, pro-tumour factor uPAR, pro-inflammatory cytokine IL-8, Chitinase-3-like protein 1, pro-angiogenesis factor MMP-9 (Figure 76). Whilst this analysis indicates that a complex network of various factors is involved, it is important to note that CCL2 and CXCL10 have been detected in conditioned medium from both M2 and mature adipocytes. Furthermore, a number of studies have provided evidence to show that CCL2 plays an important role in the migration, invasion and metastasis of PCa cells

(Loberg et al., 2006, van Golen et al., 2008, Mizutani et al., 2009, Lin et al., 2013). Thus, future *in vitro* studies should examine if knockdown of CCL2 and its CXCL10 receptor in mouse and human mature adipocytes and/or highly metastatic human and mouse PCa cells affect their *in vitro* interactions of tumour growth and advanced metastasis in obese mice.

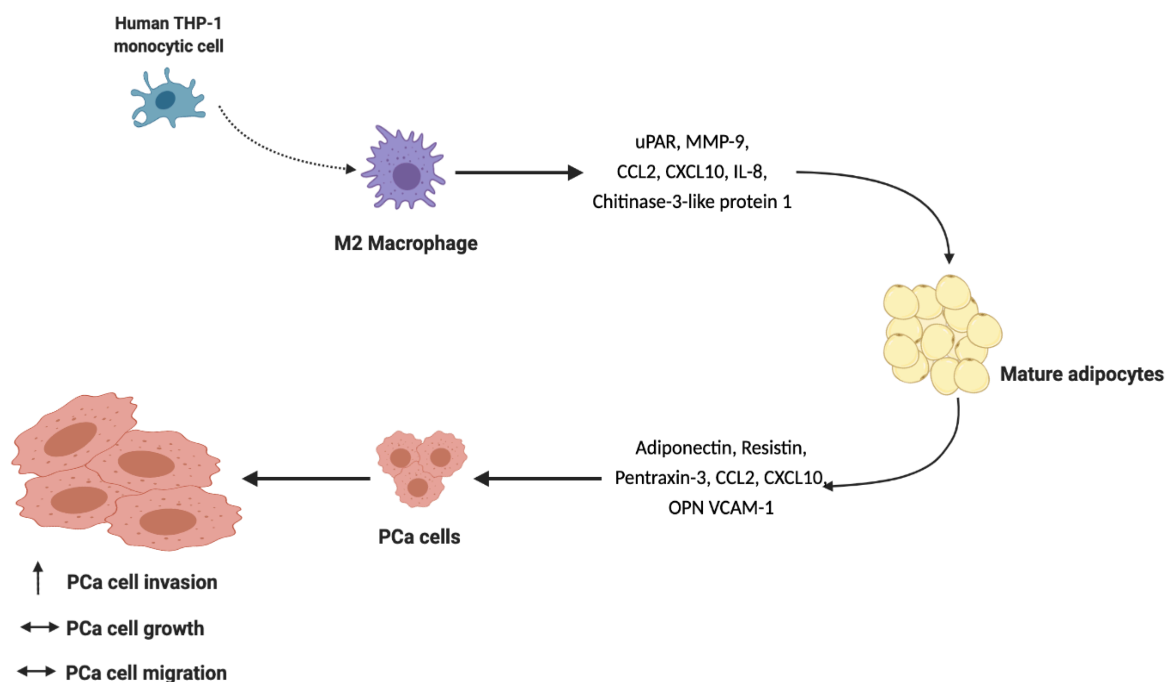


Figure. 76. Schematic summary of generation of M2 macrophage and regulation of PCa cell invasion by mature adipocyte derived factors primed with M2 conditioned medium. Refer to text or chapter 6 for details.

Future studies should also test if pharmacological inhibition of these receptors using agents such as anti-CCL2 and anti-CXCL10, which are currently being tested for treatment of inflammatory disease such as Rheumatoid arthritis (Haringman et al., 2006, Kim et al., 2014), can be of value in the reduction of PCa growth and metastasis in obese mice. Although the effects of anti-CXCL10 on PCa tumour growth in bone are poorly understood, a preclinical study has shown that CCL2 blockade slows PCa tumour growth in the skeleton (Kirk et al., 2013). Thus, I suggest future studies should test the effect of these agents on mouse models of PCa bone metastasis (Park et al., 2019). If successful, the findings of these studies may provide support for a strategy that targets the CCL2/CXCL10 axis for the treatment of advanced PCa metastasis in obese patients.

The present bioinformatic analysis in chapter 7 confirmed that the gene encoding IκBα (NFKBIA) - an essential mediator of canonical NFκB signalling – is the most amplified NFκB related gene in patients with metastatic PCa, when compared to other NFκB related gene (Inoue et al., 2007, Jin et al., 2008b, Marino et al., 2019). My data also verified the positive correlation between gene alteration of NFKBIA and the expression level of the standard markers of adipogenesis PPARγ and C-EBPβ. which are known to be associated with PCa progression. Canonical NFκB signalling is regulated by IKK-activated IκBα proteasomal degradation that leads to the translocation of p65/RelA to the nucleus (Andrea et al., 2011, Ting et al., 2017). The present meta-analysis of included animal studies revealed an association between the expression of NFκB with obesity, and also revealed that the expression of IL-1β and p65/RelA are increased in prostatic tissue from obese mice (Vykhovanets et al., 2011, Hayashi et al., 2018).

Follow up mechanistic studies that involved Western Blot analysis of IκBα activation in mature adipocytes and PCa cells showed that total IκBα expression is significantly reduced during adipocyte maturation in the mouse 3T3-L1 model, indicating elevated IκBα degradation and p65/RelA nuclear translocation. Furthermore, increased ratio of active p-IκBα to total IκBα was detected in the mature adipocytes and PCa cells primed with M2- or adipocyte-derived factors, respectively. These findings are consistent with previous studies (Berg et al., 2004, Jin et al., 2014b, Ellulu et al., 2017) and implicate the NFκB – ubiquitination – proteasome axis in the regulation of M2 - PCa cell - adipocyte crosstalk, thereby leading us to hypothesise that inhibition of canonical IκBα degradation reduces the ability of mature adipocytes to influence the *in vitro* and *in vivo* behaviour of PCa in the presence of pro-inflammatory mediators such as exogenous TNFα and endogenous M2 derived factors.

BTZ was the first inhibitor of proteasomal degradation of IκBα to be used in clinical practise to treat patients with relapsed and refractory multiple myeloma (Robert et al., 2006, Mateos et al., 2006). Previous studies have shown that BTZ inhibits NFκB activity and reduces growth

and metastasis of PCa cells (Papandreou and Logothetis, 2004). The present mechanistic *in vitro* studies in chapter 8 showed that BTZ attenuated the ability of mature adipocytes to increase the *in vitro* growth and invasion of castration-insensitive PCa cells (Figure 77). These studies provide pharmacological evidence to suggest that inhibition of canonical IκBα/NFκB activation in PCa cells reduces the ability of mature adipocytes to support PCa cell behaviour *in vitro*. Thus, *in vivo* studies are needed to test the effect IκBα/NFκB inhibition on PCa metastasis in obese mice.

A number of studies have also reported the efficacy of other inhibitors of canonical NFκB activity in reducing the growth and motility of PCa cells *in vitro* and suppressing metastasis *in vivo* (Hagemann et al., 2008, Mahato et al., 2011, Lung et al., 2019). Work by my colleagues and I in 2019 have shown that pharmacological inhibition of IκBα phosphorylation using Parthenolide reduced the ability of PCa cells to move and cause osteolytic bone damage (Marino et al., 2019). Other investigators have reported that treatment of PCa cells with this agent inhibited PCa cell growth and restored sensitivity to docetaxel chemotherapy and anti-androgen hormone therapy (Hehner et al., 1998, Shanmugam et al., 2006). Whilst these studies indicate IκBα may be of value as a therapeutic target for the treatment of advanced PCa, studies also showed that IκBα inhibition using compounds that target IKK kinase activity such as BMS-345541, PS-1145 and TPCA1 caused on-target systemic toxicity and immunodeficiency (Gamble et al., 2012, Prescott and Cook, 2018). Thus, the aforementioned *in vivo* studies should be restricted to testing the efficacy of the proteasome inhibitors such as BTZ and Parthenolide in PCa metastasis in obese mice.

