DISSECTING THE PROSTATE CANCER STEM CELL NICHE INSIDE THE BONE MARROW

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Summary

Prostate cancer frequently metastasizes to the bone and becomes incurable. Recent studies have suggested that prostate cancer stem cells (CSCs) play key roles in the initiation, progression and treatment failure of the disease and therefore represent an ideal therapeutic target. Prostate CSCs share many similarities with normal stem cells, including dependency on a stem cell niche. Prostate CSCs that disseminate into the bone marrow are believed to manipulate the hematopoietic stem cell (HSC) niche to initially maintain a quiescent state before producing a new niche to support the development of bone metastasis. Therefore, a better understanding of the bone marrow stem cell niche and its role in supporting bone metastasis may aid the development of effective treatments.

The main aim of my project was to investigate the role of osteoblasts and adipocytes, two major cellular components of the bone marrow, in the formation of a CSC-specific niche during the development of prostate tumor bone metastasis. Specifically, I have studied the role of angiopoietin-1, a well-studied osteoblast-secreted paracrine factor, in regulating the quiescence of prostate CSCs. cDNA microarray and real-time PCR (RT-PCR) analysis were used to elucidate the downstream mechanisms underlying the actions of Ang-1 in prostate CSCs. I have subsequently confirmed my findings by demonstrating that Ang-1 receptor (Tie-2) promotes prostate tumor bone metastasis *in vivo*.

In the second part of my project, I studied whether prostate CSCs manipulate adipocytes within the bone marrow to promote the formation of a CSC-specific

niche. I first established a novel 3D spheroid co-culture model to study the interactions between adipocytes and prostate CSCs. Using this model, I have tested the effects of adipocytes on CSC self-renewal and identified the key paracrine factors that mediate the crosstalk between these two cell types.

The successful completion of this study has lead to the identification of a number of potential prognostic and therapeutic targets against prostate tumor bone metastasis. Further translation of my work may therefore help in improving the treatment outcome of prostate cancer patients.

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List of Abbreviations

3D	Three-dimensional/three dimensions
α2β1	Alpha 2 Beta 1 integrin
Adi	Adipocytes
APC	Allophycocyanin
AR	Androgen receptor
ATCC	American Type Culture Collection
Ang-1	Angiopoietin-1
BAD	BclxL/Bcl-2-associated death promoter
BAT	Brown adipose tissues
CA-074ME	CTSB Inhibitor
CARN	Castrate-resistant Nkx3-1 expressing cell
CCK	Cholecystokinin
CD133	Prom1
CK	Cytokeratin
CM	Conditioned Medium
CRPC	G protein-coupled receptor
CSC	Cancer stem cell
CTC	Circulating tumor cell
CTSB	Cathepsin B
DMEM	Dulbecco's modified Eagle's medium
ECGS	Endothelial cell growth factor
ECM	Endothelial culture medium
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
eIF4A	Eukaryotic Translation Initiation Factor-4A
eIF4B	Eukaryotic Translation Initiation Factor-4B
eIF4E	Eukaryotic Translation Initiation Factor-4E
eIF4G	Eukaryotic Translation Initiation Factor-4-Gamma
EMT	Epithelial to mesenchymal transition
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FOXO1	Forkhead box O1 family transcription factors
GSK3β	Glycogen synthase kinase 3β
HGF	Hepatocyte growth factor
Hh	Hedgehog
HO	Hoechst33342
HSC	Hematopoietic stem cell
hTERT	Human telomerase reverse transcriptase
HUVEC	Human Umbilical Vein Endothelial Cell

ICAM1	Intercellular adhesion molecule 1
IGFR	Insulin-like growth factor receptor
IL	Interleukin
MDM2	Murine double minute
MEM	Minimum Essential Medium
MMP	matrix-metalloproteinase
MSC	Mesenchymal stem cell
mSIN1	mammalian stress-activated protein kinase-interacting protein1
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor-κB
P/S	Penicillin-streptomycin
PAI-1	Plasminogen activator inhibitor-1
PAP	Prostatic acid phosphatase
PBS	Phosphate-buffered saline
PCD	Promote programmed cell death
PDGFR	Platelet-derived growth factor receptor
PDPK1	Putative 3-phosphoinositide-dependent kinase 1
PE	Phycoerythrin
PECAM1	Platelet endothelial cell adhesion molecule 1
PH	Pleckstrin homology
PHAS1	Phosphorylated Heat-stable and Acid-Stable protein 1
PI3K	Phosphatidylinositol-3-kinase
PI3KCA	Phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit alpha
PI3P	Phosphatidylinositol-3-phosphate
PI4P	Phosphatidylinositol-4-phosphate
PIP2	Phosphatidylinositol-4,5-bis phosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PMSF	Phenylmethylsulfonyl fluoride
PRAS40	proline-rich Akt substrate 40 kDa
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
RTK	Receptor tyrosine kinase
PY	Pyronin Y
Rac1	Ras-related C3 botulinum toxin substrate
RANKL	Receptor activator of nuclear factor kappa-β ligand
Raptor	Regulatory-associated protein of mTOR
RhoA	Ras homolog gene family, member A
S6K	Ribosomal protein S6 kinase
S6K1	ribosomal protein p70S6K
Sca-1	Stem cell antigen 1
SCF	Membrane-bound stem cell factor
siRNAs	Small interfering RNAs

STAT3	Signal transduction and activator of transcription 3
TGFβ	Transforming growth factor beta
THPO	Thrombopoietin
TIC	Tumor-initiating cells
TNF-α	Tumor necrosis factor alpha
TSC2	Tuberous sclerosis complex 2
UCPI	Mitochondrial uncoupling protein 1
VCAM1	Vascular cell adhesion protein 1
VEGF	Vascular epithelial growth factor
VPS34	Homologue of the yeast vacuolar protein sorting-associated protein 34
WAT	White adipose tissues
γ-T3	Gamma-tocotrienol
YAT	Yellow adipose tissues/Bone marrow fat
YM022	CCKBR Inhibitor

Awards

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Publications

Manuscripts related to my PhD

Accepted Manuscript

1. **Tang KD** and Ling MT (2014). Targeting drug-resistant prostate cancer with dual PI3K/mTOR inhibition. Curr Med Chem. [Epub ahead of print].
2. **Tang KD**, Holzapfel BM, Liu J, Lee TKW, Ma S, Jovanovic L, An J, Russell PJ, Clements JA, Hutmacher DW and Ling M (2015). Tie-2 regulates the stemness and metastatic properties of prostate cancer cells. Oncotarget

Manuscript to be submitted

1. **Tang KD**, Liu J, Jovanovic L, An J, Russell PJ, Clements JA, and Ling MT. Adipocytes promote prostate cancer stem cell self-renewal through a vicious cycle of cathepsin B and cholecystokinin secretion.

Peer-Reviewed Publications on which I am an author but unrelated to my PhD

1. Liu J, Lau EY, Chen J, Yong J, **Tang KD**, Lo J, Ng IO, Lee TK, Ling MT. (2014) Polysaccharopeptide enhanced the anti-cancer effect of gamma-tocotrienol through activation of AMPK. BMC Complement Altern Med. 14:303.doi: 10.1186/1472-6882-14-303.
2. Kwan PS, Lau CC, Chiu YT, Man C, Liu J, **Tang KD**, Wong YC and Ling MT. (2013) Daxx regulates mitotic progression and prostate cancer predisposition. Carcinogenesis 34(4):750-9. doi: 10.1093/carcin/bgs391.
3. Chiu Y-T, Liu J, **Tang KD**, Wong Y-C, Khanna KK and Ling MT. (2012) Inactivation of ATM/ATR DNA Damage Checkpoint Promotes Androgen Induced Chromosomal Instability in Prostate Epithelial Cells. PLoS ONE 7(12): e51108. doi:10.1371/journal.pone.0051108.

Published conference abstracts (*are related to my PhD)

1. ***Tang KD**, Ling MT. (2014) Tie-2 regulates stemness and metastasis of prostate cancer cells. [abstract]. In: Proceedings of the 105th Annual Meeting of the American Association for Cancer Research; San Diego, CA. Philadelphia (PA): AACR; Cancer Res 2014;74(19 Suppl):Abstract nr 3871. doi:10.1158/1538-7445.AM2014-3871.
2. ***Tang KD**, Ling MT. (2014) Tie-2 regulates stemness and metastasis of prostate cancer cells. European Journal of Cancer. Vol 50 Supplement 4:e8.
3. ***Tang KD**, Ling MT. (2014) Tie-2 regulates the stemness of prostate cancer cells. European Journal of Cancer, 50, Supplement 6(0): p. 33.

4. **Tang KD**, Ling MT. (2013) Inactivation of ATM/ATR DNA damage checkpoint promotes androgen induced chromosomal instability in prostate epithelial cells. BJU International, 2013; 112 S1:30-53.

Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

QUT Verified Signature

Signature:

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Chapter 1: Introduction

1.1 Introductory Statement

Prostate cancer is one of the most common solid tumors in men and is the second leading cause of morbidity and mortality in men worldwide [1]. When the tumor is localized, the disease is curable by prostatectomy. However, patients with advanced prostate cancer are normally treated with androgen ablation therapy. The therapy is effective initially as prostate cancer cells require androgen to grow and survive; however, the cancer cells eventually become androgen independent and develop metastatic, castration-resistant tumors [2]. At this stage, chemotherapy and radiotherapy exhibit only small benefits. As a result, advanced prostate cancer remains an incurable disease by current treatment strategies [3, 4].

Most primary tumor cells enter the circulation and seed at secondary tissues during the formation of metastasis. In the secondary site, the primary tumor cells have to adapt to a new microenvironment, which normally lack nutrients and oxygen, before they can redevelop as macro metastases [5]. Bone is the most common site of cancer metastasis, especially in breast and prostate cancer. Ample evidence supports the idea that tumor metastasis originates from a rare subpopulation of cancer cells known as cancer stem cells (CSCs) [6]. CSCs are similar to normal stem cells, in that they can undergo an unlimited self-renewal [7, 8] and differentiate into heterogeneous lineages of cells [9]. Furthermore, the unique plasticity of CSCs also allows them to undergo a phenotypic switch known as epithelial to mesenchymal transition (EMT) which is believed to be the key event during cancer metastasis. EMT is a process in which epithelial cells with regular cell-cell junctions and adhesions convert into

motile and invasive cells with mesenchymal phenotypes [10]. Recent studies have reported a close relationship between CSCs and EMT [11]. More importantly, substantial analysis of tumor cells that disseminate into bone marrow has confirmed that most of these cells possess a CSC phenotype. What is not yet clear is why CSCs preferentially disseminate into the bone marrow.

One possible reason is the presence of a stem cell niche in the bone marrow, which, under normal circumstances, helps to maintain the stemness of hematopoietic stem cells (HSCs). As demonstrated in previous studies, mesenchymal stem cells (MSCs) that reside in the bone cavity are responsible for differentiating into different types of marrow stromal cell lineages, including obsteoblasts, adipocytes, fibroblasts, chondrocytes, myocytes and endothelial cells [12]. Osteoblasts, in particular, are responsible for the formation of the osteoblastic niche that regulates the homing and self-renewal of HSCs [13]. This has been supported by *in vivo* studies which showed that HSC and progenitor cells mobilized to the periphery when there is a depletion of osteoblasts [14, 15]. Like HSCs, the stemness of CSCs is highly dependent on the presence of a stem cell niche. Interestingly, according to previous studies, CSCs are capable of creating their own CSC niche by recruiting MSCs derived from bone marrow, resulting in expansion of the CSC population within the primary tumor [16]. On the other hand, CSCs can also 'hijack' the already established normal stem cell niches [5]. This has been demonstrated recently in a mouse model, where cancer cells have been shown to form micrometastases within the HSC niches by competing with the HSCs [17]. Therefore, a better understanding on the role of the bone marrow stem cell niche during development of prostate cancer metastasis may offer opportunities for new treatment strategies.

Adipocytes represent another major component within bone marrow, which contributes to more than half of the bone marrow. Although the role of adipocytes in prostate tumor bone metastasis is largely unclear, obesity, a condition associated with abnormal bone marrow adiposity, has been shown to correlate with prostate cancer development [18-20] and disease progression [21]. In a number of large cohorts, a higher body mass index was consistently found to increase the risk of prostate cancer. Obesity has also been found to correlate with prostate cancer relapse after prostatectomy [22] or radiation therapy [23].

Evidence from previous studies has demonstrated that prostate cancer cells are attracted to an adipocyte-rich metabolically active red bone marrow [24, 25]. Recently, bone marrow adipocytes have also been reported to stimulate prostate tumour growth in bone via an FABP4-dependent (Fatty acid binding protein 4) mechanism [26]. In addition, prostate tumor cells are able to infiltrate into the periprostatic adipose tissues, which influences the phenotypic behaviour of malignant cells through modulation of adipokine secretion (adipokines are factors secreted by adipose tissues), extracellular matrix components and also via direct cell-cell contact [27]. Adipocytes isolated from periprostatic adipose tissues were also found to induce invasiveness of prostate cancer cells [28]. Therefore, these findings support the idea that abnormal adipocyte function, possibly due to adjacent cancer cells or as a result of obesity, may contribute to a CSC niche that favours prostate tumor metastasis. In summary, understanding the role of osteoblasts and adipocytes in the formation of a CSC-specific niche may help in the development of new and effective treatments against metastatic prostate cancer, and as a result decrease the mortality and morbidity associated with these patients.

1.2 Hypothesis and Aims

Research questions 1: Does the osteoblast-secreted paracrine Ang-1 promote prostate tumor bone metastasis by supporting the homing and colonization of prostate CSCs into the bone marrow?

Osteoblasts within the bone marrow actively secret Ang-1 to maintain the quiescence and stemness of HSCs. Here, I hypothesize that Ang-1 also functions as a key stem cell factor which maintains the quiescence state of prostate CSCs during prostate tumor metastasis and enrichment of Ang-1 receptor, Tie-2 in prostate cancer cell population supports the homing and colonization of prostate CSCs into the bone marrow. These hypotheses will be tested by the following aims:

- Determine the role of Ang-1/Tie-2 in the maintenance of prostate CSCs.
- Determine the role of Ang-1/Tie-2 in the regulation of prostate CSCs quiescence.
- Determine the role of Ang-1/Tie-2 in prostate tumor bone metastasis.

Research Question 2: Do adipocytes contribute to the formation of a prostate CSC niche during the development of prostate tumor bone metastasis.

Prostate tumor frequently metastasizes to the bone marrow, where half of the microenvironment is composed of adipocytes. However, it is not clear whether these fat storing cells play any roles in bone metastasis. Here, I hypothesize that during prostate tumor metastasis, prostate CSCs are the architects of their own stem cell niches through manipulation of adipocytes within the bone marrow. I will test this hypothesis by carrying out the following aims:

- Determine the role of adipocytes in the maintenance of prostate CSCs.
- Determine the underlying mechanism that drives the active self-renewal of prostate CSCs by adipocytes

1.3 Overview of research contributing to publication output

In this PhD project, I have focused on studying the role of the two major components with in the bone marrow (i.e. osteoblasts and adipocytes) in prostate CSC self-renewal and prostate tumor metastasis. As will be discussed in Chapter 2, prostate CSCs have been suggested to play an important role in the development, progression and treatment failure of prostate cancer. Meanwhile, signaling cascades such as the PI3K/Akt/mTOR pathway, which plays an important role in promoting the stemness of prostate CSCs, have been demonstrated in both pre-clinical and clinical studies as a potential therapeutic target for the treatment of advanced prosetate cancer (as discussed in the published review article in Chapter 2). Furthermore, celluar components (e.g. osteoblasts and adipocytes) within the bone marrow have also been suggested to contribute to a CSC niche and as a result promote cancer development and progression [22, 23, 29]. Nevertheless, the exact roles and mechanisms of these cellsin promoting the homing and colonization of prostate CSCs into the bone marrow during prostate tumor metastasis is still far from clear.

Ang-1 is one of the osteoblast-secreted paracrines that was found to play an important role in regulating HSC stemness and quiescence [30]. As described in Chapter 3, I have uncovered a key role of Ang-1/Tie-2 in supporting the homing of prostate CSCs into the bone marrow. This work has been accepted for publication by the journal Oncotarget.

In Chapter 4, I have used a novel 3D spheroid co-culture model to help identify the role and underlying mechanism of action of adipocytes in promoting prostate CSCs self-renewal. This work will be submitted as a research article shortly.

Chapter 2: Literature Review

2.1 CSC in the development and progression of prostate cancer

2.1.1 Anatomy of the human prostate

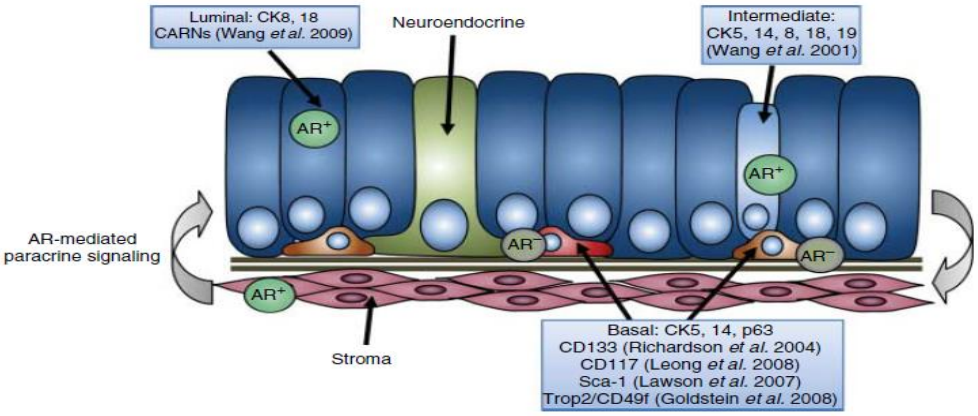

Figure 2.1: Anatomy of the human prostate. Schematic of the cellular architecture of the human prostate (taken from [31]).