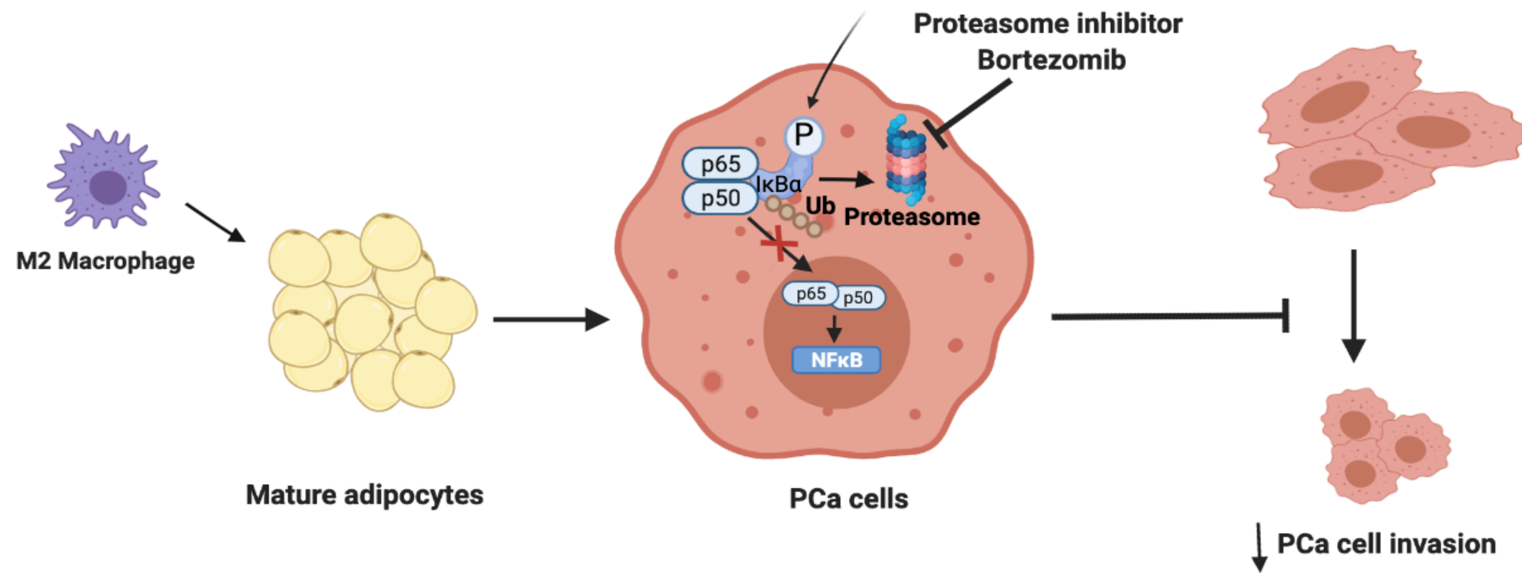


Figure. 77. Schematic summary showing inhibition of M2 – mature adipocyte - PCa cell interactions by the proteasome inhibitor Bortezomib. Refer to text or chapter 8 for details.

Approximately, 80% of PCa patients with advanced disease develop bone metastasis (Gingrich et al., 1996, Park et al., 2019). Thus, I propose that future *in vivo* studies should use the syngeneic, bone-seeking RM1 model of PCa described in figure 78, to investigate if inhibition of canonical NFκB activation affects the ability of adipocytes to enhance advanced PCa metastasis. Briefly, wild type immune-competent C57BL/6 mice will be fed either standard or high-fat diet for 3 weeks, and then receive intracardiac injection of a luciferase-expressing, bone-seeking mouse RM1-BT PCa cells. The mice will be divided into two groups; sham group that will receive a vehicle and inflammatory group that will be injected with the pro-inflammatory mediator LPS, which is known induce a classic pro-inflammatory response in immune-competent mice (Omabe et al., 2014, Capulli et al., 2019, Jain et al., 2019). The mice will be further divided in groups of 10 mice each, and then treated with vehicle control (PBS) or the proteasome inhibitor BTZ or Parthenolide (Idris et al., 2008, Idris et al., 2010, Marino et al., 2017, Marino et al., 2019).

In summary, the present findings from meta and bioinformatic analysis and *in vitro* functional and mechanistic studies provide a novel insight into the interactions of macrophages, adipocytes and PCa cells, and indicate that cancer-specific inhibition of canonical NFκB activation using IκB inhibitors reduces the ability of mature adipocytes to enhance the *in vitro* invasion of highly metastatic, castration-insensitive human and mouse PCa cells in the presence of pro-inflammatory mediators. Whilst the findings from the planned *in vivo* studies (Figure 78) are to come, the evidence from the present *in vitro* studies imply that therapeutic agents that are in clinical practise such as the proteasome inhibitor BTZ - or possibly anti-CCL2 inhibitors - show promise for the treatment of PCa in obese patients. Thus, further preclinical testing of the effects of these agents – alone or in combination with chemotherapy – on the spread and growth at metastatic sites of PCa cells in obese mice in both immunocompetent (Figure 78) or nude mouse models bearing mouse and human PCa cells are needed.

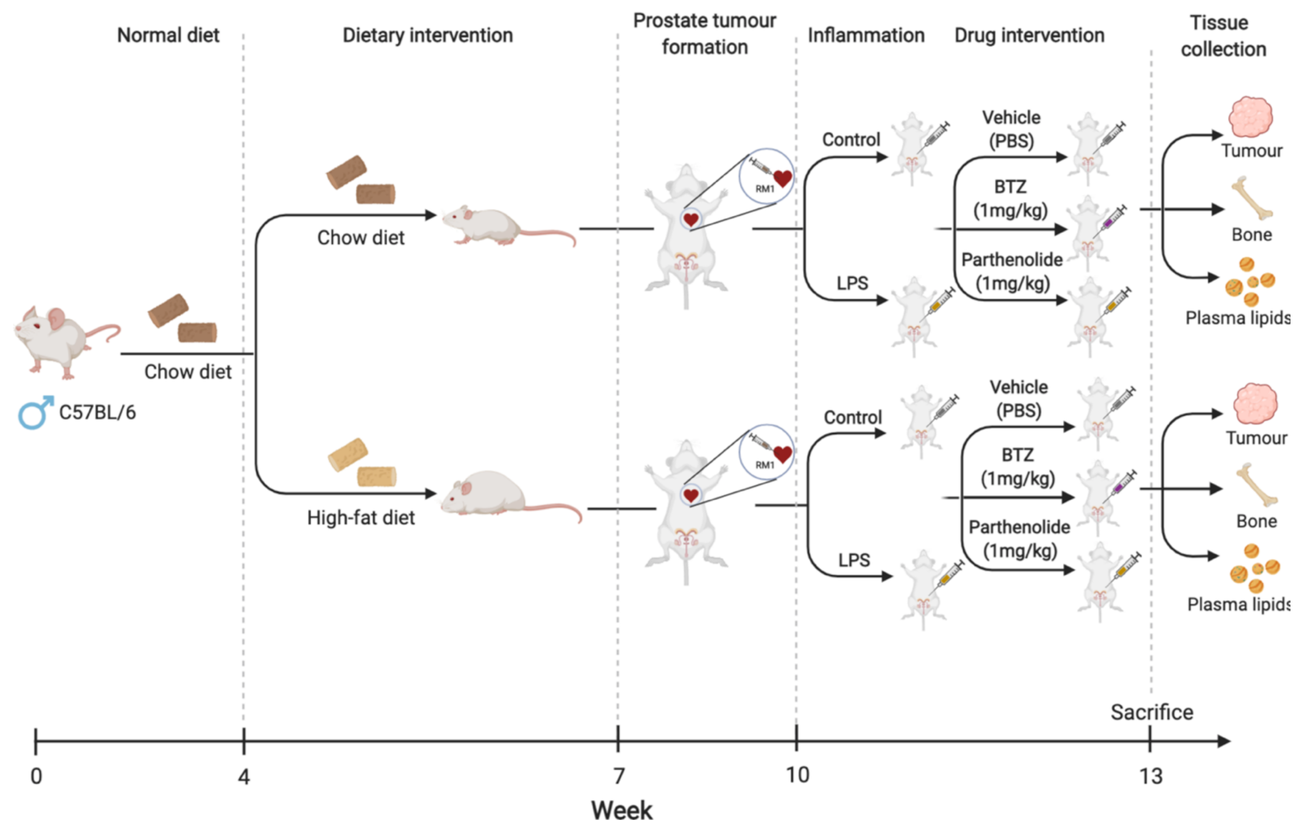


Figure. 78. The proposed in vivo models to study the effect of inhibition of canonical IκBα/NFκB activation on PCa bone metastasis, skeletal tumour growth and osteolysis. Immunocompetent mice C57BL/6 will be divided into groups (10 mice/group) and injected intra-cardiacally with luciferase-expressing, bone-seeking RM1-BT cells and given weekly i.p injections of bortezomib or parthenolide (1mg/kg). Mice will be monitored for body weight and development of metastases thrice-weekly using a IVIS Spectrum CT In Vivo Imaging System (PerkinElmer, UK). Mice were sacrificed 2-3 weeks post injection. Bones will be excised and analysed by ex vivo IVIS imaging and micro–computed tomography (MicroCT, Skyscan 1172 scanner). Skeletal tumour growth will be measured on images from 2D microCT and histological sections using Image J (1.34s; NIH, Bethesda, MD, USA). Histological analyses of Macrophage tumour infiltration and bone parameters including osteoclast and osteoblast numbers and activity will be performed in formalin fixed sections. Serum samples will be collected, and levels of pro-inflammatory mediators will be measured as described in Chapter 2.

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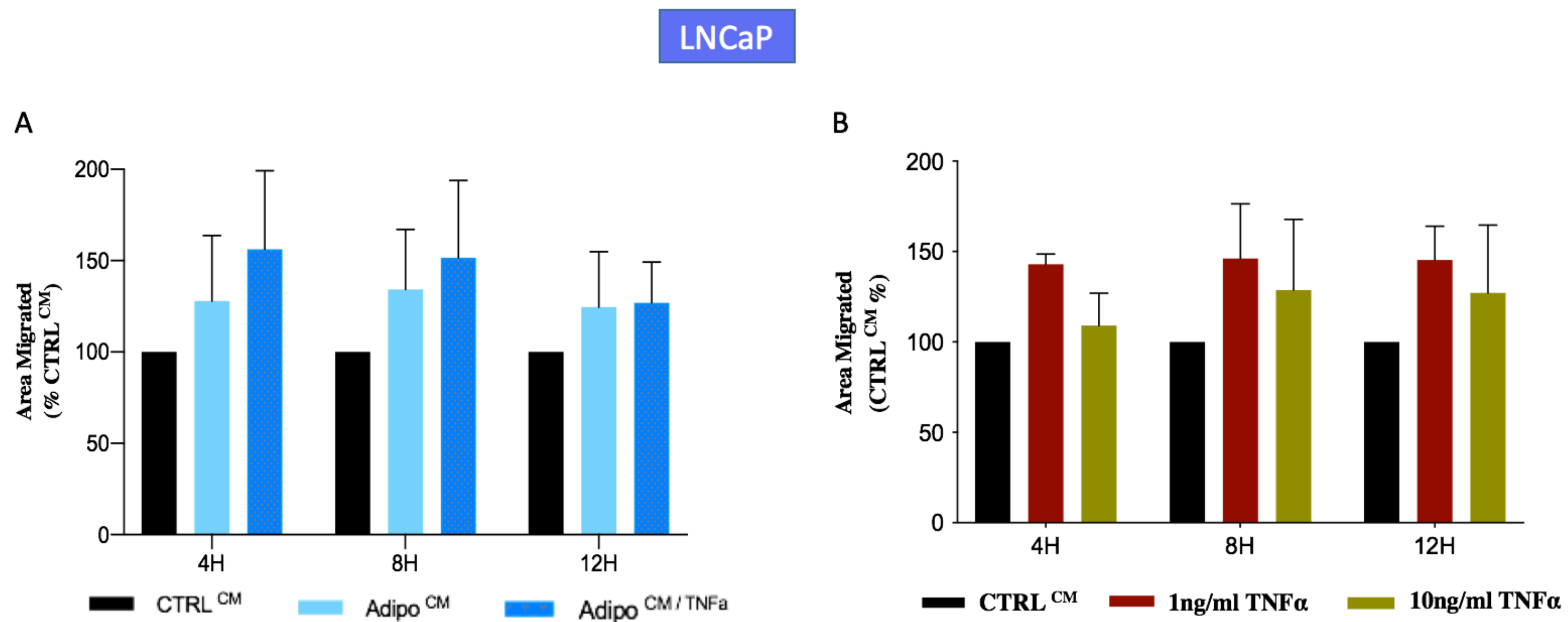
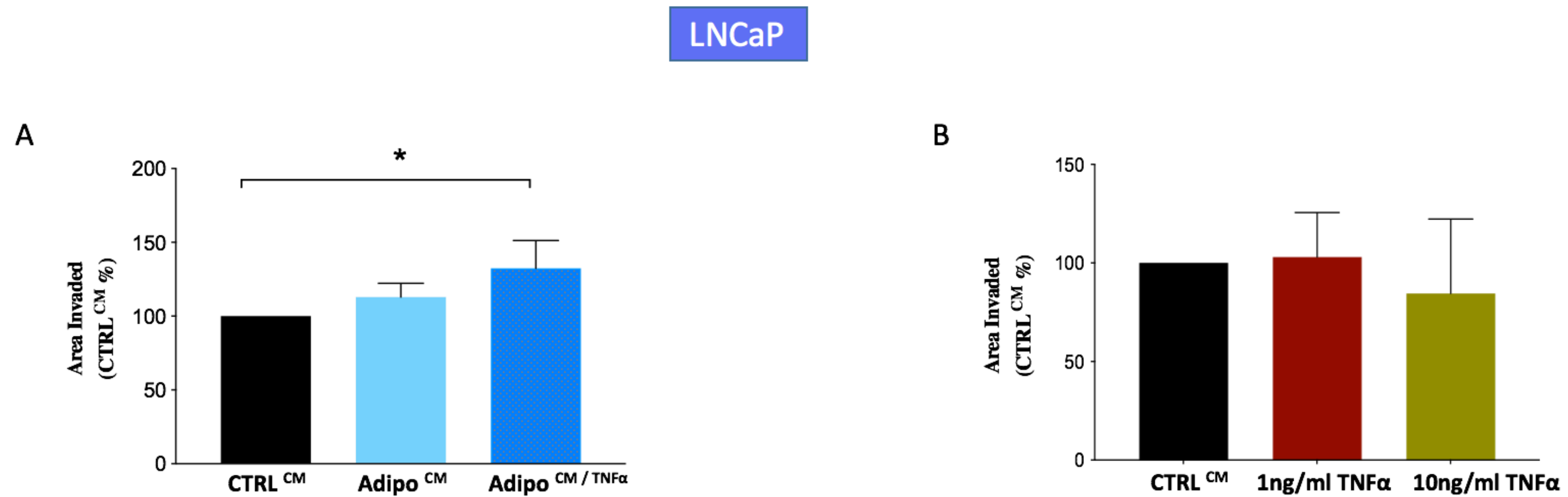
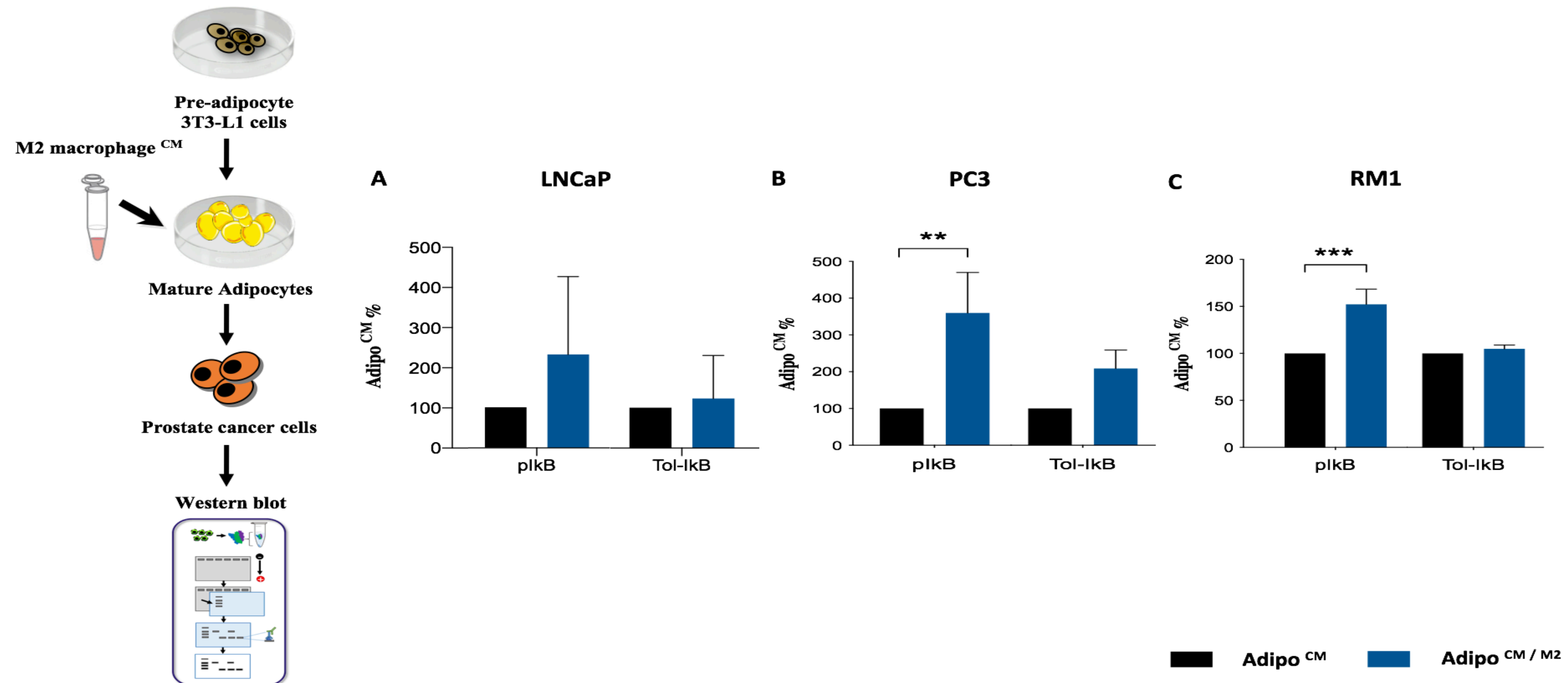
Supplementary Figures