There are different cell types within normal and mature prostatic epithelium: luminal, basal and neuro-endocrine (Figure 2.1). There is an intermediate cell type within the normal prostate which shares the properties of both basal and luminal cells. The luminal cells constitute the exocrine portion of the prostate which secretes prostatic acid phosphatase (PAP) and prostate specific antigen (PSA) into glandular lumina. They are dependent on androgens for their survival and hence, they express high levels of androgen receptor (AR). Besides that, basal cells reside as one or two layers connected to the basement membrane below the luminal cells. Unlike luminal cells, basal cells express a low level of AR and also exclusively express p63 (a homolog of the tumor suppressor gene p53). Hence, luminal and basal cells can be differentiated by different markers. Luminal cells express cytokeratins (CKs) CK8 and CK18, whereas, basal cells express CK5 and CK14, but not CK8 or CK18. Besides that,

intermediate cells express CKs of both basal and luminal cells (CKs 5, 14, 8, and 18) [3, 31]. Neuroendocrine cells are considered as rare cells which are often located in the luminal layer of the epithelium and is found to regulate the growth of prostate and development through endocrine–paracrine actions. Serotonin and thyroid-stimulating hormone can be found in the major types of neuro-endocrine cell. They are terminally differentiated, post-mitotic cell types that are androgen-insensitive. Fibroblasts, myofibroblasts, and smooth muscle cells are the stromal cells that found in prostate which support the growth and differentiation of the epithelium. Blood cell elements like vascular and stromal endothelial cells are also present in the gland [32].

2.1.2 Localization of stem cells in the prostate

Currently, the three proposed models of stem cell differentiation in normal tissue include the linear, bidirectional, and independent lineages.

2.1.2.1 Linear differentiation model

Biologically, basal cells contain many characteristics of stem cells including their relatively undifferentiated state, high proliferative capacity, resistance to apoptosis, and a long life span [33]. This linear model is defined by Isaacs & Coffey [34], where stem cells within the basal cell will undergo asymmetric cell division and give rise to one stem cell copy (self-renewal) and one multipotent progenitor cell (or transient amplifying cell). There are several types of marker that have been used to identify the prostate stem cells in the basal cell population: stem cell antigen-1 (Sca-1, also known as Ly6a), ALDH, CD133 (Prom1), Trop-2, and CD44. However, as reported by previous studies, most non-stem cells in prostate mouse model also express these markers. Leong *et al.* [35] identified CD117 as a new marker of a rare adult mouse prostatic stem cell population, CD117+ cells are predominantly basal

(CK14+) in the mouse and exclusively basal (p63+) in the human. This new marker is then combined with other stem cell markers which define a sub-population of cells as Lin-Sca-1+CD133+CD44+CD117+ which are capable of regenerating prostatic epithelium that consists of all epithelial cell types *in vivo*. Some studies also used Alpha 2 Beta 1 integrin ($\alpha 2\beta 1^{High}$) CD44+CD133+ and Trop2 and CD49f as the markers to define the human prostate stem cells, which are found in the basal epithelium [36].

2.1.2.2 Bidirectional differentiation model

Wang *et al.* and Kurita *et al.* proposed that CK5+8- basal cells are the 'only' source of prostatic stem cells, and indicated the possible existence of a distinct multipotent stem cell in the intermediate cell population [37, 38].

2.1.2.3 Independent lineages model

Linear and Independent models postulate that stem cells are confined to a single-cell type, either basal or intermediate cell. However, according to Wang *et al.*, stem cells may also be presented in the luminal compartment of the prostate gland [39]. They used the Nkx3-1 homeobox gene expression to identify the stem or progenitor cell in the luminal compartment. Meanwhile, the rare luminal cells that express Nkx3-1 in the absence of testicular androgens or known as castrate-resistant Nkx3-1-expressing cells, CARNs are shown to be bi-potent and maintain the capacity to self-renew *in vivo* thus proving the presence of stem cells in the prostate gland [31].

Based on these three models, it appears that at least three major sites normally contain prostate stem cells. Nevertheless, whether the stem cells in these three different sites all contribute to the development of disease such as cancer or whether they were susceptible to the same genetic instability remains unclear.

2.1.3 Origin of prostate CSCs

Prostate cancer most probably arises from the luminal cells because the bulk population of tumour cells express luminal cell-specific markers (CK 8, CK 18, AR, PSA and PAP), but lack the expression of basal cell markers, such as p63. Some studies also suggested that this disease arises from the intermediate progenitors which can undergo self-renewal [32]. On the other hand, Liu and colleagues observed that most primary tumors consist of luminal cells, whereas the majority of metastases contain basal cells [40]. Recently, the expression of prostate epithelial stem cell markers in patients with primary and metastatic prostate cancer has been examined, with the result showing that approximately 0.1% of tumor cells express this phenotype (n > 40) [3]. There are two main types of prostate CSC markers that had been identified in recent years. These include prostate CSC surface markers: CD338, CD57, CD44, CD26 (Dipeptidyl peptidase I), CD133, CD13, CD104 (integrin b 4 ,) CD10, CD138 (syndecan), CD38, α2β1, Her-2/New and Sca-1. The other types of markers are responsible for supporting the renewal of prostate CSC. These included BrdU, Ki67, Bmi-1, beta-catenin and BCL-2 [41, 42].

2.1.4 Regulation of prostate CSCs self-renewal

There are several critical signaling pathways that play an important role in the regulation of prostate CSC self-renewal including hedgehog (Hh), Wnt, Notch and phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR [43, 44]. The Hh pathway regulates adult stem cell quiescence and self-renewal. It induces the expression of a polycomb gene, Bmi-1, which is expressed at high levels in the prostate cancer cell lines. The Hh pathway has been shown to suppress the expression of p16INK4A and p14ARF

while enhancing the expression of human telomerase reverse transcriptase (hTERT) [45]. The Wnt pathway promotes self-renewal, proliferation and transient differentiation of normal stem cells and is involved in vertebrate limb development and regeneration [10, 46-48]. Based on a previous study, Wnt signaling has been shown to be active in prostate CSCs, and Bisson and Prowse [49] showed that modulation of the activity of beta-catenin, a downstream effector of Wnt, alters CSC properties as reflected by the changes in the sphere-forming capacity of prostate cancer cells. Notch signaling promotes the survival and proliferation of normal neural stem cells and inhibits differentiation. Furthermore, previous studies have also shown that the Notch pathway promotes prostate cancer development [50, 51]. Phosphatase and tensin homologue deleted on chromosome 10 (*PTEN*) is a tumor suppressor gene that helps to maintain stem cells in a quiescent state through suppression of the PI3K/Akt/mTOR pathway. *PTEN* also served as a negative regulator of both mTOR and STAT3. According to Wang *et al.* [52], deletion or mutation of *PTEN* has been reported to induce CSC development, which may be mediated by aberrant activation of the PI3K/Akt/mTOR pathway.

Recently, the effect of single or dual inhibitors of the PI3K/Akt/mTOR pathway against prostate cancer has been studied intensively. The following review article (published in Current Medicine Chemistry) summarized the key evidence from both pre-clinical and clinical studies which support the therapeutic value of targeting the PI3K/Akt/mTOR pathway.

Statement of Contribution of Co-Authors for Thesis by Published Paper

The following is the format for the required declaration provided at the start of any thesis chapter which includes a co-authored publication.

The authors listed below have certified* that:

1. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
2. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. there are no other authors of the publication according to these criteria;
4. potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit, and

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Targeting drug-resistant prostate cancer with dual PI3K/mTOR inhibition.
Accepted in Current Medicinal Chemistry.

Contributor	Statement of contribution*
Kai Dun Tang	Wrote the manuscript and prepared the figures and table.
	
30.09.2015	
Ming-tat Ling*	Edited the manuscript, figures and table.

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

Ming Tat Ling		30-09-2015
Name	Signature	Date

2.1.5 Abstract

The phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR pathway is one of the most frequently activated signaling pathways in prostate cancer cells, and loss of the tumor suppressor PTEN and amplification of PIK3CA are the two most commonly detected mechanisms for the activation of these pathways. Aberrant activation of PI3K/Akt/mTOR has been implicated not only in the survival and metastasis of prostate cancer cells but also in the development of drug resistance. As such, selective inactivation of this pathway may provide opportunities to attack prostate cancer from all fronts. However, while preclinical studies examining specific inhibitors of PI3K or mTOR have yielded promising results, the evidence from clinical trials is less convincing. Emerging evidence from the analyses of some solid tumors suggests that a class of dual PI3K/mTOR inhibitors, which bind to and inactivate both PI3K and mTOR, may achieve better anti-cancer outcomes. In this review, we will summarize the mechanisms of action of these inhibitors, their effectiveness when used alone or in combination with other chemotherapeutic compounds, and their potential to serve as the next generation therapies for prostate cancer patients, particularly those who are resistant to the frontline chemotherapeutic drugs.

2.1.6 Introduction

Prostate cancer is the most common type of solid tumor in men around the world and is a leading cause of morbidity and mortality [31]. When diagnosed at an advanced stage at which surgery is no longer feasible, the only frontline treatment available for prostate cancer is hormone ablation therapy. Unfortunately, the majority of patients will eventually relapse and develop castration-resistant prostate cancer (CRPC), a fatal and terminal stage that is regarded as incurable. Prostate cancer frequently develops resistance to conventional chemotherapy, with the most effective treatment (Docetaxel, a microtubule-disrupting agent) extending patient survival for an average of only two months and is associated with significant side effects [53]. Thus, there is an urgent need for a better therapy for CRPC that shows improved treatment efficacy and minimal side effects.

Phosphatidylinositol 3-kinase (PI3K) belongs to a family of lipid kinases responsible for phsophorylating phosphatidylinositol within the cell membrane (Figure 2.4). It regulates a wide range of cellular functions such as metabolism, proliferation, survival and motility [53, 54] . The PI3K are grouped into three different classes (Class I-III) based on their functional and structural features. Aberrant activation of PI3K, particularly members of class IA, is commonly detected in various types of cancer. In prostate cancer, deregulation of PI3K and its downstream targets has been linked to tumor growth, angiogenesis and metastasis as well as the development of drug resistance, making PI3K a potential therapeutic target for improving the treatment of advanced prostate cancer patients. Over the past decade, a number of PI3K inhibitors have been under clinical development, with dual PI3K/mTOR inhibitors such as NVP-BEZ235 and PI103 currently in the spotlight due to their

successful application in many pre-clinical studies [55, 56]. In this review, we describe the rationale for targeting PI3K with dual PI3K/mTOR inhibitors in the treatment of prostate cancer and the safety issues that must be overcome before these compounds can eventually be brought to the clinic.

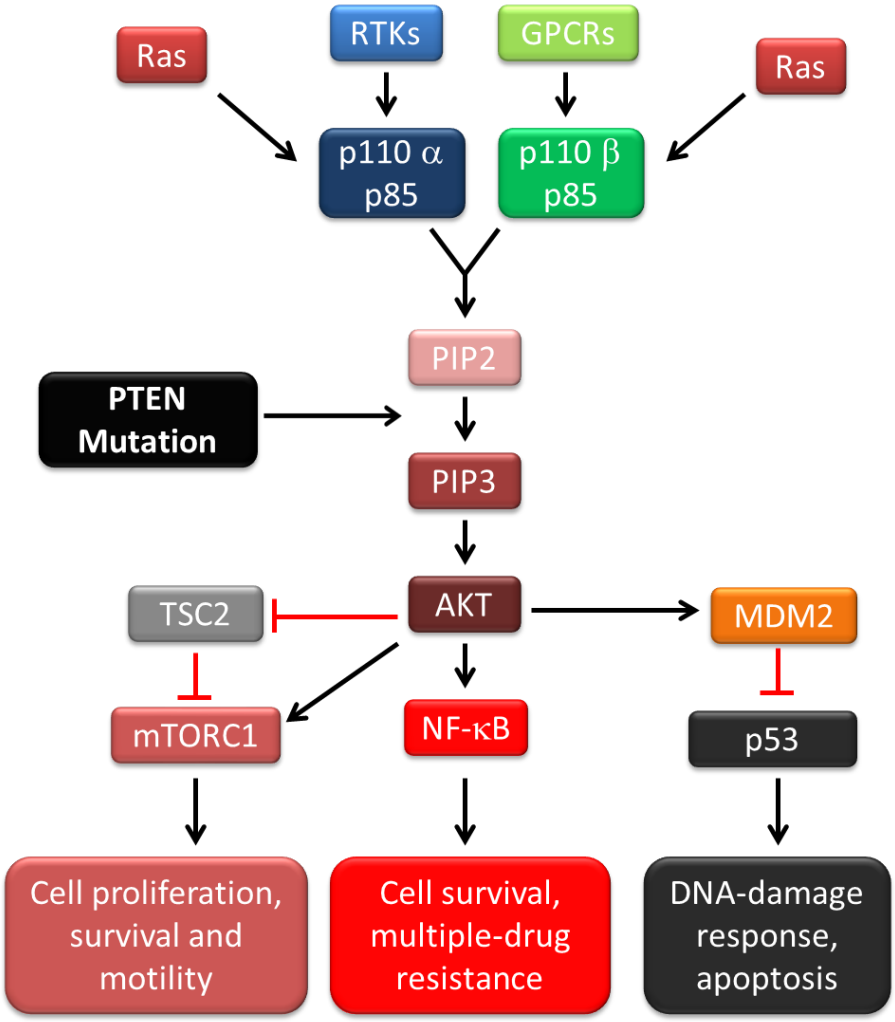

Figure 2.2: The PI3K/Akt/mTOR pathway. Phosphatidylinositol 3-kinase (PI3K) is activated when growth factors bind to receptor tyrosine kinases (RTKs, such as epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR), and platelet-derived growth factor receptor (PDGFR). G protein-coupled receptors (GPCRs) can also cause activation of PI3K. These events promote the conversion of phosphatidylinositol-4,5-bis phosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3) and the subsequent activation of Akt. Akt activation elicits a broad range of downstream signaling events to regulate cellular processes, which include the phosphorylation of nuclear factor-κB (NF-κB), murine double minute (MDM2), tuberous sclerosis complex 2 (TSC2) and

mammalian target of rapamycin (mTOR). Besides that, mutation of the tumor suppressor *PTEN*, which is commonly detected in prostate cancer, also contributes to aberrant Akt activation in cancer cells.

2.1.7 The PI3K/Akt/mTOR Signalling Cascade

PI3Ks. There are three classes of PI3K which can be grouped according to their functional and structural characteristics. Class I PI3Ks are the best-studied members of the PI3K family. These kinases are composed of a 110 kDa catalytic subunit (p110) and an 85 kDa regulatory subunit (p85). Depending on the composition of the heterodimer, class I PI3Ks can be further divided into subclass 1A (p110α, p110β and p110δ) or 1B (p110γ) [57, 58]. Class I PI3Ks are mainly activated through the p85 subunit, which binds to the phosphotyrosine residue of the ligand-activated receptor tyrosine kinases (RTKs) [59], although a previous study demonstrated that p85 also exerts inhibitory effects on p110 [60]. Among the RTKs, epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR) and platelet-derived growth factor receptor (PDGFR) have all been shown to activate PI3K [61-63]. Additionally, both Ras and G protein-coupled receptors have been found to bind to p110 and activate PI3K activity [61, 64]. Once activated, the p110 catalytic subunit will convert phosphatidylinositol‑4,5‑bis phosphate (PIP2) at the membrane into phosphatidylinositol‑3,4,5‑trisphosphate (PIP3) by phosphorylating the 3'OH position [61]. PIP3 provides docking sites for signaling proteins by recruiting two pleckstrin homology (PH) domains: a putative 3‑phosphoinositide‑dependent kinase 1 (PDPK1) and the serine–threonine protein kinase Akt (also known as protein kinase B) to the cell membrane, where Akt is phosphorylated and activated by PDPK1 [62, 63]. This process is inhibited by the tumor suppressor *PTEN*, which promotes the conversion of PIP3 into PIP2, thus inactivating the PI3K pathway [62].

The class IB PI3Ks consist of catalytic subunit p110γ and the regulatory subunit p10; which are activated directly by G-protein coupled receptors through interaction of its

regulatory subunit with the Gβγ subunit of trimeric G proteins [61, 65, 66]. Class II PI3Ks are monomeric catalytic subunits which preferentially use phosphatidylinositol‑4‑phosphate (PI4P) as substrates [67, 68]. There are currently three class II isoforms being identified: PI3KC2α, PI3KC2β and PI3KC2γ. PI3KC2α and PI3KC2β are almost ubiquitously expressed; while PI3KC2γ are restricted to liver, pancreas and prostate [69, 70]. Class III PI3K are heterodimeric enzymes composed of adaptor (p150) and a single catalytic subunit, VPS34 (homologue of the yeast vacuolar protein sorting-associated protein 34, also known as PI3KC3 [61].VPS34 produces phosphatidylinositol‑3‑phosphate (PI3P) which takes part in membrane trafficking [67]. Besides that, it has been implicated in regulating the cell growth and autophagy [61, 71]. Intriguingly, it also plays an important role in cellular stress response [71].