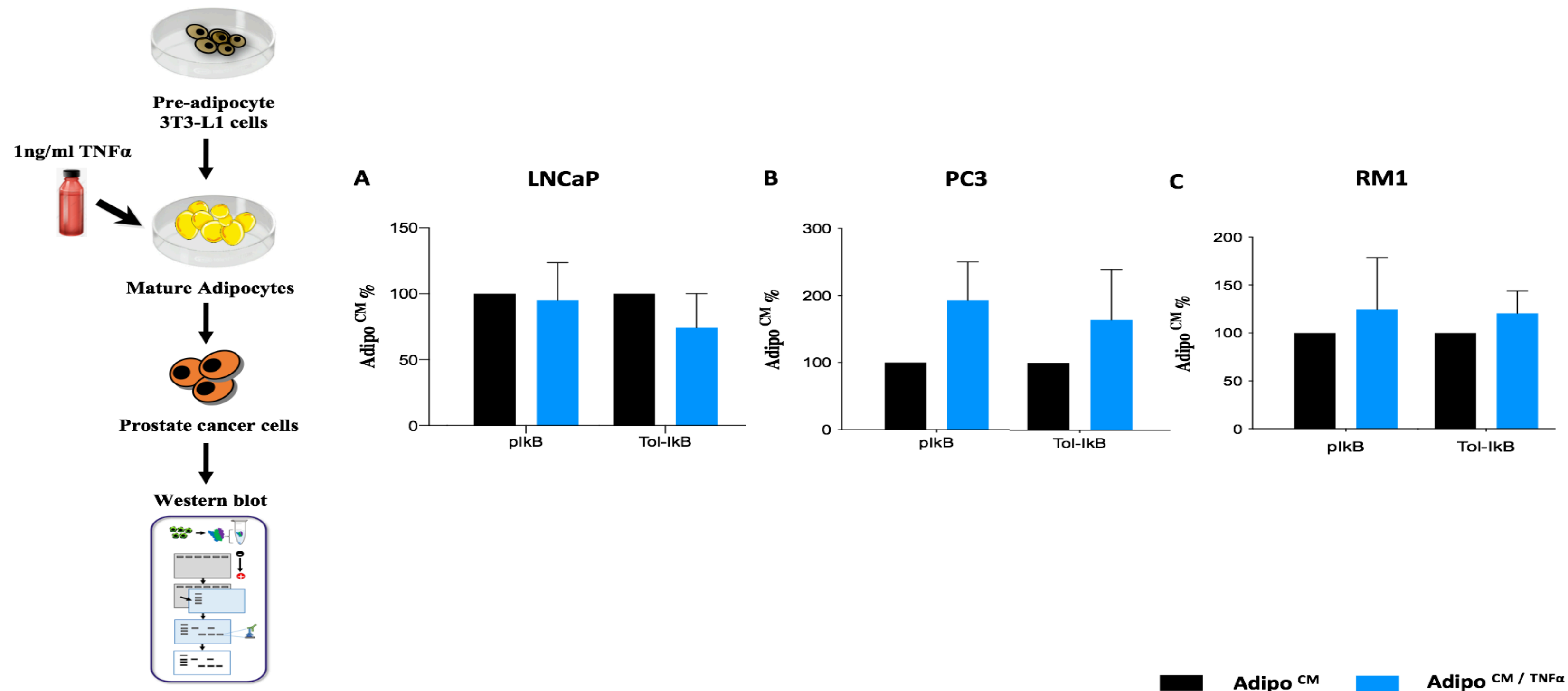
Supplementary figure 1. Pre-treatment of mature adipocytes to TNFα failed to enhance the migration of human LNCaP prostate cancer cells in vitro. *The effects of TNFα on the migration of the human prostate cancer cells LNCaP after 1-24 hours was assessed by wound healing assay. Plotted values for the area migrated as the percentage of each time CTRL ^{CM} in 4, 8, and 12 hours. Results are from three independent experiments (N=3). Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Magnifications is 10x.*



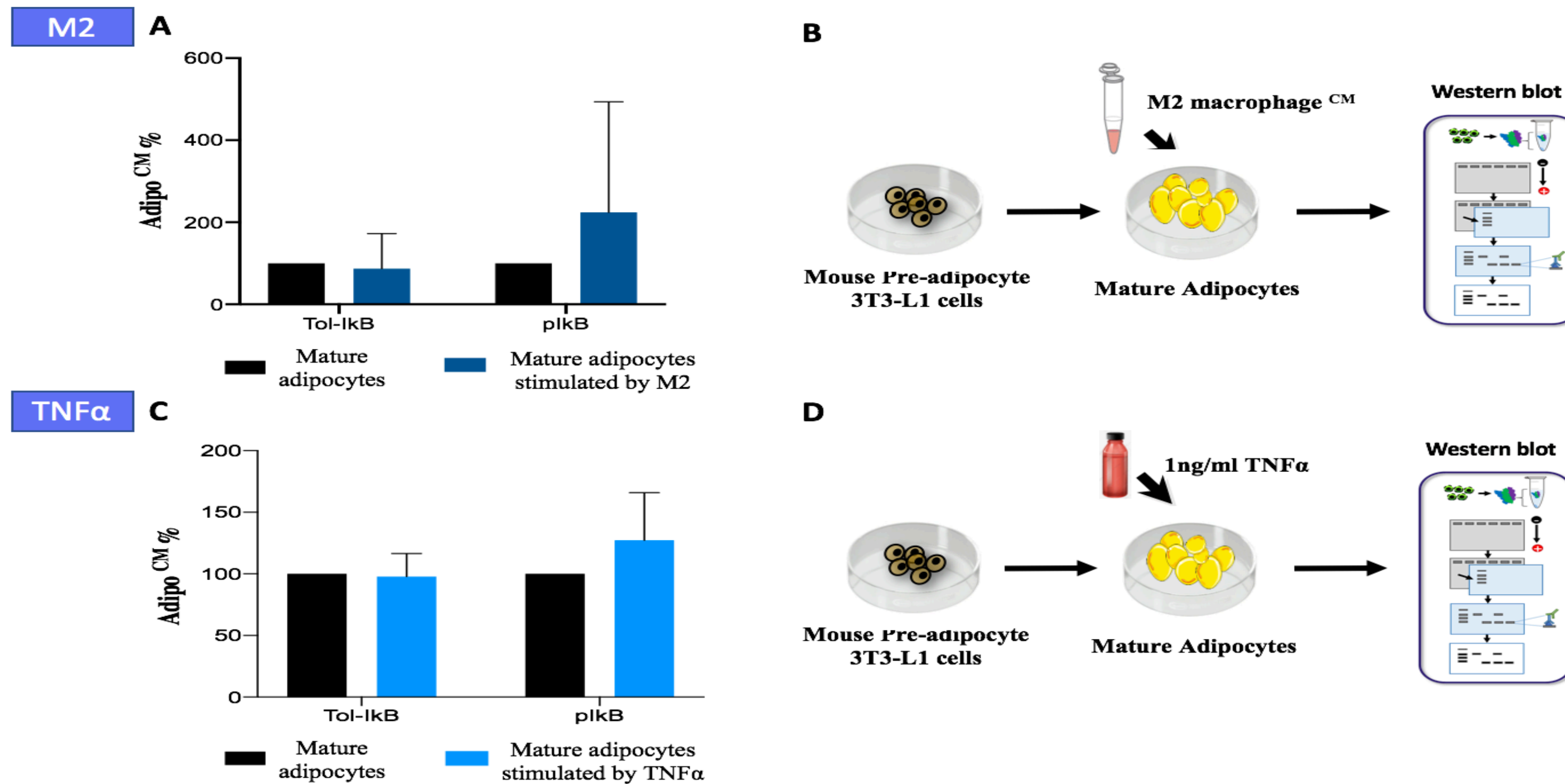
Supplementary figure 2. Pre-exposure of mature adipocytes to TNFα failed to enhance the invasion of human prostate cancer cells LNCaP *in vitro*. The effects of the TNFα on the invasion of the human prostate cancer cells LNCaP after 72 hours were assessed by Transwell® invasion. Results are from three independent experiments (N=3). Data were analysed using one-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.



Supplementary figure 3. ***Expression changing of canonical NFκB subunits in the pre-exposure of prostate cancer cells to conditioned medium collected from mature adipocytes stimulated by M2 macrophage conditioned medium.*** *Western blot quantification represents the NFκB subunits expression, IκBα and p-IκBα, relative to β-actin and normalized to Adipo ^{CM} - treated group. Results are from three independent experiments (N=3). Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

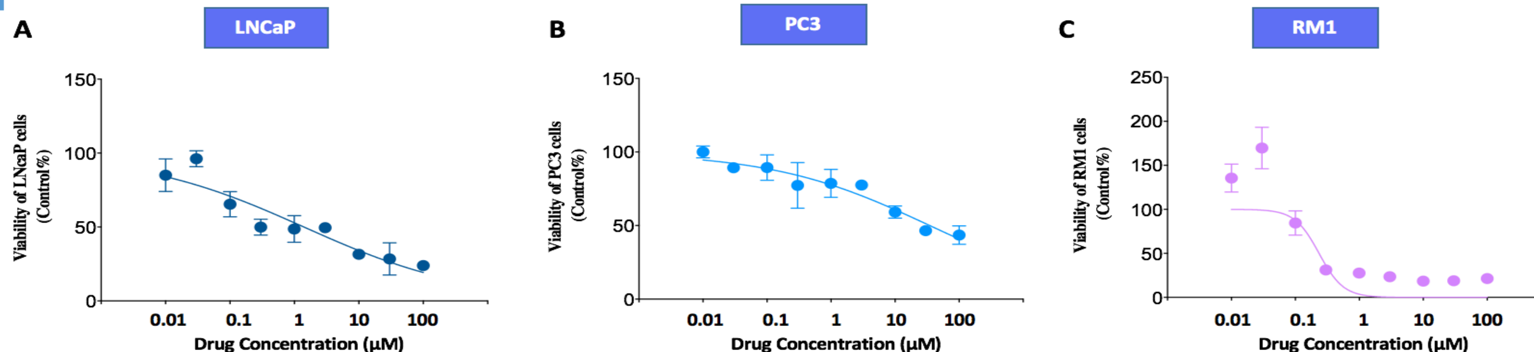


Supplementary figure 4. Expression changing of canonical NFκB subunits in the pre-exposure of prostate cancer cells to conditioned medium collected from mature adipocytes stimulated by TNFα. Western blot quantification represents the NFκB subunits expression, IκBα and p-IκBα, relative to β-actin and normalized to Adipo ^{CM} - treated group. Results are from three independent experiments (N=3). Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

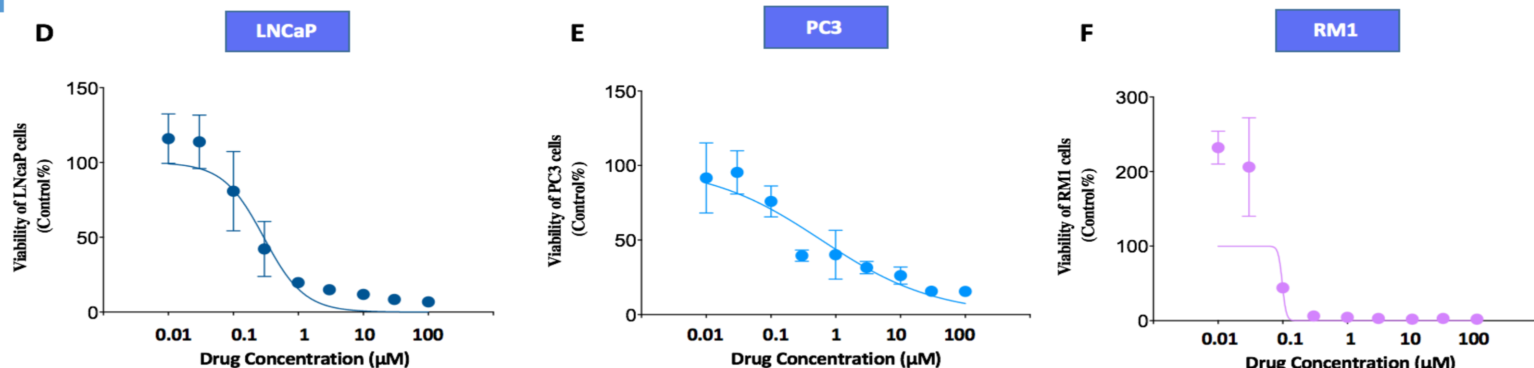


Supplementary figure 5. Expression changing of canonical NFκB subunits in the mouse mature adipocytes stimulated with TNFα or M2 macrophage conditioned medium. Western blot quantification represents the NFκB subunits expression, IκBα and p-IκBα, relative to β-actin and normalized to protein expression in mature adipocytes. Results are from three independent experiments (N=3). Data were analysed using two-way ANOVA test (Prism 7.0). Error bars indicate mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

24H



48H



Supplementary figure 6. Prostate cancer cell viability for proteasome inhibitor used in IC50 calculations. Dose-response curves of the proteasome inhibitor bortezomib on the viability of human LNCaP and PC3 and mouse RM1 prostate cancer cells after 24 hours (A-C) or 48 hours (D-F) assessed by Alamar Blue assay. Data obtained from triplicate experiments. Error bars indicate mean ± SD.

Supplementary Tables

Supplementary table 1. Raw data for mouse XL cytokine microarray used in section. Mature mouse 3T3-L1 cells were cultured with 1% DMEM for 16hrs before probing Adipo CM using Proteome Profiler^{TM} Mouse XL Cytokine Array Kit (R&D systems) followed by chemiluemiscence detection and analysis

Probed factor (spotted antibody duplicates)	CTRL ^{CM} (1% DMEM) (mean pixel density)		Adipo ^{CM} (mean pixel density)		Significance (multiple t-test) non-significant results highlighted in grey
Adiponectin	5432	0	1220712	1168131	<0.000001
Angiopoietin-1	0	116	134656	128256	<0.000001
CCL2	560	0	378784	394336	<0.000001
CCL5	300	8100	656620	749120	<0.000001
CCL11	176	312	386164	371476	<0.000001
Chemexin	380	0	1014140	905300	<0.000001
Chitimase 3-like 1	200	2012	286232	270272	<0.000001
Coagulation Factor III	320	0	924208	888052	<0.000001
Complement Factor D	0	0	319080	321316	<0.000001
CX3CL1	0	32	2258266	2177728	<0.000001
CXCL1	200	472	2441287	2395228	<0.000001
CXCL10	1848	324	130060	116420	<0.000001
Cystain C	344	0	402960	403344	<0.000001
Flt-3 Ligand	0	0	73308	75436	0.000616
Gas6	0	0	175964	177212	<0.000001
HGF	0	0	573424	582696	<0.000001
IGFBP-6	1072	180	1034880	954524	<0.000001
Lipocalin-2	0	0	694000	795932	<0.000001
LIX	0	32	2104377	2124094	<0.000001
M-CSF	0	176	343232	398044	<0.000001
MMP2	9488	10016	2070915	2046445	<0.000001
MMP3	0	0	2174652	2193028	<0.000001
MMP9	0	0	253224	185572	<0.000001
Osteopotin (OPN)	0	0	534852	553992	<0.000001
Pentraxin 3	2820	1876	653904	678552	<0.000001
Periostin	160	0	435940	363492	<0.000001
Prof-1	0	1280	690552	667004	<0.000001
Proliferin	448	0	455144	399624	<0.000001
Proprotein Convertase 9	160	120	207016	216016	<0.000001
Resistin	0	424	445500	473280	<0.000001
Serpin E1	0	64	1791087	1804950	<0.000001
VCAM-1	236	56	160120	204040	<0.000001