Akt. Akt is a serine/threonine kinase that plays a major role in cell survival. Activation of Akt elicits a broad range of downstream signaling events by phosphorylating a number of substrates, which include BclxL/Bcl-2-associated death promoter (BAD), forkhead box O1 (FOXO1) family transcription factors, nuclear factor‑κB (NF‑κB), murine double minute (MDM2), glycogen synthase kinase 3β (GSK3β), tuberous sclerosis complex 2 (TSC2), ribosomal protein S6 kinase (S6K) and mammalian target of rapamycin (mTOR). [61, 62]. Phosphorylation of the pro-apoptotic protein BAD and FOXO1 by Akt leads to inhibition of their function, thus protecting the cell from apoptosis [61, 72-74]. In addition, Akt activation antagonizes p53-mediated apoptosis by inducing the phosphorylation of MDM2, which ultimately leads to destabilization of the p53 protein [75]. Akt also negatively regulates GSK3β, thereby reducing glucose metabolism and cell cycle progression

[76]. Phosphorylation of TSC2 by Akt inhibits the rheb GTPase activity of TSC1/TSC2 dimers. As a result, rheb is activated and stimulates the downstream mTOR pathway.

mTOR. There are two distinct mTOR complexes: mTORC1 (mTOR Complex-1) and mTORC2 (mTOR Complex-2). mTORC1 consists of the mTOR catalytic subunit, Raptor (regulatory-associated protein of mTOR), PRAS40 (proline-rich Akt substrate 40 kDa) and the mLST8/GbL protein [57, 77]. The best characterized effectors downstream of mTORC1 involve phosphorylation of the ribosomal protein p70S6K (or S6K1) and 4EBP1 (also known as Phosphorylated Heat-stable and Acid-Stable protein, PHAS1). Both of these effectors take part in regulating mRNA translation [61, 78-80]. S6K1 is a serine/threonine kinase that phosphorylates and activates the 40S ribosomal S6 protein and facilitates the recruitment of the 40S ribosomal subunit to actively translating polysomes [78, 79, 81]. Additionally, S6K1 kinase can act as a negative regulator that phosphorylates and inhibits the adaptor protein insulin receptor substrate 1, thereby inhibiting insulin or insulin like growth factor 1 and causing inactivation of the PI3K pathway [63]. On the other hand, 4EBP1 is a low molecular weight protein that plays a role in mRNA translation. Phosphorylation of 4EBP1 by mTORC1 reduces the affinity of 4EBP1 for eIF4E (eukaryotic Initiation Factor-4E). As a result, eIF4E associates with eIF4G (Eukaryotic Translation Initiation Factor-4-Gamma), the adenosine triphosphate-dependent RNA helicase eIF4A (Eukaryotic Translation Initiation Factor-4A), and eIF4B (Eukaryotic Translation Initiation Factor-4B) to form an active complex. This complex then binds to 5'-capped mRNAs and initiates mRNA translation [78-80].

mTORC2 consists of mTOR, Rictor (rapamycin-insensitive companion of mTOR), mSIN1 (mammalian stress-activated protein kinase-interacting protein 1) and mLST8/GbL [61, 82, 83]. Unlike mTORC1, mTORC2 can be phosphorylated or activated in the absence of Rheb. However, its activation requires PI3K and the TSC1/TSC2 complex [84]. When the mTORC2 complex binds to Rictor, it can facilitate the function of PDK1, which phosphorylates Akt on Ser 473 and Thr 308. It also plays a role in cytoskeleton organization by regulating actin polymerization and the activation of Ras homolog gene family, member A (RhoA) and Ras-related C3 botulinum toxin substrate 1 (Rac1) [84, 85].

2.1.8 Aberrant activation of PI3K/Akt/mTOR in prostate cancer

2.4.4.1 Genetic inactivation of PTEN

Impaired negative regulatory feedback of the PI3K pathway caused by genetic alterations is one of the major mechanisms underlying cancer development [63]. *PTEN*, a negative regulator of the PI3K/Akt/mTOR pathway, is subjected to several different types of genetic alterations that are commonly found in prostate cancer. For an instance, *PTEN* mutations, which include both germline and somatic mutations, occur at a frequency of up to 50% in prostate cancer [61, 86-90]. Deletion of the *PTEN* gene on chromosome 10q23 is commonly observed in prostate cancer [91, 92]. Inactivation of *PTEN* via homozygous and hemizygous deletion has been reported to correlate with the Gleason score, presence of lymph node metastasis, development of hormone-refractory disease and the presence of the *ERG* gene fusion in prostate cancer [73, 86]. On the other hand, somatic *PTEN* mutations have been detected in both localized and metastatic protate cancer tissues in at least one

metastatic site [93]. The missense mutation P95S in exon 5, D223N in exon 7 or deletion of an adenine leading to a premature stop codon in amino acid 164 (exon 5) of the *PTEN* gene have also been reported [94]. The combination of somatic mutations, LOH and decreased expression due to methylation of the *PTEN* gene displays a high frequency in prostate tumors [65].

2.4.4.2 PIK3CA mutation/amplification

Phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit alpha isoform (PI3KCA) is encoded by the PIK3CA oncogene and catalyzes the production of phosphatidylinositol-3, 4, 5-triphosphate (PIP3), resulting in the activation of downstream Akt signaling. Somatic mutation and amplification of the gene encoding the catalytic subunit of PI3KCA are the two most frequent genetic alterations observed in PI3KCA. A variety of human tumors, including breast, colon, endometrial and prostate cancer, have been reported to harbor these genetic alterations [74, 95]. Genetic analyses of prostate cancer have demonstrated that the PI3KCA mutation occurs in approximately 5% of patients; while the increase in copy number can be detected in up to 10% of patients [86, 88, 96].

The missense mutations *E545K* and *H1047R*, in exons 9 and 20, corresponding to the helical and kinase domains of p110α, respectively, are two "hotspot" mutations clustered in PI3KCA. These somatic mutations have been shown to mediate aberrant Akt activation by enhancing the levels of PIP3, thus leading to cellular transformation [61, 63]. On the other hand, amplification of PI3KCA has been reported during the progression from hormone-dependent to hormone-refractory prostate cancer and was found to correlate with Gleason score in human prostate tumors [15]. Another comprehensive study based on direct sequencing,

pyrosequencing, quantitative mRNA analysis and fluorescence *in situ* hybridization performed in over 100 prostate tumors also revealed an association of high-grade prostate tumors with PIK3CA mRNA overexpression, resulting in activation of the PI3K signaling pathway and increased pAkt protein expression [97].

2.1.9 Role of PI3K/Akt/mTOR in the development and progression of prostate cancer

2.4.5.1 Tumor initiation

The PI3K/Akt/mTOR signaling pathway, which is activated via different mechanisms in human cancers, plays critical roles in tumor initiation and disease progression. Alterations in key players in this pathway have been reported in malignant prostate cancer and are associated with increasing tumor stage, grade, and the risk of biochemical recurrence [98]. Specifically, loss of *PTEN* function and genetic alterations of PI3KCA are common mechanisms resulting in changes in the PI3K/Akt/mTOR signaling pathway in cancer cells. Loss of *PTEN* function is known to downregulate cell cycle inhibitors such as p27, p18ink4c and p14arf, whereas it activates key components of the PI3K/Akt/mTOR signaling pathway, such as Akt, Rheb and TSC2. Overall, *PTEN* inactivation promotes cancer cell invasiveness, proliferation, angiogenesis and drug resistance during prostate cancer development [99]. Activation of the mTOR pathway due to the loss of *PTEN* also results in overexpression of the eIF4E and S6K1 proteins, which have both been linked to prostate cancer progression [100]. Loss of *PTEN* function also leads to increased eIF4E phosphorylation via activation of the PI3K/Akt/mTOR signaling pathway [101]. Knockdown of PTEN in a transgenic mouse model was found to induce tumor

invasion through eIF4E phosphorylation [101]. Another study verified the important role of eIE4F in a prostate cancer model via the expression of a non-phosphorylatable form of eIF4E in knock-in mice, which became resistant to tumorigenesis induced by PTEN knockout [101]. The non-phosphorylatable form of eIF4E was also shown to be associated with decreases in the levels of the MMP3, CCL2, VEGFC, BIRC2 and NFKBIA (IκBα) proteins, which have been implicated in prostate cancer [101]. Additionally, PI3K3CA mutation or amplification has been reported to promote prostate tumorigenesis both *in vitro* and *in vivo*. PI3KCB, which is the second class of PI3K isoform, has also been found to play roles in the development of prostate cancer [102].

2.4.5.2 Tumor metastasis

Tumor metastasis occurs when a tumor cell invades into the surrounding tissues through the production of a series of proteases, leading to the degradation of the extracellular matrix (ECM) and the dissemination of tumor cells into the circulation. This phenomenon is believed to involve a process known as epithelial-to-mesenchymal transition (EMT), where tumor cells acquire a mesenchymal phenotype to become more mobile and invasive [103]. The PI3K/Akt/mTOR signaling pathway has been shown to activate a variety of proteases that may take part in cell invasion and migration. For example, urokinase plasminogen activator-1 is an important protease involved in degrading plasminogen in the ECM and is activated by PI3K [104]. Other proteases downstream of PI3K include matrix-metalloproteinase 9 (MMP-9), which degrades collagen IV, and matrix-metalloproteinase 1 (MMP-1), which drives cell division, motility, and invasion, as well as matrix-metalloproteinase 3 (MMP-3), which triggers EMT [105, 106].

Further evidence supporting the role of PI3K signaling in prostate tumor metastasis comes from a study by Mulholland *et al.,* which demonstrated the cooperation of RAS/MAPK activation with the *PTEN* inactivation during prostate cancer progression. Crossing mice carrying a K-ras mutation with *PTEN*-null mice resulted in frequent macrometastasis with 100% penetrance, which was associated with the induction of EMT [107].

2.4.5.3 Cancer stem cell (CSC) survival

Recent evidence supports the existence of prostate CSCs, which are likely to arise from normal stem cells located in the basal, intermediate or luminal compartment of prostate gland [108]. *PTEN* has long been suggested to regulate hematopoietic stem cell renewal. Recent studies have demonstrated that it may play a similar role in the maintenance of CSCs. Deregulation of the PI3K/Akt/mTOR signaling pathway caused by *PTEN* inactivation was found to promote CSC properties in a variety of solid tumors, including glioblastomas, hepatocellular carcinomas, breast carcinomas, lung adenocarcinomas, and prostate carcinomas [84]. Deletion of *PTEN* in luminal cells expressing Nkx3.1 (a stem cell marker regulating prostate epithelial differentiation) results in rapid tumor development [109]. Moreover, the loss of *PTEN* negatively regulates $p63^{+}$ prostatic basal cell proliferation, without blocking differentiation, causing the expansion of a prostate stem/progenitor-like subpopulation [110-112]. Dubrovska *et al.* also reported that *PTEN* knockdown in DU145 cells increases their sphere-forming ability, supporting the inhibitory function of *PTEN* in prostate CSC self-renewal [113].

2.4.5.4 Development of therapeutic resistance

The PI3K/Akt/mTOR signaling pathway also plays a significant role in promoting drug resistance [84]. West *et al.* demonstrated that loss of *PTEN* was strongly associated with a poor prognosis and chemoresistance in a number of types of cancer [99, 114]. Meanwhile, the restoration of *PTEN* expression or suppression of Akt phosphorylation restored the doxorubicin sensitivity of doxorubicin-resistant PC3 cells [115]. Interestingly, the effect of the PI3K/Akt/mTOR pathway on drug resistance appears to be related to its functional role in CSC maintenance. A study by Dubrovska *et al.* revealed that the PI3K pathway plays an extensive role in maintaining $CD133^{+}/CD44^{+}$ prostate cancer progenitor cells [116]. Meanwhile, combining a PI3K-mTOR inhibitor with the chemotherapeutic drug Taxotere was found to reduce the CSC population in human prostate cancer models. Surprisingly, cancer progenitor cells carrying a *PTEN* missense mutation (E91D) repopulated after the combined treatment, further highlights the importance of the PI3K/Akt/mTOR pathway in maintaining the survival of CSCs under chemotherapy [116].

2.4.5.5 Development of castration-resistant prostate cancer

The PI3K/Akt/mTOR signaling pathway has been found to be critical for maintaining the growth and proliferation of prostate cancer cells under low-androgen conditions [117]. Moreover, preclinical studies have suggested that loss of *PTEN* function, which activates the PI3K/Akt/mTOR signaling pathway, may result in androgen-independent prostate cancer [118]. For example, prostate tumors formed in prostate-specific *PTEN* knockout mice initially respond to androgen ablation, as demonstrated by tumor regression. However, the tumor cells were found to continue to propagate under androgen-deprived conditions [98, 119]. Additionally, the

progression of an androgen-dependent prostate cancer cell line to androgen independency after long-term culturing under androgen-deprived conditions was found to lead to resistance to PI3K/Akt inhibition [120]. These findings suggest that aberrant activation of the PI3K/Akt/mTOR signaling pathway may play a crucial role in maintaining the survival of prostate cancer cells during the development of CRPC.

2.1.10 DUAL INHIBITORS OF PI3K/AKT/MTOR FOR THE TREATMENT OF PROSTATE CANCER

2.4.6.1 Mechanisms of action

Over the past decade, numerous synthetic inhibitors have been developed to target different layers of the PI3K/Akt/mTOR pathway (Figure 2.5) [63]. These agents exhibit different degrees of suppression of cell proliferation, survival, motility and angiogenesis as well as metastasis [121]. Based on their general action, these inhibitors can be categorized into four different types: dual PI3K–mTOR inhibitors, PI3K inhibitors (not targeting mTOR), Akt inhibitors and mTOR inhibitors. In this review, we will focus on dual PI3K-mTOR inhibitors, as they have recently been recognized as an essential strategy for maximizing the inhibition of the PI3K/Akt/mTOR pathway [121, 122]. These inhibitors take advantage of the structural similarity between the PI3K subunit p110α and mTOR (both belong to the phosphatidylinositol kinase–related kinase family), which allows them to inhibit PI3K mTOR activities simultaneously [63, 123]. The efficacy of some of these inhibitors in the treatment of prostate cancer is currently being evaluated under pre-clinical and clinical settings (As summarized in Table 2.1), which merits detailed discussion in the following sections.

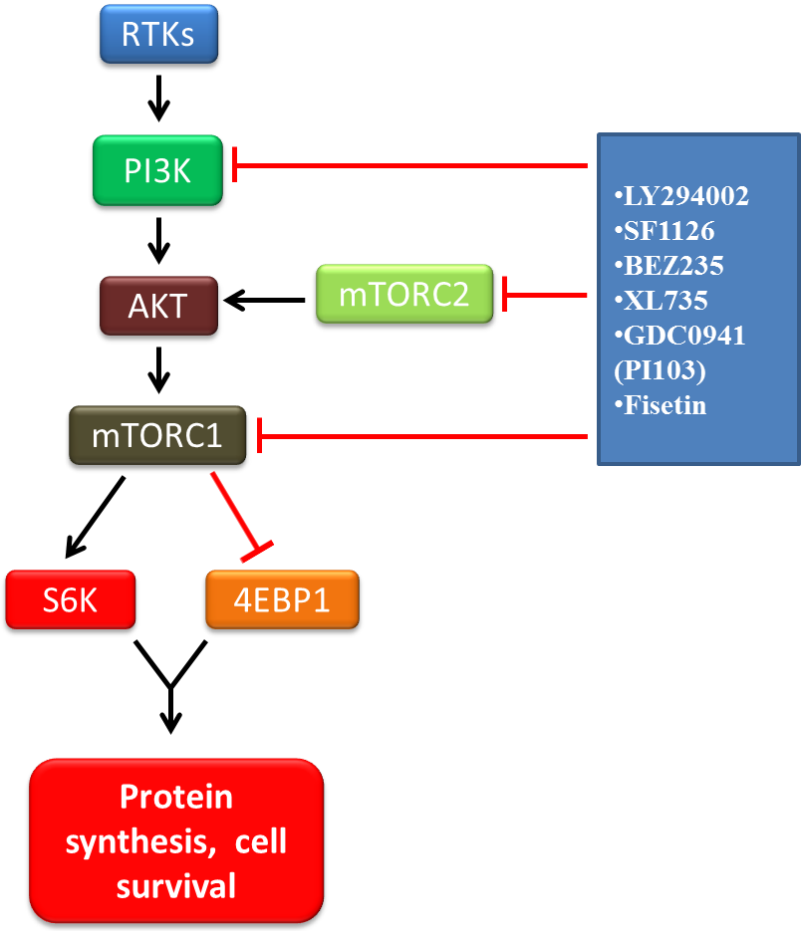

Figure 2.3: Targeting of dual PI3K-mTOR inhibitors in the PI3K/Akt/mTOR pathway. Dual PI3K-mTOR inhibitors target both PI3K and mTOR (mTORC1 and mTORC2) and, thus, have the potential of achieving better clinical effect against drug resistant prostate cancer.

Table 2.1: Summary of the use of dual PI3K-mTOR inhibitors in prostate cancer treatment.

Dual PI3K-mTOR inhibitors	Company	Phase	Cancer type or condition	Side effects
LY294002	N/A	Animal Studies	advanced solid tumours	small bowel obstruction, dry scaly skin and respiratory depression in mice
SF1126	Semafore	Phase I	advanced or metastatic solid tumours	diarrhoea
BEZ 235	Novartis	Phase I-II	advanced solid tumouts; castration-resistant prostate cancer	mucositis, AST/ALT elevation, anorexia and diarrhea
XL 735	Exelixis	Phase I	advanced solid tumours	elevated liver enzymes, nausea and diarrhoea.
GDC0941	Piramed or Genentech	Phase I	advanced solid tumours or multiple myeloma	nausea, diarrhoea, fatigue, vomiting, dysgeusia and decreased appetite.
Fisetin	N/A	Animal Studies	prostate cancer	N/A

LY294002 was one of the first few dual inhibitors to be described and is currently the most studied [124-126]. LY294002 was shown to suppress the invasion of prostate cancer cells through downregulation of HIF1-α and VEGF [127]. In addition, treatment with LY294002 was also found to sensitize prostate cancer cells to radiation-induced apoptosis [128]. Renner *et al.* were the first research group to utilize a transgenic model to study the effect of LY294002 in prostate cancer. They expressed a constitutively active form of the p110-alpha subunit in the epithelial cells of the prostate. The phosphorylation of Akt and eIF4G as well as the elevation of the Mst1 and RanBP2 protein levels were all found to be inhibited by LY294002 treatment *in vivo* [100]. LY294002 has been extensively tested in different preclinical studies. However, despite its effectiveness in animal models, this compound has failed to function efficiently in patients, possibly due to non-specific effects against non-PI3K kinases [63].

SF1126 is a prodrug developed commercially through modifying LY294002 via conjugation with an RGD (arg-gly-asp) tetra-peptide. This modification not only enhanced the solubility of the compound, but also improved its effect in targeting tumor vasculature [129]. In preclinical studies, administration of SF1126 was shown to inhibit the growth and angiogenesis of prostate tumors *in vivo*, but with lower toxicity compared to LY294002 [61, 129]. Reverse-phase protein array analysis revealed downregulation of Akt, S61 kinase and p27 levels following SF1126 treatment, indicating that the compound represses the PI3K/Akt/mTOR pathway and leads to tumor suppression [130]. In a phase I study addressing solid and B cell malignancies, SF1126 was found to inhibit Akt phosphorylation and induce apoptosis of the cancer cells *in vivo* [131]. The compound was also well tolerated by

the patients, which indicates that it warrants further investigation in additional clinical trials.

NVP-BEZ235 is an imidazaoquinazoline derivative that binds to ATP-binding pockets, thus inhibiting PDK1, PI3K isoforms and mTOR kinase. NVP-BEZ235 was found to reduce Akt phosphorylation and increase FOXO3a nuclear localization and transcriptional activity. The efficacy of NVP-BEZ235 against the CSC population was compared with that of different conventional drugs, including Taxotere, fluorouracil and Oxaliplatin, and was found to be the most effective agent in targeting CSCs [116]. Preclinical data also demonstrated that NVP-BEZ235 possesses strong anti-proliferative activity against tumor xenografts displaying a loss of *PTEN* or harboring a gain-of function-PI3K mutation [61]. Additionally, a recent study showed that the combination of NVP-BEZ235 with the chemotherapy drug Taxotere suppressed the tumor growth *in vivo* through targeting of prostate cancer progenitor cells [116]. Currently, this compound is being tested in a Phase Ib clinical trial together with Abiraterone (an FDA-approved compound that inhibits androgen production) in patients with CRPC.

XL765 is a potent inhibitor of Class I PI3K isoforms and mTOR that is available for oral administration for the treatment of solid tumors. A preclinical study demonstrated its ability to inhibit all class I isoforms of PI3K and mTOR, leading to inhibition of the growth of tumors generated from breast, lung, ovarian and prostate cancer [129]. However, a phase I study showed that XL765 was ineffective in patients with metastatic or unresectable solid tumors.

GDC0941 acts through inhibition of the class I PI3Ks and mTOR, and its anti-cancer effect has been demonstrated in pre-clinical xenograft tumor models. GDC0941 is currently undergoing a Phase I trial in patients with locally advanced or metastatic solid tumors. *In vitro* and *in vivo* investigations have suggested that GDC0941 is effective against prostate cancer when it is administered in combination with the allosteric mTOR inhibitors rapamycin and RAD001 [122].

Fisetin (3,7,30,40-tetrahydroxyflavone) belongs to the flavonol subgroup of flavonoids along with quercetin, myricetin and kaempferol. It can be found in a variety of fruits and vegetables, including strawberry, apple, persimmon, kiwi, cucumber and onion. Fisetin has been studied for many years and is known to exhibit diverse biological properties, ranging from antibacterial to cancer therapeutic effect. This compound was recently redefined as a dual PI3K/mTOR inhibitor that targets not only the mTOR signaling pathway, but also Akt phosphorylation. Haddad *et al.* compared the effect of two flavonoids, 2,20- dihydroxychalcone (DHC) and fisetin, in prostate cancer cells. The results demonstrated that these two compounds were able to induce apoptosis and decrease the clonogenic survival of prostate cancer cells [132]. Another study revealed a similar result, showing that fisetin was able to decrease the viability of hormone-dependent prostate cancer cells, but not that of normal prostate epithelial cells [133]. Treatment of PC-3 cells with fisetin had inhibitory effects on mTOR targets including S6, eIF4B and 4EBP1, which led to autophagic-programmed cell death [134, 135].

2.4.6.2 Pharmacokinetic/pharmodynamic studies

Among the known dual PI3K/mTOR inhibitors, LY294002 appears to be the most toxic, as shown in several *in vivo* studies. Two out of 12 animals died after receiving 2 mg of LY294002 due to either obstruction of the small bowel or unknown causes [136]. Moreover, approximately 80% of the animals were found to develop dry scaly skin that was a reversible following cessation of LY294002 treatment. Although, some studies did not note any toxicity associated with LY294002 or mortality of mice when treated with LY294002, side effects such as respiratory depression after injecting 2 mg of LY294002 into mice have been reported [137].

PI3K/mTOR inhibitors with reduced side effects in patients are urgently needed to cure patients suffering from prostate and other solid tumors. SF1126 is a modified form of LY294002 that has been developed that shows reduced side effects in patients. The SF1126 prodrug has been tested in a phase I clinical trial. SF1126 was administered twice weekly to patients who presenting with stable disease for more than 8 weeks. Pharmacodynamic evaluation revealed that the tolerable dosage was up to 630 mg/m^2. Apart from transient grade 3 diarrhea, no consistent effects on blood glucose levels were detected, but disease stabilization was observed in some patients [58].

2.1.11 Conclusions

The PI3K/Akt/mTOR signaling pathway plays a critical role in the development and progression of human prostate cancer. Extensive *in vitro* and *in vivo* studies have revealed that PI3K/Akt/mTOR is aberrantly activated either through the loss of *PTEN* function or genetic alteration of PI3KCA. These studies have provided a

strong basis for the development of targeted therapies against this signaling pathway. Currently, a number of dual PI3K/Akt/mTOR inhibitors are being study extensively in phase I/II clinical trials, with a few demonstrating promising anti-cancer effects while showing reduced side effects compared to the earlier generation of PI3K inhibitors. Strikingly, combinations of dual PI3K/mTOR inhibitors with current chemotherapeutic agents tend to display improved efficacy compared to the use of a single agent alone. Further pharmacokinetic/pharmodynamic studies may provide additional support for the development of dual PI3K/mTOR inhibitors for use as new treatments for management of prostate and other solid tumors.

2.1.12 Role of CSCs in the development of bone metastasis

EMT is believed to be the key event during cancer metastasis. EMT is a process in which epithelial cells with regular cell-cell junctions and adhesions convert into motile and invasive cells with mesenchymal phenotypes [10, 138]. Recent studies have reported a close relationship between CSCs and EMT [11, 139]. For example, Sampieru and Fodde [11] have shown that differentiated cancer cells acquired the CSC phenotype upon induction of an EMT. Other studies have demonstrated that growth factors released from the bone microenvironment may induce an EMT and cooperate via the Wnt-signaling pathway to promote the invasion of CSCs, which may lead to the development of bone metastasis [40, 103]. Interestingly, CXCR4, a protein implicated in cellular migration and the metastatic potential of cancer cells, was recently shown to induce the migration of CSCs [140, 141]. CXCL12 is the ligand for CXCR4 and is highly expressed at sites of prostate cancer metastases including the lungs, bone and liver. CXCR4 is highly expressed in primary prostate

tumors and prostate metastases but not in normal prostate tissues [142, 143], which further supports its role in prostate cancer metastasis.

2.2 Bone marrow stem cell niche

2.2.1 CSC specific niche

Most primary tumor cells enter the circulation and seed at secondary tissues during the formation of metastasis. In the secondary site, the primary tumor cells have to adapt to a new microenvironment, which normally lacks nutrients and oxygen, before they can redevelop as macrometastasis [5]. It is now believed that the CSCs may reside in a microenvironment known as the CSC niche which regulates their differentiation and proliferation. This niche has a complex cellular and molecular components which include stromal, endothelial, mesenchymal and immune cells, as well as the extracellular matrix (ECM) and soluble factors derived from the cellular component [144]. Interestingly, according to previous studies, CSCs are capable of creating their own cancer stem cell niche by recruiting mesenchymal stem cells derived from bone marrow, resulting in expansion of the CSC population within the primary tumor [16]. On the other hand, CSCs can also ‘hijack’ the already established normal stem cell niches [5]. This has been demonstrated in both human and mouse models for prostate and breast cancer where the cancer cells were shown to form micrometastases within hematopoietic stem cell (HSC) niches in the bone marrow by competing with the HSCs [17]. According to Shiozawa et al. [17], the CXCL12/CXCR4 pathway, which mediates the interaction between HSCs and the niche, was adopted by prostate cancer cells to gain access to the HSC niche and cause bone metastasis. By blocking this pathway, prostate cancer bone metastasis

was significantly suppressed. Interestingly, while disseminated prostate cancer cells can be detected in the bone marrow of prostate cancer patients, bone metastasis may take years to develop. This may reflect the time required for the disseminated cancer cells to articulate a suitable tumor microenvironment before the cells can grow exponentially.

2.2.2 Regulation of HSC by the stem cell niche

Bone plays an extensive role in maintaining the structure and movement of our body [145]. As demonstrated in previous studies, MSCs that residing in the bone cavity are responsible for differentiating into different types of marrow stromal cell lineages, including obsteoblasts, adipocytes, fibroblasts, chondrocytes, myocytes and endothelial cells [12]. The most critical role for bone marrow is to provide a cellular and molecular microenvironment (i.e. the niche) to regulate HSC function. This includes maintaining the balance between dormancy and active self-renewal as well as HSC mobilization and early lineage decision [5]. Undifferentiated HSCs localize to the endosteal region and are closely associated with osteoblasts. This niche (known as an osteoblastic niche) mediates the balance between HSC quiescence, self-renewal and trans-marrow migration [13]. On the other hand, a vascular niche composed of the endothelial cells of the bone marrow sinusoids regulates proliferation, differentiation and trans-endothelial migration of HSCs [146].

2.2.1.1 The Endosteal/Osteoblastic Niche

Osteoblasts are the major cellular components of the endosteal niche, which reside on the bone surface at the endosteum [147]. Evidence from *in vivo* studies has shown that HSC and progenitor cells mobilize to the periphery when there is a depletion of

osteoblasts, supporting their role in the homing of HSC [14, 15]. Osteoblasts are believed to be derived from bone marrow mesenchymal progenitor cells and have been found to produce paracrine factors such as CXCL12, osteopontin and N-cadherin which are involved in HSC retention and maintenance in bone marrow. Moreover, they also can secrete a variety of molecules such as angiopoietin-1 (Ang-1), thrombopoietin (THPO) and membrane-bound stem cell factor (SCF) which are involved in maintaining the quiescence of HSCs. These paracrine factors act through numerous signalling pathways (e.g. Hh, Notch and Wnt signalling pathways) to regulate cell cycle progression and self-renewal of HSCs [147, 148].

2.2.1.2 The Vascular Niche

The vascular niche represents another HSC niche within the bone marrow, which is located in the extravascular spaces between the vascular sinuses [147]. Adhesion molecules such as E-selectin, P-selectin, vascular cell adhesion protein 1 (VCAM1), Intercellular adhesion molecule 1 (ICAM1), platelet endothelial cell adhesion molecule 1 (PECAM1) and vascular endothelial cadherin are all found in endothelial cells of the vascular niche [149]. Besides that, a population of Nestin-expressing cells expresses CXCL12, SCF, Ang-1, Interleukin (IL) 7, VCAM-1 and osteopontin. These cells can be found in the perivascular environment and are suggested to play an extensive role in perivascular distribution through their ability to regulate HSC mobilization by associating with the nerve fibers of the sympathetic nervous system [147]. In addition, the endothelial cells in the bone marrow also take part in megakaryocyte maturation and thrombopoiesis via the MPL/THPO signalling pathway [146].

2.2.3 Ang-1/Tie2 signalling pathway in the maintenance of HSC quiescence

Ang-1 is a pro-angiogenic protein that regulates endothelial cell proliferation and blood vessel formation. It has also been found to be one of the key osteoblast-secreted paracrine factors responsible for the maintenance of HSCs [4]. It binds to Tie-2 receptors expressed in HSCs and activates downstream targets that include AKT and Bmi-1. Tie-2 belongs to the tyrosine kinase receptor family, which also binds to other ligands such as Ang-2, Ang-4 [49]. According to Arai *et al.* [30], Tie2 receptors are expressed on the quiescent HSCs which adhere to osteoblasts that line the inner surface of the bone. They further demonstrated that osteoblasts secrete large amounts of Ang-1 which promotes the adhesion of HSCs to osteoblasts while at the same time maintains HSC quiescence by preventing the cells from undergoing cell division and differentiation.

2.3 Adipocytes in the development and progression of prostate cancer

2.3.1 Adipocytes

Adipocytes also defined as lipocytes traditionally have been viewed as fat storage cells. When viewed microscopically, these cells appear full of triglycerides, the chemical form of fats that exist in food and the body. There are three types of adipocytes: white adipose tissues (WAT), brown adipose tissues (BAT) and yellow adipose tissue (YAT/marrow fat). The white lipocyte is the most abundant in the body which involved in fat storage; whereas, brown lipocytes produce heat. The marrow fat or the yellow adipose tissue (YAT) constitutes a third category of fat tissue and its metabolic activity is largely unknown [150]. On the other hand, they play an important role in regulating the endocrine and immune systems. Furthermore, they also help to maintain proper energy balance. Meanwhile, adipocytes are formed through the process of adipogenesis (Figure 2.4). There are several cell types in adipose tissues; however, only one third of the tissue is constituted by adipocytes and the rest is represented by fibroblasts, macrophages, stromal cells, monocytes and preadipocytes.

2.3.1.1 White Adipose Tissues (WAT)

White adipose tissue (WAT) is a major secretory and fat storage. It plays an important role in cell function through regulation of a complex network of endocrine, paracrine, and autocrine signals, which will affect the response of different tissue types, including the hypothalamus and metabolic organs like the pancreas, liver, skeletal muscle, kidneys, adrenal glands, or the cardiovascular system.

2.3.1.2 Brown Adipose Tissue (BAT)

Unlike WAT, brown adipose tissue (BAT) enables the body to release excess energy as heat. Brown adipose tissue (BAT) is also known as the baby fat, which mostly found in babies; however, it will be converted into white adipose tissues in adulthood by a specialized molecule called mitochondrial uncoupling protein 1 (UCP1), which is activated in the mitochondria by diet. Interestingly, the induction of UCP1 in white adipose tissues can lead to resistance in obesity development, although the exact mechanism is unknown.

2.3.1.3 Yellow adipose tissue (YAT)

Yellow adipose tissue is often referred as bone marrow fat. It has a yellowish appearance which is due to the presence of a moderate number of mitochondria and is suspected to have a combined phenotype of white and brown fat. The origin of marrow fat is the same as for WAT, which is from the marrow MSCs which can also differentiate into osteoblasts. Besides that, it plays an extensive role in regulating the lipid metabolism by clearing and storing circulating triglycerides, which can provide a localized energy reservoir for emergency situations like osteogenesis or bone fracture healing [150].

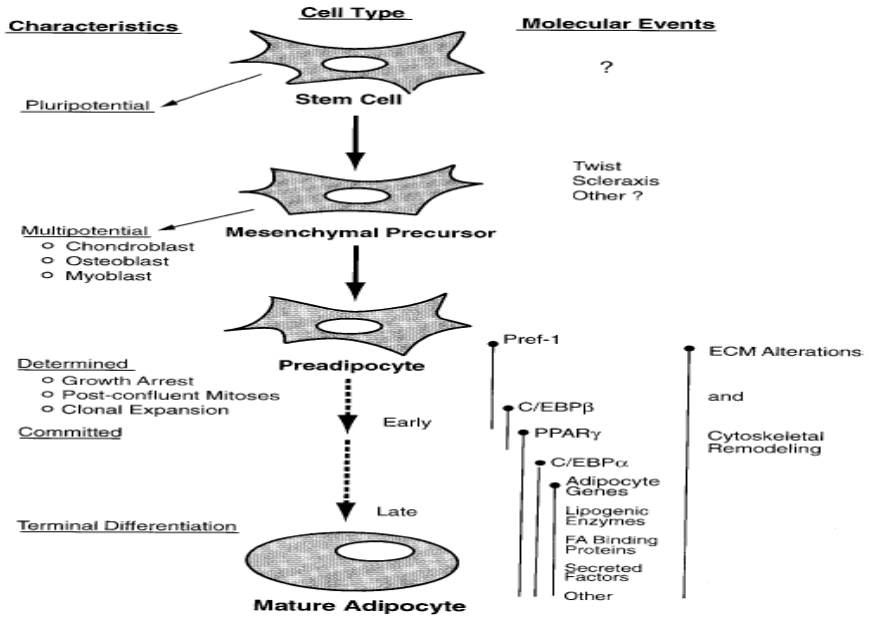

Figure 2.4: Overview of adipocyte differentiation. MSCs differentiated from pluripotent stem cell precursors can further differentiate into myoblasts, chondroblasts, osteoblasts, and preadipocytes. Given the appropriate environmental and gene expression cues, preadipocytes undergo clonal expansion and turn into a mature adipocytes (taken from[151]).