VEGF	220	0	1988604	2022567	<0.000001
WISP-1	104	0	261732	269112	<0.000001
CCL6	28348	6376	113152	91932	0.000103
CCL17	28	528	60840	60340	0.004972
Endostain	428	0	82256	84844	0.000142
IL-12	8	0	44968	34612	0.060523
LDLR	392	0	29816	25028	0.196551
LIF	10944	4772	20316	25256	0.477388
IL-1α	0	0	32348	31476	0.130792
IL-4	0	0	31504	21368	0.209728
IL-28A/B	696	140	17280	13836	0.471174

Supplementary table 2. Mature mouse 3T3-L1 cells stimulated with or without TNFa were cultured with 1% DMEM for 16hrs before probing factors derived from conditioned medium using Proteome ProfilerTM Mouse XL Cytokine Array Kit (R&D systems) followed by chemiluemiscence detection and analysis.

Probed factor (spotted antibody duplicates)	Adipo ^{CM} (mean pixel density)		Adipo $^{CM / TNFa}$ (mean pixel density)		Significance (multiple t-test) significant results highlighted in yellow
Adiponectin	1220712	1168131	1446240	1412025	<0.000001
Angiopoietin-1	134656	128256	252164	258300	0.000303
CCL2	378784	394336	1417659	1309137	<0.000001
CCL5	656620	749120	1436144	1527731	<0.000001
CCL11	386164	371476	1838124	1789646	<0.000001
Chemexin	1014140	905300	931460	944976	0.514693
Chitimase 3-like 1	286232	270272	440736	433136	0.000006
Coagulation Factor III	924208	888052	539496	530240	<0.000001
Complement Factor D	319080	321316	54448	45512	<0.000001
CX3CL1	2258266	2177728	2109550	2076068	0.000261
CXCL1	2441287	2395228	2463559	2459739	0.190237
CXCL10	130060	116420	171164	80696	0.934956
Cystain C	402960	403344	326260	322876	0.018978
Flt-3 Ligand	73308	75436	57960	61904	0.661492
Gas6	175964	177212	130804	124912	0.141799
HGF	573424	582696	595704	560360	0.999322
IGFBP-6	1034880	954524	1293801	1254070	<0.000001
Lipocalin-2	694000	795932	591168	637924	0.00015
LIX	2104377	2124094	2011964	1912491	0.000013
M-CSF	343232	398044	437444	422040	0.075623
MMP2	2070915	2046445	1455097	1462237	<0.000001
MMP3	2174652	2193028	2708610	2665498	<0.000001
MMP9	253224	185572	322024	273012	0.019672
Osteopotin (OPN)	534852	553992	872728	855960	<0.000001
Pentraxin 3	653904	678552	1458714	1533342	<0.000001
Periostin	435940	363492	320752	307968	0.011055
Prof-1	690552	667004	469372	443460	<0.000001
Proliferin	455144	399624	344676	342068	0.012336
Proprotein Convertase 9	207016	216016	337944	277944	0.004288
Resistin	445500	473280	582488	603200	0.000108
Serpin E1	1791087	1804950	1749640	1540534	0.000012
VCAM-1	160120	204040	226384	278044	0.035689

VEGF	1988604	2022567	1833467	1833922	0.000001
WISP-1	261732	269112	185932	169444	0.00908
CCL6	113152	91932	151444	160976	0.106127
CCL17	60840	60340	150492	143656	0.010074
Endostain	82256	84844	2296	796	0.0145
IL-12	44968	34612	119780	102228	0.03301
LDLR	29816	25028	49316	34296	0.662722
LIF	20316	25256	39708	33924	0.670519
IL-1α	32348	31476	27868	34892	0.987122
IL-4	31504	21368	12544	9260	0.637651
IL-28A/B	17280	13836	17740	14248	0.989446

Supplementary table 3. Mature mouse 3T3-L1 cells stimulated with or without M2 conditioned medium were cultured with 1% DMEM for 16hrs before probing factors derived from conditioned medium using Proteome ProfilerTM Mouse XL Cytokine Array Kit (R&D systems) followed by chemiluemiscence detection and analysis

Probed factor (spotted antibody duplicates)	Adipo CM (mean pixel density)		Adipo CM / M2 (mean pixel density)		Significance (multiple t-test) significant results highlighted in yellow
Adiponectin	1220712	1168131	1740327	1574761	<0.000001
Angiopoietin-1	134656	128256	580080	558064	<0.000001
CCL2	378784	394336	1619906	1416505	<0.000001
CCL5	656620	749120	1856017	1900605	<0.000001
CCL11	386164	371476	1290326	1298033	<0.000001
Chemexin	1014140	905300	1647063	1481437	<0.000001
Chitimase 3-like 1	286232	270272	1014480	936100	<0.000001
Coagulation Factor III	924208	888052	827236	822556	0.06671
Complement Factor D	319080	321316	193452	162624	0.001648
CX3CL1	2258266	2177728	2502072	2506494	<0.000001
CXCL1	2441287	2395228	2934319	2812549	<0.000001
CXCL10	130060	116420	1049940	1117768	<0.000001
Cystain C	402960	403344	539280	569100	0.000862
Flt-3 Ligand	73308	75436	97768	94464	0.62038
Gas6	175964	177212	477852	441304	<0.000001
HGF	573424	582696	982584	904148	<0.000001
IGFBP-6	1034880	954524	1436768	1346865	<0.000001
Lipocalin-2	694000	795932	270340	273340	<0.000001
LIX	2104377	2124094	1560105	1641291	<0.000001
M-CSF	343232	398044	516720	664768	0.000003
MMP2	2070915	2046445	2018902	2003013	0.278308
MMP3	2174652	2193028	2507344	2524195	<0.000001
MMP9	253224	185572	341696	203800	0.225919
Osteopotin (OPN)	534852	553992	1085644	1079832	<0.000001
Pentraxin 3	653904	678552	926844	871228	<0.000001
Periostin	435940	363492	167600	153476	<0.000001
Prof-1	690552	667004	603812	565344	0.034071
Proliferin	455144	399624	330960	346508	0.04579
Proprotein Convertase 9	207016	216016	197596	156092	0.430152
Resistin	445500	473280	697040	757728	<0.000001
Serpin E1	1791087	1804950	752992	712548	<0.000001
VCAM-1	160120	204040	333748	558100	<0.000001

VEGF	1988604	2022567	2137135	2145314	0.002608
WISP-1	261732	269112	201704	180396	0.092691
CCL6	113152	91932	554664	591216	<0.000001
CCL17	60840	60340	105684	105776	0.304971
Endostain	82256	84844	114428	118076	0.456722
IL-12	44968	34612	94852	91388	0.226092
LDLR	29816	25028	36560	49792	0.719605
LIF	20316	25256	7832	8288	0.73719
IL-1α	32348	31476	32316	41068	0.913235
IL-4	31504	21368	27212	23720	0.982359
IL-28A/B	17280	13836	26144	21956	0.846521

Supplementary table 4. Raw data of human XL Cytokine Microarray used in section. M2 macrophages were cultured with 1% DMEM for 16 hours before probing M2 CM using Proteome ProfilerTM Mouse XL Cytokine Array Kit (R&D systems) followed by chemiluemiscence detection and analysis

Probed factor (spotted antibody duplicates)	Adipo CM (mean pixel density)		Adipo CM / M2 (mean pixel density)		Significance (multiple t-test) significant results highlighted in yellow
IL8	0	0	5896519	5809823	<0.000001
CCL2	0	0	1863356	2531553	<0.000001
MIC-1	0	0	605896	656412	0.000626
CXCL10	0	0	7630327	7305104	<0.000001
CCL3/4	0	0	2662572	2250992	<0.000001
OPN	2240	1556	72108	54204	0.693115
Chitimase 3-like 1 (CHI3L1)	1927224	1750336	5423490	5183540	<0.000001
MMP9	0	472	577476	577476	0.001374
uPAR	1092	124	644780	506716	0.001417

Supplementary Table 5. Summary table for effects of differentially regulated factors produced by adipocytes stimulated with TNFα or M2 macrophage conditioned medium on prostate cancer cells. Effects on cells (reported in the literature) are indicated by: ↑ = positive; ↓ = negative; ↑↓ = positive and / or negative; ↔ = an ambiguous association; — = no association found.