2.3.2 Functions of adipocytes

Adipose tissue produces different types of molecules such as cytokines, hormones, complement factors, prostacyclin, growth factors and enzymes. These molecules exhibit a numerous range of physiological functions including taking part in regulating the immune system (Tumor necrosis factor alpha (TNF-α), IL-1: IL-6, IL8, IL10), formation and maintenance of the vascular structure (vascular epithelial growth factor (VEGF), hepatocyte growth factor (HGF), angiotensin, plasminogen activator inhibitor-1 (PAI-1)), insulin sensitivity (resistin) and body weight (leptin and adiponectin) [152] .

2.3.3 Role of adipocytes in obesity

The development of obesity not only changes the morphology of adipocytes, but also alters their secretion profiles as shown in Figure 2.5. Adipocytes during the development of obesity are hypertrophic and most of the molecules that are produced by adipocytes are changed in terms of quantity or amount. Adipocytes secrete high amounts of fatty acids as well as adipokines, which promote insulin resistance and the ectopic accumulation of lipids in pancreas, liver and skeletal muscle. Moreover, obese adipose tissues may change the non-adipose tissues to adipose tissue-released by infiltrating to other organs. Lipid deposition in non-adipose tissue further contributes to metabolic stress, leading to loss of glucose homeostasis and resulting in tissue damage. For example, during the development of fatty liver, increased expression of proinflammatory cytokines and activation of Kupffer cells represents potential causes and sources of liver damage. During obesity, macrophages infiltrate into pancreatic islets and are likely to contribute to the local production of

proinflammatory cytokines. It has been reported that obesity is associated with endoplasmic reticulum stress in neurons leading to central leptin and insulin resistance, thereby further promoting obesity and loss of glucose homeostasis [46] .

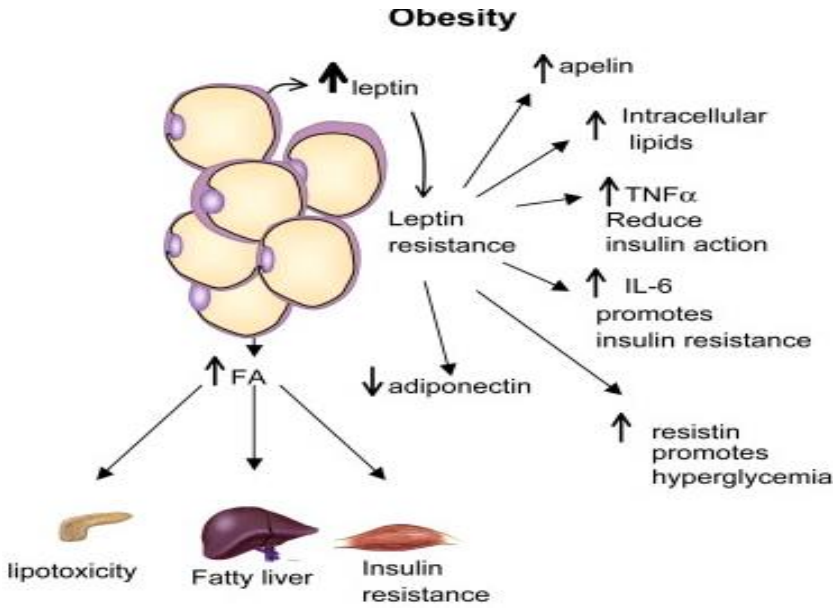

Figure 2.5: The roles of adipocytes during the development of obesity. Obesity induces the secretion of apelin, intracellular lipids, TNF-α, IL-6 and resistin in adipocytes, while suppressing the level of adiponectin. Besides that, obesity also contributes to hormonal changes such as an increase in the levels of oestrogen and a decrease in the level of testosterone (taken from [153]).

2.3.4 Influence of obesity and adipocytes on prostate cancer progression

There is evidence to suggest that obesity is associated with prostate cancer development [18-20] and disease progression [21-23]. In a number of large cohort studies [18, 19] a higher body mass index was consistently found to increase the risk of prostate cancer. Obesity has been found to correlate with prostate cancer relapse after prostatectomy [22] or radiation therapy [23]. Besides that, adipocytes have long been suggested as a key component of the tumor microenvironment. Adipocytes isolated from periprostatic adipose tissues were found to induce invasiveness of prostate cancer cells [28] and it has been shown that prostate tumors frequently metastasizes to bone marrow, where half of the microenvironment is composed of adipocytes. Interestingly, adipocyte lineage cells were recently found to contribute to a functional skin stem cell niche, which stimulates follicular stem cell expansion [154]. Adipocytes adjacent to cancer cell were found to secrete cytokines (known as adipokines) such as IL-6 or IL-8 [155, 156], which have been shown to promote CSC self-renewal [157, 158]. These findings support the idea that cancer cells may architect their own stem cell niches through manipulation of the adipocyte, and that targeting of this adipocyte CSC niche provides therapeutic opportunities for the treatment of prostate cancer.

2.3.5 Influence of leptin and adiponectin on prostate cancer progression.

2.3.5.1 Leptin

Leptin synthesis is mostly occurs in white adipose tissues, however, it still can be found in other tissues including the placenta, skeletic muscle, gastric epithelium, mammary gland. It not only plays an important role in regulating energy

homeostasis, but also has biological effects in several cellular processes such as reproduction, hematopoiesis, angiogenesis, and immunity. Leptin concentration is positively associated with the level of total body fat. Meanwhile, the induction of leptin concentration have been shown to correlate with testosterone and PSA levels in subjects with prostate cancer compared with subjects with benign prostate hyperplasia and the control group. Chang *et al,* [40] reported that the high leptin concentrations are positively associated with tumor volume. Furthermore, adipokine also increases the production of other cytokines and growth factors such as VEGF, which is involved in tumor progression [152].

2.3.5.2 Adiponectin

Adiponectin is also produced by WAT and is the most abundant circulating adipokine which accounts for 0.05% of the total plasma proteins. Unlike other adipokines, the circulating levels of adiponectin are inversely proportional to obesity. Patients with breast, endometrium, colon and prostate cancer were found to have lower concentrations of adiponectin when compared to normal individuals. Adiponectin receptors have been found in prostate cancer cell lines (LNCaP-FGC, DU145 and PC-3). As reported by Miyasaky *et al,* [159], JNK and signal transducer and activator of transcription 3 (STAT3) were both involved in adiponectin-induced intracellular signalling. Besides that, it also found to play major roles in obesity, insulin resistance and as well in the tumor development. As a result, low adiponectin concentration can lead to the induction of cancer cells proliferation [152].

2.3.6 Influence of aromatase and estrogens on prostate cancer progression.

Aromatase is a P450 mono-oxygenase enzyme produced by adipocytes and increases during the development of obesity. In this situation, estrogens will also increase, however, androgens will decrease. This is because, in males, estrogens are derived from circulating androgens (testosterone) which is catalyzed by the aromatase enzymes. Estrogen signalling via ERα has been shown to induce inflammation and promote prostate cancer development. Furthermore, aberrant aromatase expression in prostate cancer has been suggested to contribute to the induction of inflammation and probably the development of an altered local hormonal milieu within prostate tumors. Besides that, based on previous studies, elevated levels of estrogen also enhance the growth of cancer cells [29].

2.3.7 Influence of adipocyte-secreted growth factors on prostate cancer progression.

2.3.7.1 Transforming growth factor beta (TGFβ)

TGFβ is the growth factor that is synthesized in the WAT [160] and can be mediated by heterodimeric type I and type II serine/threonine kinase receptors (TGFβ-RI or TGFβ RII). Some clinical studies have shown that TGFβ overexpression and loss of expression of TGFβ-RI or TGFβ RII are associated with greater pathological progression to metastasis. It has been suggested that in the absence of inhibitory autocrine TGFβ signalling, the high levels of secreted TGFβ promote prostate tumor growth and metastasis by acting as a paracrine factor on the tumor microenvironment [161]. Meanwhile, TGFβ is also known to regulate adipocyte function as well as obesity. As expected, TGFβ mRNA level was drastically higher in adipose tissue of

both *ob/ob* and *db/db* transgenic mice when compared with their lean counterparts. TGFβ expression has also been found to be increase during maturation of adipocytes [162]. These data suggest that obesity is associated with a higher expression of TGFβ in adipose tissues. Although, there is no strong evidence to prove the role of TGFβ in prostate cancer bone metastasis; however, previous study suggested that it may enhance an osteolytic bone response in a xenograft prostate cancer model system [163].

2.3.7.2 TNF-α

TNF-α is another example of a growth factor that is synthesised in WAT. Similarly, it also increases during the obesity development and surprisingly, it was found to promote TGFβ synthesis [160]. As reported by previous studies, they suggested that the role of TNF-α in regulating the formation of bone metastasis. It was found to enhance DKK1 secretion (known to promote programmed cell death (PCD), which inhibits MSC derived osteoblastogenesis and lowers osteoprotegerin (OPG) levels and thus, results in reduced bone accretion. Furthermore, DKK1 secretion also enhances receptor activator of nuclear factor kappa-B ligand (RANKL) levels and thus increases the RANKL:OPG ratio and activates the osteoclast activity , leading to bone resorption [164].

Chapter 3: Research Paper 1: Tie-2 regulates the stemness and metastatic properties of prostate cancer cells

Within the bone marrow, there are two major cellular components: osteoblasts and adipocytes, which have been suggested to regulate the development and progression of prostate cancer, however, the underlying mechanisms remain largely unknown. As noted in the published review in Chapter 2, receptor tyrosine kinases are considered key therapeutic targets for prostate cancer. In this chapter, I have reported the discovery of a rare population of prostate cancer cells that express the Tie-2 protein, a receptor tyrosine kinase activated by the osteoblast-secreted cytokine Ang-1. Moreover, I have identified a novel Ang-1/Tie-2 autocrine loop in prostate cancer cells that promotes CSC quiescence and stemness. More importantly, I have demonstrated that Tie-2 positive prostate cancer cells are more metastatic compared to Tie-2 negative population. Based on these findings, I believe that my work has broadened the knowledge on how obsteoblasts contribute to a CSC niche in the bone marrow and regulate the stemness of prostate CSCs. Therefore, inactivation of the receptor tyrosine kinase, Tie-2 with currently available inhibitors may have the potential of developing into effective therapy for the advanced stage prostate cancer patients. This work has been accepted as a research article in the journal Oncotarget .

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2. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. there are no other authors of the publication according to these criteria;
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Publication title and date of publication or status:
Tie-2 regulates the stemness and metastatic properties of prostate cancer cells. Accepted in Oncotarget.

Contributor	Statement of contribution*
Kai Dun Tang	Conceived, designed and performed the experiments. Wrote the manuscript.
	
30.09.2015	
Boris M. Holzapfel *	Contributed to the mouse intra-cardiac injection and provided the PC-3 and human bone metastatic tumor sections.
Ji Liu*	Contributed to the generation of stable Tie-2 overexpressing human prostate cancer cell lines and technical support.
Terence Kin-Wah Lee*	Contributed to the data analysis and provided the feedback on the manuscript.
Stephanie Ma*	Contributed to the data analysis and provided the feedback on the manuscript.

Lidija Jovanovic*	Performed the cDNA microarray analysis.
Jiyuan An*	Contributed to the cDNA microarray data analysis.
Pamela J. Russell*	Provided feedback on the manuscript.
Judith A. Clements*	Provided feedback on the manuscript.
Dietmar W Hutmacher*	Provided the PC-3 and human bone metastatic tumor sections and feedback on the manuscript.
Ming-Tat Ling*	Conceived and designed the experiment. Edited the manuscript.

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

Ming Tat Ling		30-09-2015
Name	Signature	Date

3.1 Abstract

Ample evidence supports that prostate tumor metastasis originates from a rare population of cancer cells, known as cancer stem cells (CSCs). Unfortunately, little is known about the identity of these cells, making it difficult to target the metastatic prostate tumor. Here, for the first time, we report the identification of a rare population of prostate cancer cells that express the Tie-2 protein. We found that this Tie-2^{High} population exists mainly in prostate cancer cell lines that are capable of metastasizing to the bone. These cells not only express a higher level of CSC markers but also demonstrate enhanced resistance to the chemotherapeutic drug Cabazitaxel. In addition, knockdown of the expression of the Tie-2 ligand angiopoietin (Ang-1) led to suppression of CSC markers, suggesting that the Ang-1/Tie-2 signaling pathway functions as an autocrine loop for the maintenance of prostate CSCs. More importantly, we found that Tie-2^{High} prostate cancer cells are more adhesive than the Tie-2^{Low} population to both osteoblasts and endothelial cells. Moreover, only the Tie-2^{High}, but not the Tie-2^{Low} cells developed tumor metastasis *in vivo* when injected at a low number. Taken together, our data suggest that Tie-2 may play an important role during the development of prostate tumor metastasis.

3.2 Introduction

Prostate cancer is one of the most common solid tumors in men and is the second leading cause of morbidity and mortality in men worldwide [31, 165]. Patients with advanced prostate cancer are normally treated with hormone ablation therapy. This therapy is effective initially, as prostate cancer cells require androgen to grow and survive; however, the cancer cells eventually become androgen-independent and develop metastatic, castration-resistant tumors [2]. At this stage, chemotherapy and radiotherapy have only minor benefits. As a result, advanced prostate cancer remains incurable with current treatment strategies [4].

Recent studies suggested that prostate tumor-initiating cells (TICs)/cancer stem cells (CSCs) may play key roles in the initiation, progression and treatment failure of the disease [166-168]; however, their exact identity remains unclear. These cells share many similarities with normal stem cells, including the ability to differentiate into cells of different lineages and the dependence on a stem cell niche for the maintenance of their stemness. Interestingly, according to previous studies, CSCs are capable of creating their own CSC niche by recruiting mesenchymal stem cells derived from bone marrow [16]. CSCs can also 'hijack' the established normal stem cell niches [5]; as has been demonstrated in both human and mouse models of prostate and breast cancers. According to Shiozawa et al. [17], prostate CSCs are capable of competing with hematopoietic stem cells (HSCs) for the bone marrow niche. Moreover, prostate cancer cells that occupy the niche were found to express the CXCR4 receptor, which is responsible for mediating the interaction between HSCs and the niche. By blocking the CXCL12/CXCR4 pathway, prostate cancer bone metastasis was significantly suppressed, supporting the hypothesis that prostate

tumors metastasize to the bone by adopting this bone-homing signaling pathway. However, although disseminated prostate cancer cells can be detected in the bone marrow of prostate cancer patients, bone metastasis may take years to develop. This may reflect the time required for the disseminated cancer cells to establish a suitable tumor microenvironment before the cells can grow exponentially. Therefore, a better understanding of the bone marrow stem cell niche and its role in supporting bone metastasis may aid the development of effective treatments.

Tie-2 is a membrane receptor commonly expressed by HSCs, osteoblasts and endothelial cells. It binds to and is activated by angiopoietin-1 (Ang-1), a cytokine actively secreted by osteoblasts within the bone marrow niche [169-171]. Similar to the KITLG/c-Kit, CXCL12/CXCR4 and FGF1/FGFR chemokine axes, the Ang-1/Tie-2 signalling pathway also plays a key role in regulating the homing and stemness of HSCs [30, 172-175]. According to Arai et al. [30], Tie-2 receptors are expressed by the quiescent HSCs that adhere to osteoblasts within the inner surface of the bone. The same group further demonstrated that osteoblasts secrete a large amount of Ang-1, which promotes the adhesion of HSCs to osteoblasts while maintaining the cells in a quiescent state. Recently, the Ang-1/Tie-2 signalling pathway was also found to promote muscle satellite cell self-renewal [176]. Apart from regulating normal stem cells, Tie-2 was also found to play a role in cancer progression. As reported by Lee et al. [177], Tie-2 was found to be expressed by neoplastic glial cells, and its expression level was significantly associated with disease progression. Tie-2 activation was also found to correlate with chemoresistance [178]. Further research by the same group demonstrated that Tie-2

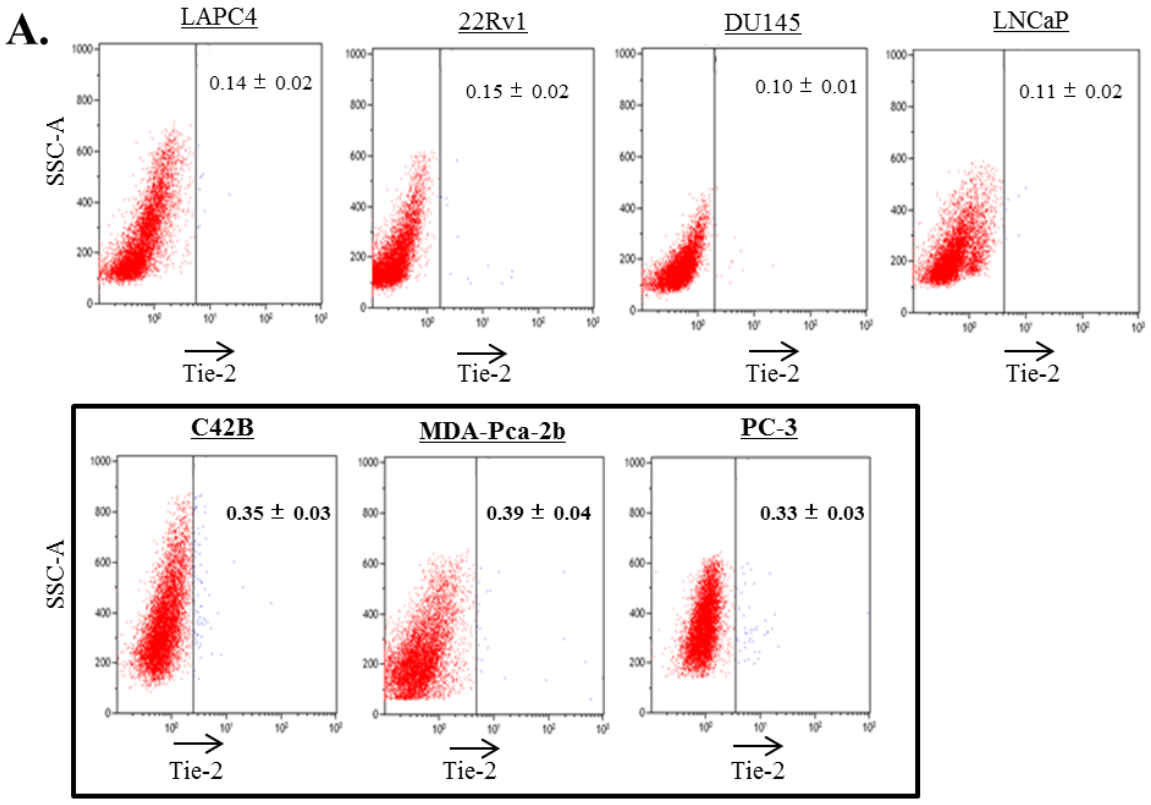
promoted glioma cell invasion and modulated the interaction of glioma tumor stem cells with endothelial cells [179].

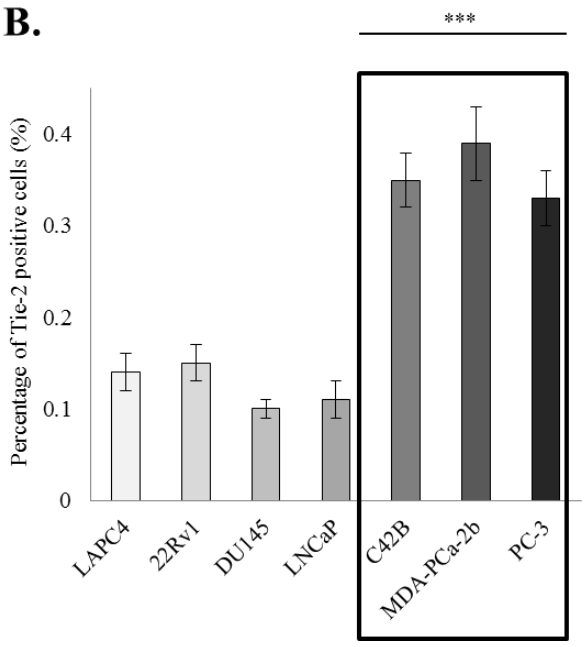
In this study, we show that Tie-2 is expressed by a rare population of prostate cancer cells that co-express several CSC markers. Compared to the Tie-2^{Low} population, Tie-2^{High} prostate cancer cells demonstrate not only enhanced chemoresistance but also an increased ability to adhere to stromal cells such as endothelial cells and osteoblasts. Intra-cardiac injection of the cells into immune-incompetent mice confirmed that Tie-2^{High} cells, but not Tie-2^{Low} cells, actively developed into metastatic tumors *in vivo*. Our data, therefore, have demonstrated a novel role for Tie-2 in the development of prostate tumor metastasis.

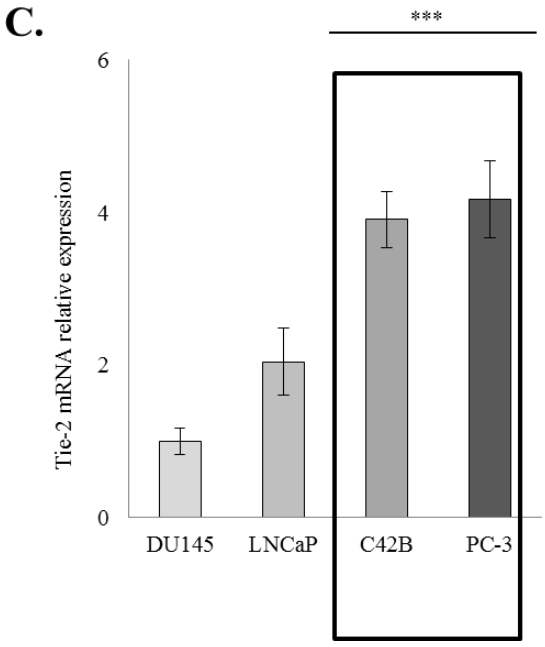
3.3 Results

3.3.1 Identification of a rare population of Tie-2^{High} prostate cancer cells

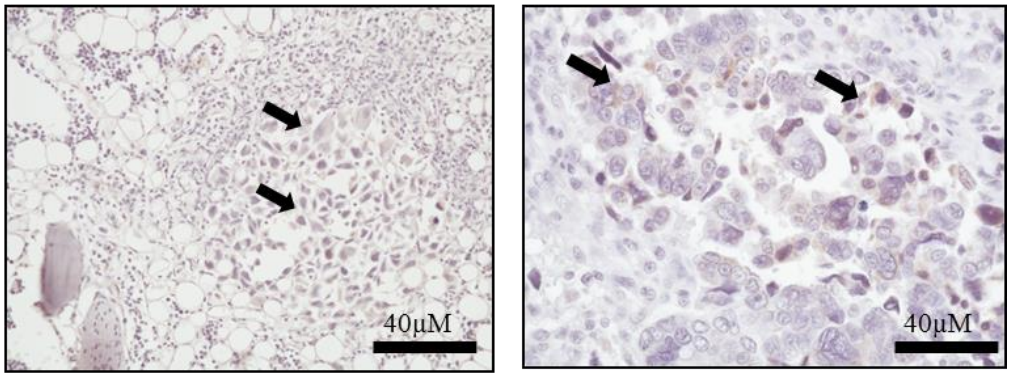
Although Ang-1/Tie-2 is one of the key signalling pathways involved in HSC maintenance, it is currently unknown whether it plays any role in prostate cancer progression. To address this question, we first examined whether prostate cancer cells express Tie-2. Using a PE-conjugated anti Tie-2 antibody, the expression level of the Tie-2 receptor in a panel of prostate cancer cell lines with different metastatic potential (LAPC4, 22Rv1, DU145, LNCaP, C42B, MDA-PCa-2b and PC-3) was determined by flow cytometry. As shown in Figure 3.1A and 3.1B, Tie-2 positive cells exist at a very low level in soft tissue metastatic prostate cancer cell lines (between 0.1 - 0.15%). However, the Tie-2 positive population was found higher in C42B (a bone metastatic cell line derived from LNCaP) (0.35%), and two bone metastatic prostate cancer cell lines (0.33% and 0.39% in PC-3 and MDA-PCa-2b respectively). Consistently, qRT-PCR analysis revealed that Tie-2 mRNA was expressed at a higher level in the bone metastatic prostate cancer cell lines (Figure 3.1C). More importantly, Tie-2 was also found to be expressed not only in PC-3 tumors metastasized into a humanized bone scaffold (left panel) established in our previous study [180], but also in bone metastatic tumor of human prostate cancer patient (right panel), further suggesting that Tie-2 may play roles in the development of prostate tumor bone metastasis (Figure 3.1D).


A.
LAPC4
22Rv1
DU145
LNCaP
0.14 ± 0.02
0.15 ± 0.02
0.10 ± 0.01
0.11 ± 0.02
SSC-A
Tie-2
C42B
MDA-Pca-2b
PC-3
0.35 ± 0.03
0.39 ± 0.04
0.33 ± 0.03


B.
***
Percentage of Tie-2 positive cells (%)
0
0.1
0.2
0.3
0.4
LAPC4
22Rv1
DU145
LNCaP
C42B
MDA-PCa-2b
PC-3


C.
***
Tie-2 mRNA relative expression
0
2
4
6
DU145
LNCaP
C42B
PC-3

D.



PC-3 tumor xenograft within the human bone cells scaffold

Human prostate cancer bone metastasis tumors section

Figure 3.1: Expression of Tie-2 in prostate cancer cells. (A&B) Flow cytometry analysis of Tie-2 expression in a panel of prostate cancer cell lines (LAPC4, 22Rv1, DU145, LNCaP, C42B, MDA-PCa-2b and PC-3). The results are presented as the mean ± SD from triplicate experiments. (C) Tie-2 mRNA was analyzed in these different prostate cancer cell lines using qRT-PCR. Note that Tie-2 protein and mRNA expression were both increased in bone metastatic prostate cancer cell lines (highlighted). Results quantified represented as fold change normalized to DU145. (D) Immunohistochemical staining was performed on humanized bone scaffold containing the PC-3 metastastic tumor (5 cases) (left panel) and human bone section containing the metastatic prostate tumor (1 case) (right panel). All the sections were stained with antibody against Tie-2. Note that, all the sections were positive for the Tie-2 proteins and the arrows showed the positive staining within the tumor cells (40X magnifications). (p values: *** < 0.005).

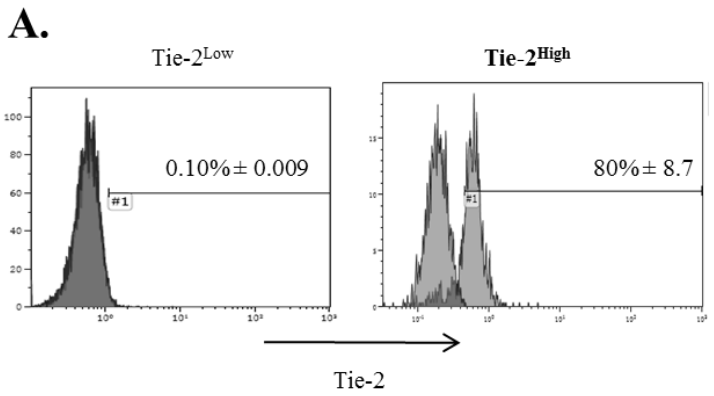
3.3.2 Co-expression of stem cell markers with Tie-2 in prostate cancer cells

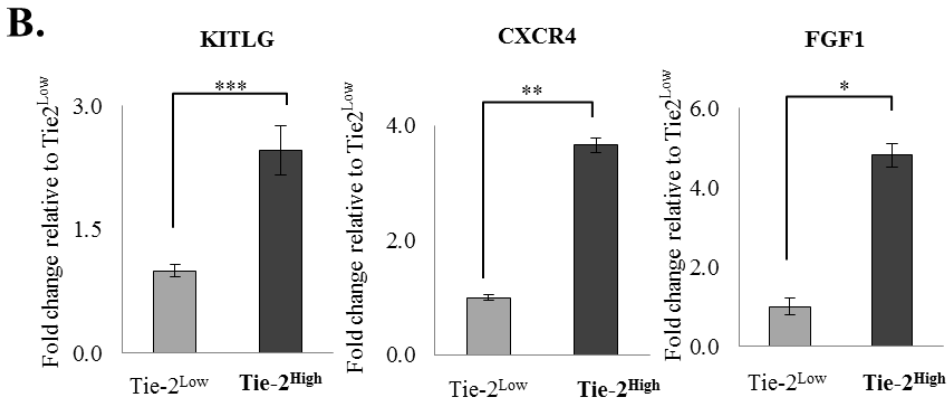
To further characterize the Tie-2 positive prostate cancer cells, we first performed FACS to enrich the Tie-2-positive population from PC-3 cells. Analysis of the population sorted by flow cytometry confirmed the successful enrichment of Tie-2^{High} cells (Figure 3.2A) by cell sorting. cDNA microarray analysis was then performed to examine the gene expression profile of Tie-2^{High} cells. As shown in in Suppl. Table A.2, several stem cell factors/markers commonly found in HSCs (e.g. FGF1, KITLG and CXCR4) were found to be upregulated in the Tie-2^{High} population, which was further confirmed by qRT-PCR analysis (Figure 3.2B), demonstrating that Tie-2 expression is associated with upregulation of HSC markers.

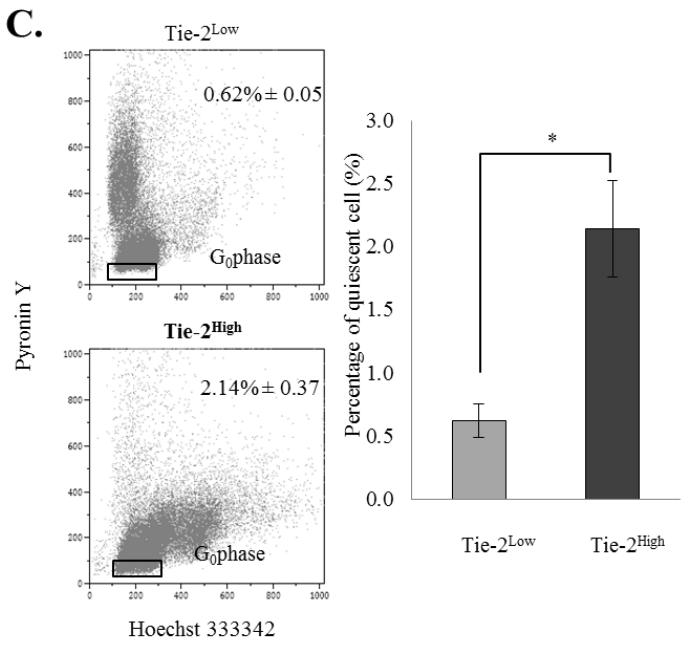
3.3.3 Tie-2 regulates the quiescence of prostate cancer cells

One of the key roles of Tie-2 is to regulate the quiescence state of HSCs. To determine if expression of Tie-2 is associated with cellular quiescent, HO/PY staining was performed to quantitate quiescent population in both Tie-2^{High} and Tie-2^{Low} prostate cancer cells. As expected, the population of quiescent cells was increased more than 3-fold in the Tie-2^{High} population when compared to the Tie-2^{Low} population (Figure 3.2C), suggesting that Tie-2 expression plays an important role in maintaining the quiescent state of prostate cancer cells. Cellular quiescence has been shown to contribute to the chemodrug resistance of CSCs. We therefore examined the sensitivity Tie-2^{High} prostate cancer cells to Cabazitaxel, a chemotherapeutic drug commonly used for the treatment of prostate cancer. As shown in Figure 3.2D, treatment of the Tie-2^{Low} population with Cabazitaxel led to induction of apoptosis of 48% cells, as evidenced by Annexin V staining. However, the apoptotic population

was significantly lower in Tie-2^{High} cells under the same conditions (<35%), clearly demonstrating that Tie-2 expression is associated with Cabazitaxel resistance.