Protein	M2 macrophage conditioned medium	TNFα	Refs
Adiponectin	↑	↓	(Luo and Liu, 2016, Hector et al., 2007)
Resistin	—	↓	(Fasshauer et al., 2001)
Angiopoietin-1	↑↓	↑	(Seok et al., 2013, Atanasov et al., 2018, Kabala et al., 2019)
CCL2	↑	↑	(Loberg et al., 2006, Ho et al., 2008, Roca et al., 2009, Sha et al., 2015)
CCL5	↑	—	(Izumi and Mizokami, 2019, Huang et al., 2020)
CXCL1	↔	↔	(Hardaway et al., 2015, Hu et al., 2018)
M-CSF	↑	—	(Caux et al., 1992, Chambers et al., 1995, Silzle et al., 2003)
MMP2	↑	↑	(Yang et al., Han et al., 2001, Zheng et al., 2017)
MMP3	—	↔	(Guo et al., 2018)
MMP9	↑	↑	(Tsai et al., 2014, Zheng et al., 2017, Xu et al., 2019)
OPN	↑	↑	(Zeyda et al., 2011, Kahles et al., 2014, Schuch et al., 2016)
VCAM-1	↔	↔	(Chen et al., 2011, Lim et al., 2012)
VEGF	↑	↑	(Ferrer et al., 1997, Hellawell and Brewster, 2002, Chen et al., 2014)
CCL17	↑	—	(Simona et al., 2014, Rani et al., 2019)
Endostatin	↔	—	(Guo et al., 2016, Foguer et al., 2016)

IL-12	↓	—	(Edwards and Mosser, 2010)
IL-4	↑	—	(Goldstein et al., 2011, Choi et al., 2020)
Pentraxin 3	↑	↑	(Inforzato et al., 2012, Hsiao et al., 2013)

Appendices

Appendix 1: Materials, Reagents, Apparatus and Software

Appendix 1.1 Materials and Reagents used in this study

Materials and reagents	Supplier
1.5ml Eppendorf tubes with cap	Starlab, Milton Keynes, UK
12% Criterion™ TGX™ Precast Midi Protein Gel, 12+2 well	Bio-Rad Laboratories, Hertfordshire, UK
3T3-L1	ATCC, Manassas, VA-USA
5-flurouracil	Sigma Aldrich, Dorset, UK
Acetic Acid Glacial	Sigma Aldrich, Dorset, UK
AlamarBlueTM reagent	Invitrogen, Paisley, UK
Alizarin Red S	Sigma Aldrich, Dorset, UK
Ampicllin	Fisher Scientific,Leicestershire, UK
BD microlance needles (19, 21 and 25G)	Fisher Scientific, Leicestershire, UK
Bicinchoninic acid (BCA) solution	Sigma Aldrich, Dorset, UK
Bovine Insulin	Sigma Aldrich, Dorset, UK
Bovine serum albumin	Sigma Aldrich, Dorset, UK
Bromophenol Blue	BDH Laboratory Supplies, Poole, Dorset, UK
C4-2	ATCC, Manassas, VA-USA
C4-2B4	ATCC, Manassas, VA-USA
Centrifuge tubes 15ml	Scientific laboratory supplies (SLS), Nottingham UK
Centrifuge tubes 50ml	Fisher Scientific, Leicestershire, UK
Cetyl pyridinium chloride monohydrate	Sigma Aldrich, Dorset, UK
Clarity Western ECL Substrate	Bio-Rad Laboratories, Hertfordshire, UK
Collagenase (type 1A)	Sigma Aldrich, Dorset, UK
Copper (II)-sulfate	Sigma Aldrich, Dorset, UK
Corning™ Transwell™ Multiple Well Plate with Permeable Polycarbonate Membrane Inserts	Corning, Flintshire, UK
Cover slips	Fisher Scientific, Leicestershire, UK
Cyclophosphamide	Sigma Aldrich, Dorset, UK
DAKO	Agilent Technologies, Wokingham, UK
Dexamethasone	Sigma Aldrich, Dorset, UK

Diethanolamin	Sigma Aldrich, Dorset, UK
DL-Dithiothreitol (DTT)	Sigma Aldrich, Dorset, UK
DMSO	Sigma Aldrich, Dorset, UK
Docetaxel	Sigma Aldrich, Dorset, UK
Doxorubicin	Sigma Aldrich, Dorset, UK
DPX mounting medium	Sigma Aldrich, Dorset, UK
EDTA	Sigma Aldrich, Dorset, UK
Electrophoresis power supply	Bio-Rad Laboratories, Hertfordshire, UK
Ethanol Absolute	Sigma Aldrich, Dorset, UK
Fetal calf serum (FCS)	Fisher Scientific, Leicestershire, UK
Filter Tips any size	Starlab, Milton Keynes, UK
Forceps watchmaker's	Fisher Scientific, Leicestershire, UK
Glycine	Acros organics, Geel, Belgium
Hanks buffer (HBSS)	Sigma Aldrich, Dorset, UK
Human recombinant RANKL	Gift from Dr. Patrick Mollat (Proskelia SASU)
Isobutylmethylxanthine	Sigma Aldrich, Dorset, UK
Isopropanol	Fisher Scientific, Leicestershire, UK
Jackson ImmunoResearch Anti-rabbit secondary ab	Stratech Scientific Unit, Newmarket Suffolk, UK
Kaleidoscope Pre-stained standards	Bio-Rad Laboratories, Hertfordshire, UK
LNCaP	ATCC, Manassas, VA-USA
Luria-Bertani (broth & agar)	Sigma Aldrich, Dorset, UK
Magic Marker	Invitrogen, Paisley, UK
Magnesium chloride	Sigma Aldrich, Dorset, UK
MC3T3-E1	ATCC, Manassas, VA-USA
M-CSF mouse recombinant	R & D Systems, Abingdon, UK
Methanol	VWR International LTD, Leicestershire, UK
Microtubes (0.5, 1.5, 2ml)	Sarstedt Ltd, Leicester, UK
Minimum Essential Medium (αMEM)	Fisher Scientific, Leicestershire, UK
Minimum Essential Medium (DMEM)	Fisher Scientific, Leicestershire, UK
N,N-Dimethylformamide	Sigma Aldrich, Dorset, UK
Napthol-AS-BI-phosphate	Sigma Aldrich, Dorset, UK
Neubauer Haemocytometer	Hawksley, Lancing, UK
Oil Red O	Sigma Aldrich, Dorset, UK
Paclitaxel	Sigma Aldrich, Dorset, UK
Paraformaldehyde	Taab Lab, Berkshire, UK
Pararosanilin	Sigma Aldrich, Dorset, UK
PC3	ATCC, Manassas, VA-USA

PC3-BT	Dr. Colby Eaton (Sheffield, UK)
PC3-NW1 (luciferase labelled)	Dr. Colby Eaton (Sheffield, UK)
Penicillin/Streptomycin	Fisher Scientific, Leicestershire, UK
Pierce™ Bovine Serum Albumin Standard Pre-Diluted Set	Fisher Scientific, Leicestershire, UK
Phosphatase inhibitor cocktail	Sigma Aldrich, Dorset, UK
Phosphate buffered saline	Fisher Scientific, Leicestershire, UK
Pipette tips (all sizes)	Starlab, Milton Keynes, UK
Puromycin	Fisher Scientific, Leicestershire, UK
Protease inhibitor cocktail	Sigma Aldrich, Dorset, UK
Proteome ProfilerTM Human XL Cytokine Array Kit	R&D Systems, Abingdon, UK
Prostate cancer tissue microarray (PR242b)	Biomax, MD, USA
4-Nitrophenyl phosphate disodium salt hexahydrate powder	Scientific laboratory supplies (SLS), Nottingham UK
Recombinant Human Semaphorin 3A Fc Chimera Protein, CF	R & D Systems, Abingdon, UK
Saos-2	ATCC, Manassas, VA-USA
Scalpel, disposable	VWR International LTD, Leicestershire, UK
Scissors (fine points and spring bow handles)	S Murray & Co Ltd, Surrey, UK
Silver nitrate	Sigma Aldrich, Dorset, UK
Sodium acetate trihydrate	VWR International LTD, Leicestershire, UK
Sodium barbiturate	Sigma Aldrich, Dorset, UK
Sodium chloride	Sigma Aldrich, Dorset, UK
Sodium dodecyl sulphate (SDS)	Bio-Rad Laboratories, Hertfordshire, UK
Sodium hydroxide	VWR International LTD, Leicestershire, UK
Sodium phosphate	Sigma Aldrich, Dorset, UK
Sodium tartrate dibasic dihydrate	Sigma Aldrich, Dorset, UK
Starguard® laboratory gloves	Starlab, Milton Keynes, UK
Sterile filter (0.2 and 0.45µm)	Pall lifesciences, Portsmouth, UK
Stripettes (5, 10, 25 and 50ml)	Fisher Scientific, Leicestershire, UK
Superfrost Plus™ Adhesion Microscope Slides	Fisher Scientific, Leicestershire, UK
Syringes (all sizes)	Fisher Scientific, Leicestershire, UK
Tissue culture 25, 75, 175cm2 flasks	Fisher Scientific, Leicestershire, UK