A.
Tie-2Low
Tie-2High
0.10%± 0.009
80%± 8.7
#1
Tie-2


B.
KITLG
CXCR4
FGF1
Fold change relative to Tie2Low
***
**
*
Tie-2Low
Tie-2High


C.
Tie-2Low
0.62%± 0.05
G0phase
Tie-2High
2.14%± 0.37
Pyronin Y
Hoechst 333342
Percentage of quiescent cell (%)
*
Tie-2Low
Tie-2High

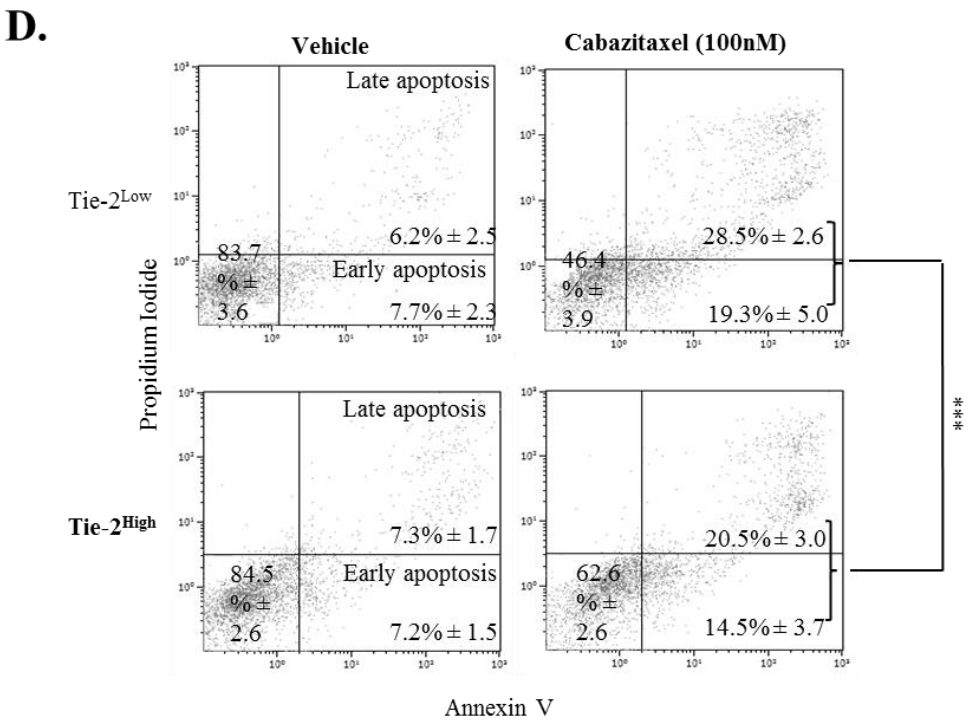


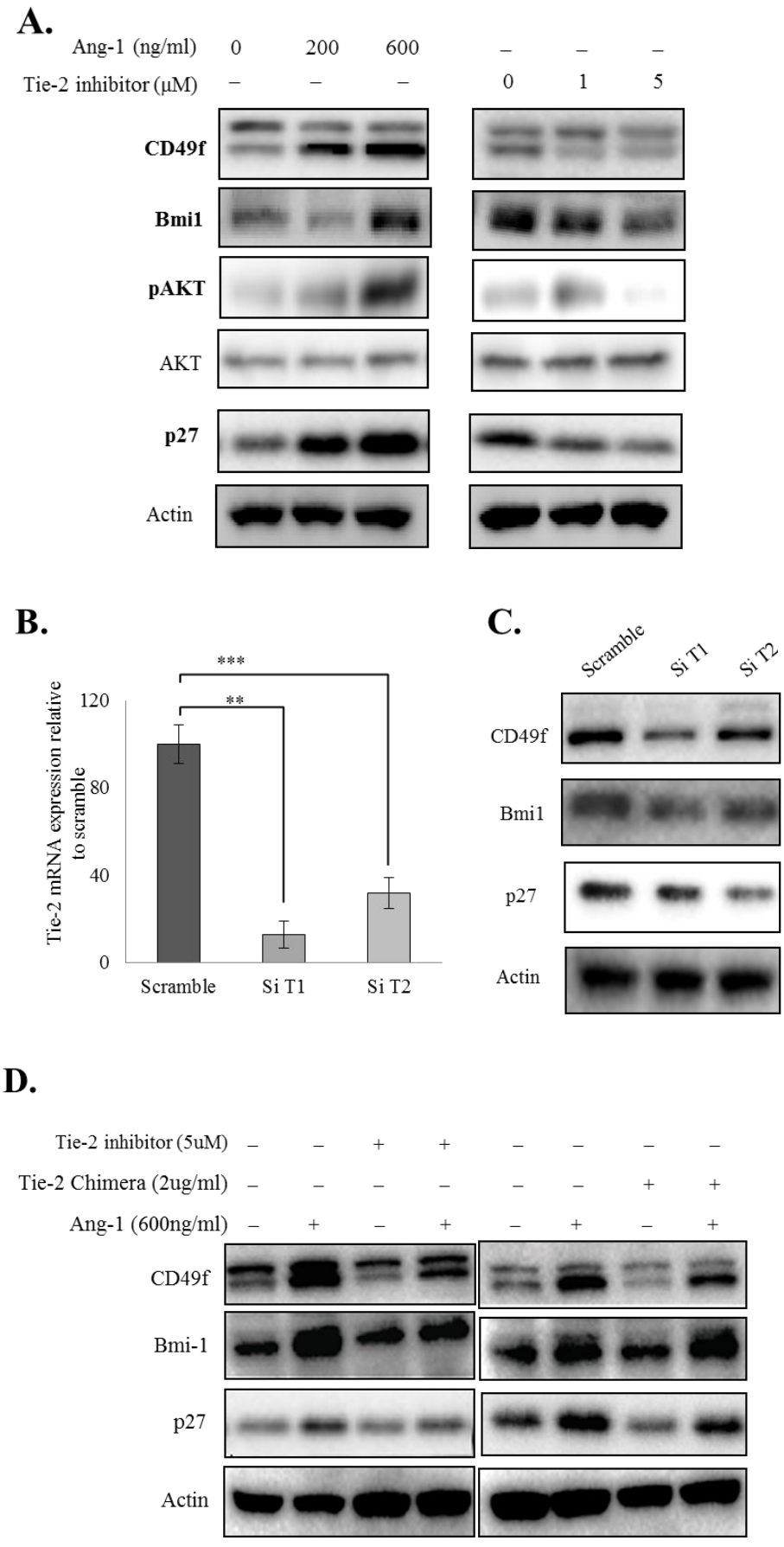
Figure 3.2: The Tie-2^{High} population possessed stem cell characteristics. (A) Analysis of the population sorted by flow cytometry confirmed the successful enrichment of Tie-2^{High} cells. (B) Validation of the selected candidate genes (i.e., KITLG, CXCR4, and FGF1) with qRT-PCR. Results were normalized with internal control and are presented as fold change relative to Tie-2^{Low} population. (C) Flow cytometry analysis revealed that quiescent cells were increased by more than 3-fold in the Tie-2^{High} population when compared to the Tie-2^{Low}population. (D) Flow cytometry analysis of apoptotic cells by Annexin V staining in Tie-2^{Low} and Tie-2^{High} cells that were treated with 100nM Cabazitaxel for 72hrs (p value = 0.0018 for apoptosis). Note that a high percentage of apoptotic cells were detected in the Tie-2^{Low} population when compared to the Tie-2^{High} prostate cancer cells. (p values: * < 0.05, ** < 0.005, *** < 0.0005).

3.3.4 Ang-1 activates the Tie-2 downstream signalling pathway in prostate cancer cells

Because the Ang-1/Tie-2 signalling cascade plays a role in the regulation of HSC stemness, we therefore questioned whether Ang-1 also regulates the stemness of prostate cancer cells. We first treated PC-3 cells with increasing doses of recombinant Ang-1 (0, 200 and 600 ng/ml) for 72 hours under serum-free conditions. The expression of a series of stem cell factors/markers known to be induced by the Ang-1/Tie-2 signalling in HSCs was then examined by Western blotting. As shown in Figure 3.3A (left panel), Ang-1 induced a dose-dependent increase in AKT phosphorylation, a direct downstream target of the Ang-1/Tie-2 signalling pathway, confirming that Tie-2 activates prostate cancer cells. More importantly, Ang-1 treatment was found to induce the expression of prostate CSC (CD49f and Bmi-1) and quiescence (p27) markers in PC-3 cells in a dose-dependent manner. When the cells were treated with a Tie-2 kinase inhibitor (0, 1 and 5 μM), a cell permeabile pyridinylimidazole found to block the kinase activity of Tie-2 [181], all the markers tested were found to be downregulated, suggesting that activation of Tie-2 is required for maintaining the levels of these markers (Figure 3.3A, right panel). To confirm our findings, cells were transfected with two different siRNAs that target different regions of the Tie-2 mRNA, which resulted in a significant decrease (>50%) in the Tie-2 mRNA level (Figure 3.3B). As shown in Figure 3.3C, knockdown of Tie-2 led to concomitant decrease in the level of CD49f, Bmi-1 as well as p27. More interestingly, the effect of recombinant Ang-1 was significantly suppressed when the cells were pre-treated with the Tie-2 inhibitor (Figure 3.3D, left panel) or Tie-2 Fc Chimera (Tie-2 neutralizing peptide) (Figure 3.3D, right panel). Furthermore, ectopic expression of Tie-2 in DU145 cells, which lack endogenous Tie-2 expression (Suppl.

Figure A.1A) and fail to respond to Ang-1 (data not shown), was found to successfully restore the response of the cells to Ang-1 treatment (Suppl. Figure A.1B), further supporting the hypothesis that activation of Tie-2 by Ang-1 is crucial for maintaining the expression of stem cell markers in prostate cancer cells.

Since Ang-1 regulates the stemness of HSCs through induction of cellular quiescence, we therefore examined if Ang-1 treatment also induces quiescent of the prostate cancer cells. PC-3 cells were first treated with Ang-1 before being stained with HO/PY for quantitation of quiescent population. As shown in Figure 3.3E&F, when cells were treated with recombinant Ang-1, a 4-fold increase in the quiescent population was observed. Consistently, expression of p27, a protein closely associated with cell cycle arrest and cellular quiescence, was also found to be induced by Ang-1 treatment in PC-3 cells (Figure 3.3A). Meanwhile, inactivation of Tie-2 using siRNA (Figure 3.3C), a Tie-2-specific inhibitor or a Tie-2 Fc chimera (Figure 3.3D) also led to suppression of p27, even in the presence of recombinant Ang-1, further suggesting that Ang-1 induces quiescence of prostate cancer cells through the activation of Tie-2.


A.
Ang-1 (ng/ml) 0 200 600 – – –
Tie-2 inhibitor (μM) – – – 0 1 5
CD49f
Bmi1
pAKT
AKT
p27
Actin
B.
Tie-2 mRNA expression relative to scramble
120
80
40
0
***
**
Scramble
Si T1
Si T2
C.
Scramble
Si T1
Si T2
CD49f
Bmi1
p27
Actin
D.
Tie-2 inhibitor (5uM) – – + + – – – –
Tie-2 Chimera (2ug/ml) – – – – – – + +
Ang-1 (600ng/ml) – + – + – + – +
CD49f
Bmi-1
p27
Actin

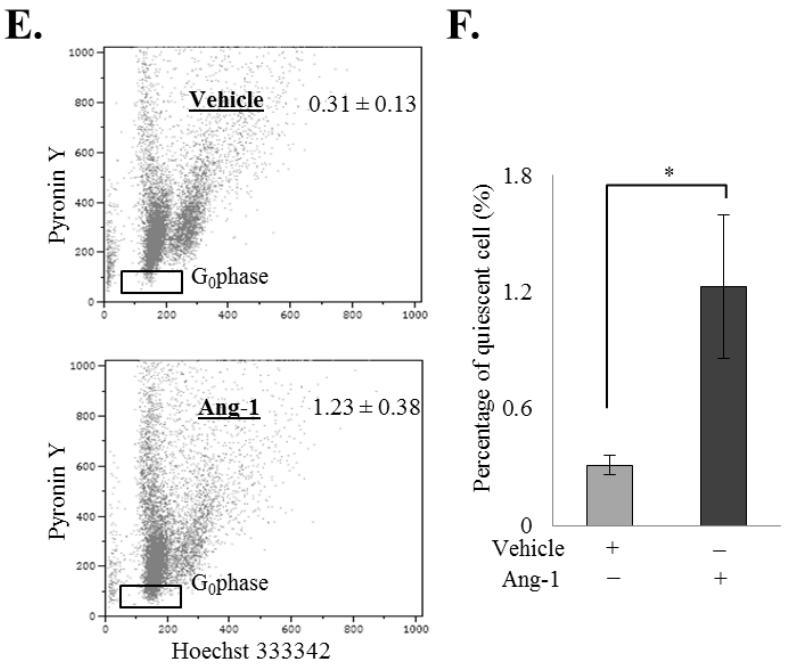
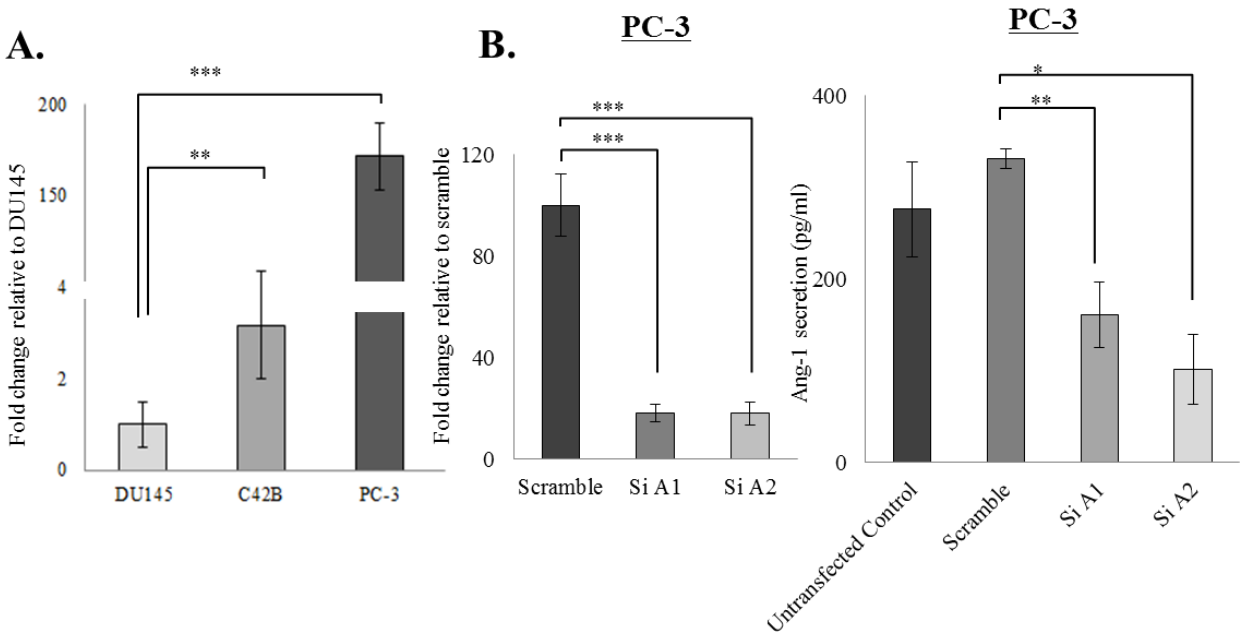


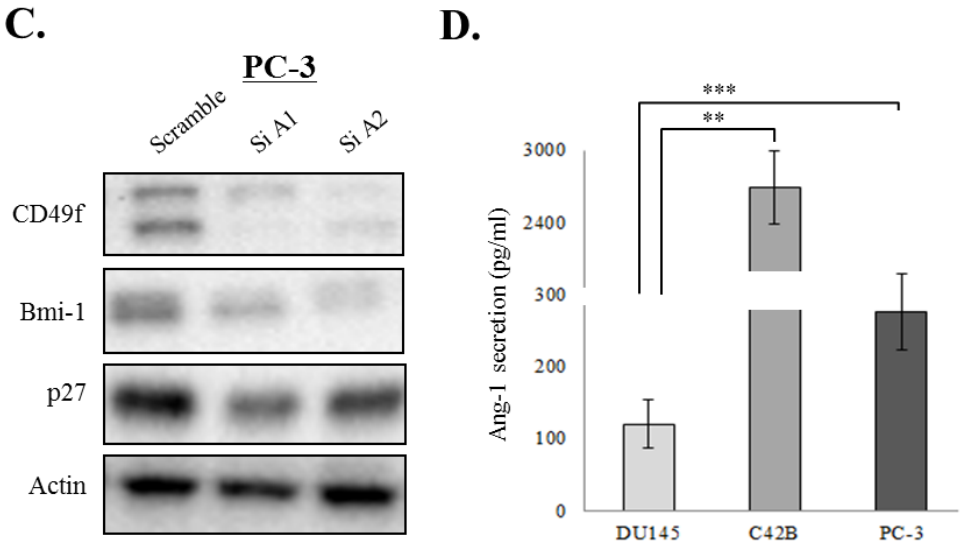
Figure 3.3: Ang-1 upregulated prostate CSC and quiescent markers in prostate cancer cell lines. Western blotting (A) of prostate CSC markers (CD49f and Bmi-1) and a quiescence marker (p27) after Ang-1 treatment in PC-3 cells (left panel). Ang-1 was found to upregulate both stem cell and quiescence markers in a dose-dependent manner. The Tie-2 inhibitor, on the other hand, suppressed both types of markers in a dose-dependent manner in PC-3 cells (right panel). (B) Transfection of Tie-2 siRNAs led to downregulation Tie-2 mRNA levels in PC-3 cells. Results were normalized with internal control and are presented as fold change relative to scramble. (C) Effect of Tie-2 knockdown on CSC and quiescence marker expression in PC-3 cells. (D) The experiment was repeated in the presence of Tie-2 inhibitor (left panel) or a Tie-2 neutralizing antibody (right panel), which showed that both the Tie-2 inhibitor and Tie-2 neutralizing antibody abolished the effect of Ang-1 on PC-3 cells. *(E)* Quantitation of the quiescent population in PC-3 cells with or without Ang-1 treatment. Note that Ang-1 treatment led to a 4-fold induction of the quiescent population in PC-3 cells when compared to the vehicle control. Each experiment was

repeated at least three times, and the results are presented as the mean ± SD. (p values: * < 0.05, ** < 0.005, *** < 0.0005).

3.3.5 Ang-1 functions as a novel autocrine factor in prostate cancer cells

Because prostate cancer cells are known to secrete Ang-1, we hypothesized that Ang-1 may indeed function as an autocrine factor in prostate cancer cells. To test this hypothesis, we first confirmed the expression of Ang-1 mRNA in the prostate cancer cell lines with qRT-PCR. Consistent with previous studies, Ang-1 mRNA was detectable in prostate cancer cell lines, and its expression is found higher in bone metastatic prostate cancer cell lines, C42B and PC-3 (Figure 3.4A). Nevertheless, transfection of PC-3 cells with two different Ang-1 siRNAs led to >80% suppression of the Ang-1 mRNA level, as shown in Figure 3.4B. More importantly, knockdown of endogenous Ang-1 resulted in suppression of both CSC (CD49f an Bmi-1) and quiescence (p27) markers in PC-3, supporting the hypothesis that Ang-1 produced by cancer cells is crucial for CSC maintenance (Figure 3.4C). Surprisingly, examination of Ang-1 secretion with ELISA revealed that C42B produced the highest level of Ang-1 among all the prostate cancer cell lines (Figure 3.4D). Meanwhile, we found that C42B cells failed to respond to treatment with Ang-1 recombinant proteins (data not shown); however, knockdown of endogenous Ang-1 not only suppressed the expression of CD49f an Bmi-1, but also restored the response of the cells to exogenous Ang-1 treatment, as evidenced by the induction of CSC and quiescence markers by recombinant Ang-1 (Figure 3.4E-G). These findings further confirm that Ang-1 regulates the stemness of prostate cancer cells by functioning as an autocrine factor.