Tissue culture microplates (6, 12, 24, 48 and 96-well plates)	Corning, Flintshire, UK
Transblot Turbo midi Size PVDF membrane	Bio-Rad Laboratories, Hertfordshire, UK
Transblot Turbo midi Size Transfer stacks	Bio-Rad Laboratories, Hertfordshire, UK
Tris	Bio-Rad Laboratories, Hertfordshire, UK
Tris-EDTA buffer	Sigma Aldrich, Dorset, UK
Tris-Glycine buffer 10x	Bio-Rad Laboratories, Hertfordshire, UK
Triton X-100TM	Sigma Aldrich, Dorset, UK
Trizma® hydrochloride	Sigma Aldrich, Dorset, UK
Trizma® base	Sigma Aldrich, Dorset, UK
Trypsin/EDTA	Sigma Aldrich, Dorset, UK
Tween-20	Acros organics, Geel, Belgium

Appendix 1.2 Antibodies in this thesis

Antibody	Supplier
Rabbit anti-p65	Cell Signalling technologies, USA
Rabbit anti-Actin	Sigma-Aldrich, UK
Rabbit anti-β-catenin	Cell Signalling technologies, USA
Rabbit anti-GAPDH	Sigma-Aldrich, UK
Rabbit anti-IκBα	Cell Signalling technologies, USA
Rabbit anti-phospho-IκBα	Cell Signalling technologies, USA

Appendix 1.3 Apparatus in this thesis

Apparatus	Supplier
AA Hoefer® protein transfer apparatus	Fisher Scientific, Leicestershire, UK
Bench-top Eppendorf centrifuge	Fisher Scientific, Leicestershire, UK
Grant OLS 200 water bath	Thistle Scientific, Glasgow, UK
Horizontal electrophoresis tanks	Fisher Scientific, Leicestershire, UK
Hotplate/stirrer	Thistle Scientific, Glasgow, UK
Ika Vortex	Thistle Scientific, Glasgow, UK
Leica AF6000	Leica Microsystems, Milton Keynes, UK
Nichiryo America Inc. Pipettes (2, 10, 100, 200 and 1000µl)	Thistle Scientific, Glasgow, UK
Origo PSU-400/200 power supply for electrophoresis	Anachem, Bedfordshire, UK
PowerPac basicTM	Bio-Rad Laboratories, Hertfordshire, UK
QImaging Retiga 4000R CCD camera	Media Cybernetics UK, Berkshire, UK
SpectraMax® M5 microplate reader	Fisher Scientific, Leicestershire, UK
Syngene GeneGenius Gel Bio-Imaging system	Fisher Scientific, Leicestershire, UK
SynSyngene GeneGnome Bio-Imaging system for chemiluminescence	Fisher Scientific, Leicestershire, UK
Vertical CriterionTM gel tanks	Bio-Rad Laboratories, Hertfordshire, UK

Appendix 1.4 Software used in this thesis

Software	Supplier
CompuSyn	ComboSyn, NJ, USA
GraphPad Prism (version 7)	GraphPad Software Inc., CA-US
ImageJ	U. S. National Institutes of Health Bethesda, MA-US
Image Lab	Biorad, Watford, UK
Osteomeasure	Osteometrics, DA, USA
SpectraMax® M5 plate reader software	Fisher Scientific, Leicestershire, UK
TScratch	ETH Zürich, Switzerland

Appendix 2: Solutions and Recipes

Appendix 2.1 Solution for cell lysis

RIPA Lysis buffer

1% Triton 100X, 0.5% (w/v) Sodium Deoxycholate, 0.1% (w/v) Sodium Dodecyl Sulphate (SDS), 50 mM Tris-HCl (pH 7.4) and 150 mM Sodium Chloride were dissolved in dH2O

Cytoplasmic fraction Lysis buffer

10 mM Tris [pH 7.5], 0.05% NP-40, 3 mM MgCl2, 100 mM NaCl, 1 mM EGTA were dissolved in dH2O

Appendix 2.2 Solutions for PAGE and western blot

Electrophoresis running buffer

100 ml of TGS buffer (10X) in 1000 ml of dH2O

Samples loading protein buffer (5X stock)

5.2 ml of 1M Tris-HCl pH adjusted to 6.8, 1 g of DL-Dithiothreitol (DTT), 3 g SDS, 6.5 ml glycerol and 130 µl of 10% (w/v) Bromophenol Blue. Stored at -20°C.

Transfer buffer

3.63 g of Tris, 14.4g of Glycine, 200 ml of Methanol and 3.75 ml of 10% (w/v) SDS made up to 1000 ml with dH2O. Stored at room temperature.

TBS

1 M of Tris and 1 M Tris-HCl. pH adjusted to 7.9 prior to addition of 3 M Sodium Chloride. Stored at room temperature.

TBST

0.1% (v/v) Tween-20 in TBS. Stored at room temperature.

Stripping buffer

1 mM DTT, 2% (w/v) SDS and 62.5 mM Tris-HCl (pH 6.7). Stored at room temperature.

Appendix 3: Informatic technology

Appendix 3.1 Search strategy – "Medline via Ovid SP"

1. exp NF-kappa B/
2. NF-kappaB$.tw.
3. (NF-kappaB* or transcription factor nf-kb* or NFκB*).mp
4. (NF-kappaB* or transcription factor nf-kb* or NFκB*).tw.
5. Transcription factor nf-kb$.tw.
6. NFκB$.tw.
7. Or/1-6
8. Exp Overweight/ or exp Humans/ or exp Adult/ or exp Obesity/ or exp Prostatic Neoplasms/ or exp Male/ or exp Body Mass Index/
9. Obes* man.mp.
10. Obese man.tw.
11. Obese man$.tw.
12. Or/8-11
13. Exp Diet, High-Fat/ or exp Animals/ or exp Adipose Tissue/ or exp Obesity/ or exp Rats/ or exp Mice/ or exp males
14. (obes* mice or "high fat diet mice").mp.
15. (obes* mice or "high fat diet mice").tw.
16. Obes* mice$.tw.
17. "high fat diet mice" $.tw.
18. Or/13-17
19. Exp Prostatic Neoplasms/
20. (prostat* cancer or prostat* neoplasm).mp.
21. (prostat*cancer or prostat* neoplasm).tw.
22. Prostat* cancer$.tw.

23. Prostat* neoplasm$.tw.

24. Or/19-23

25. 7 and 12 and 24

26. 7 and 18 and 24

27. 25 or 26

Appendix 3.2 Search strategy – “Web of Science”

# 4	29	#3 OR #1 *Databases= WOS, BCI, BIOSIS, CCC, DRCI, DIIDW, KJD, MEDLINE, RSCI, SCIELO, ZOOREC Timespan=All years Search language=Auto*
# 3	14	AB=(obes* and prostat* cancer and NF-kappaB) *Databases= WOS, BCI, BIOSIS, CCC, DRCI, DIIDW, KJD, MEDLINE, RSCI, SCIELO, ZOOREC Timespan=All years Search language=Auto*
# 2	0	TI=(obes* and prostat* cancer and NF-kappaB) *Databases= WOS, BCI, BIOSIS, CCC, DRCI, DIIDW, KJD, MEDLINE, RSCI, SCIELO, ZOOREC Timespan=All years Search language=Auto*
# 1	29	TS=(obes* and prostat* cancer and NF-kappaB) *Databases= WOS, BCI, BIOSIS, CCC, DRCI, DIIDW, KJD, MEDLINE, RSCI, SCIELO, ZOOREC Timespan=All years Search language=Auto*