A.
Fold change relative to DU145
200
150
4
2
0
***
**
DU145
C42B
PC-3
B.
PC-3
Fold change relative to scramble
120
80
40
0
***
***
Scramble
Si A1
Si A2
PC-3
Ang-1 secretion (pg/ml)
400
200
0
*
**
Untransfected Control
Scramble
Si A1
Si A2


C.
PC-3
Scramble
Si A1
Si A2
CD49f
Bmi-1
p27
Actin
D.
Ang-1 secretion (pg/ml)
3000
2400
300
200
100
0
***
**
DU145
C42B
PC-3

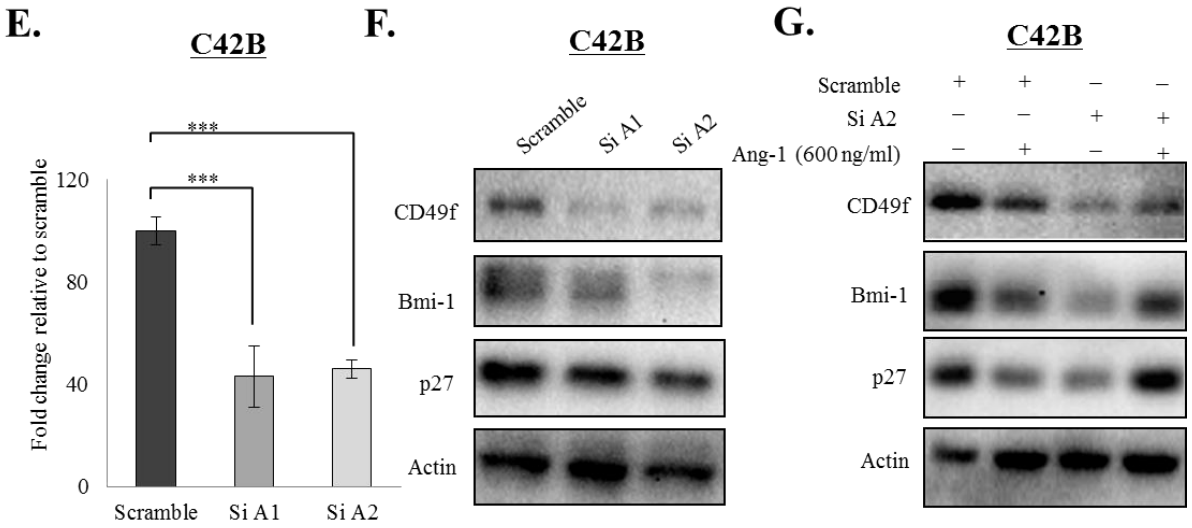

E.
C42B
Fold change relative to scramble
120
80
40
0
***
***
Scramble
Si A1
Si A2
F.
C42B
Scramble
Si A1
Si A2
CD49f
Bmi-1
p27
Actin
G.
C42B
Scramble + + – –
Si A2 – – + +
Ang-1 (600 ng/ml) – + – +
CD49f
Bmi-1
p27
Actin

Figure 3.4: Ang-1 functioned as an autocrine factor in prostate cancer cells. (A) Ang-1 mRNA expression was determined in different prostate cancer cell lines (DU145, C42B and PC-3) using qRT-PCR analysis. Results were normalized with internal control and are presented as fold change relative to DU145. (B) Knockdown of Ang-1 in PC-3 cells by siRNA transfection was confirmed with qRT-PCR and ELISA. Note that Ang-1 expression was suppressed by >80% in PC-3 cells. Results were normalized with internal control and are presented as fold change relative to scramble. *(*C) Downregulation of Ang-1 in PC-3 cells by siRNA was associated with suppression of CSC (CD49f and Bmi-1) and quiescence (p27) markers. (D) Ang-1 secretion (pg/ml) by different prostate cancer cell lines (DU145, C42B and PC-3) was determined with an ELISA. (E) Knockdown of Ang-1 for >50% was confirmed with RT-PCR in C42B cells. (F) Ang-1 knockdown suppressed both CSC (CD49f and Bmi-1) and quiescence (p27) markers in C42B cells. (G) The response of C42B cells to exogenous Ang-1 treatment (600ng/ml) was restored when endogenous Ang-1 was knocked down by siRNA. (p values: * < 0.05, ** < 0.005, *** < 0.0005).

3.3.6 Tie-2 facilitates the adhesion of prostate cancer cells to osteoblasts and endothelial cells

One of the functions of Tie-2 in HSC maintenance is to facilitate the adhesion of HSCs to osteoblasts, which is a process that is also required by prostate cancer cells during the development of bone metastasis. This led us to speculate that Tie-2 may also regulate the adhesion of prostate cancer cells to osteoblasts. The ability of the Tie-2^{High} population to adhere to osteoblasts was then determined with a cell adhesion assay using the osteosarcoma cell line (MG-63). Examination of the cells that adhered to a confluent monolayer of MG-63 cells revealed a significant increase in the adhesion ability of the Tie-2^{High} population to osteoblasts when compared to the Tie-2^{Low} cells (Figure 3.5A). This adhesion ability was completely abolished in the presence of the Tie-2 inhibitor, although the same treatment failed to affect the adhesion ability of the Tie-2^{Low}population (Figure 3.5B). A similar result was observed in another osteosarcoma cell line (SaOS-2) (Suppl. Figure A.2A & B). Strikingly, we found that the Tie-2^{High} population also showed an increased ability to adhere to endothelial cells (Figure 3.5D), which again could be abolished by the addition of the Tie-2 inhibitor (Figure 3.5E). To determine whether this effect of Tie-2 is specific to PC-3 cells, we repeated the cell adhesion assay with DU-Tie-2-GFP. Similar to Tie-2^{High} PC-3 cells, DU-Tie-2-GFP cells also demonstrated increased adhesion to both MG-63 and HUVECs when compared to the control cells (DU-GFP), as shown in Figure 3.5C&F. Meanwhile, the adhesion ability of DU-Tie-2-GFP cells to MG-63 and HUVECs was also able to abolish by the addition of Tie-2 inhibitor as shown in Suppl. Figure A.2C & D. Recently, Ang-1 has been shown to promote the bridging of intercellular Tie-2 [182]. Considering that osteoblasts express high levels of Tie-2, it is possible that the cell adhesive ability of the Tie-2

positive prostate cancer cells is mediated through bridging of the intercellular Tie-2. Nevertheless, these findings provide strong evidence that Tie-2 promotes the adhesion of prostate cancer cells to both endothelial cells and osteoblasts.

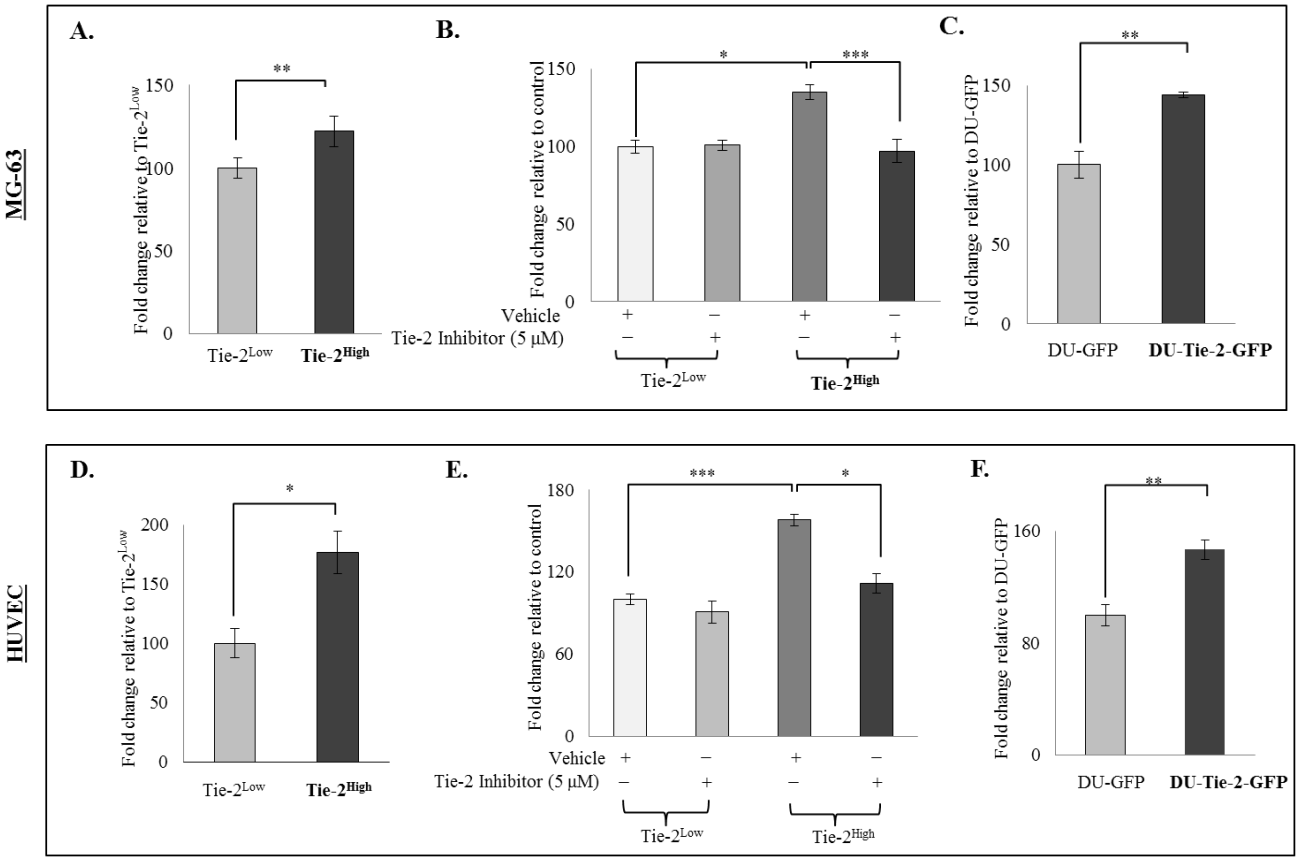
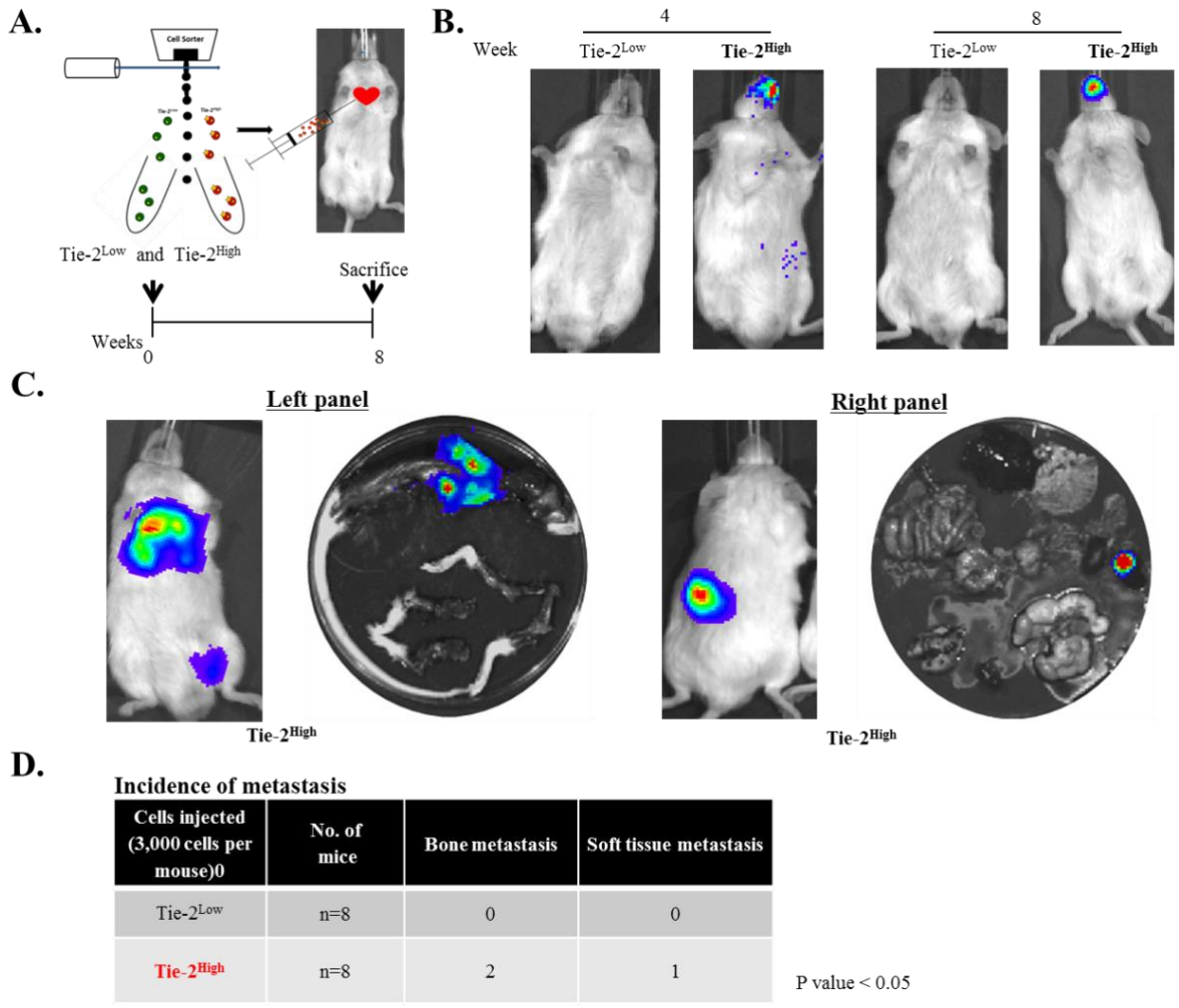


Figure 3.5: Tie-2 facilitated the adhesion of prostate cancer cells to osteoblasts and endothelial cells. A cell adhesion assay was performed with the sorted Tie-2^{High} and Tie-2^{Low} PC-3 cells prestained with Hoechst 33342. Cells that adhered to osteoblasts (MG-63) (A) or endothelial cells (HUVECs) (D) were quantified by measuring the fluorescence intensity, and the results are presented as the mean ± SD. Note that Tie-2^{High} PC-3 cells were more adhesive to MG-63 and HUVECs when compared to Tie-2^{Low}PC-3 cells. (B&E) Effect of Tie-2 inactivation on the adhesive ability of Tie-2^{High} PC-3 cells. Treatment with a Tie-2 inhibitor (5 µM) prior to the adhesion assay significantly suppressed the adhesion ability of Tie-2^{High} cells, while the same treatment failed to affect the Tie-2^{Low} population. (C&F) Ectopic Tie-2 expression promoted the adhesion of DU145 cells to osteoblast MG-63 cells and HUVECs. Each experiment was repeated at least three times, and the results are presented as the mean ± SD. (p values: * < 0.05, ** < 0.005, *** < 0.0005).

3.3.7 Tie-2 enriched cells are highly metastatic *in vivo*

Because we found that higher levels of Tie-2 were expressed in metastatic prostate cancer cell lines, and conferred the preferential ability to induce a stem cell-like phenotype and cell adhesion ability of the cancer cells, we hypothesized that Tie-2 may play an important role in prostate tumor metastasis. To test this hypothesis, we examined the ability of Tie-2^{High} prostate cancer cells to form metastatic tumors *in vivo*. Three thousand Tie-2^{High} and Tie-2^{Low} cells isolated from the PC-3-Luc cell line were injected into the mice via intra-cardiac injection as shown in Figure 3.6A. Subsequent development of metastatic tumors was then determined by bioluminescence (Figure 3.6B) and *ex vivo* (Figure 3.6C and Suppl. Figure A.3) imaging. While mice injected with the Tie-2^{Low} cells failed to develop any tumors, metastatic tumors were found in 3 out of the 8 mice that were injected with the Tie-2^{High} population. Of the 3 mice that developed tumors, two exhibited bone metastasis and one exhibited soft tissue metastasis to the kidney, as shown in Figure 3.6D. These data strongly support that Tie-2^{High} prostate cancer cells are highly metastatic and have the ability to form metastatic tumors *in vivo.*



Incidence of metastasis

Cells injected (3,000 cells per mouse)0	No. of mice	Bone metastasis	Soft tissue metastasis
Tie-2^{Low}	n=8	0	0
Tie-2^{High}	n=8	2	1

P value < 0.05

Figure 3.6: The Tie-2^{High} population was highly metastatic *in vivo*. (A) Tie-2^{High} and Tie-2^{Low} populations sorted out by FACS were injected into mice via intracardiac injection (top). An experimental regimen showing the intracardiac implantation and monitoring of tumor metastasis is shown below. (B) Representative bioluminescence images of a mouse from each group 4 and 8 weeks after the implantation. (C) *Ex vivo* imaging of the Tie-2^{High} metastatic tumor in rib cage (Left) and kidney (Right). (D) Summary of the metastatic tumors detected in each mouse. Three out of the eight mice that were injected with Tie-2^{High} cells exhibited metastasis (two in the bone and one in the kidney) ($p < 0.05$). (See also Suppl. Figure A.3)

3.4 Dissussion

In this study, we showed that a rare population of Tie-2-positive cells are present in bone metastatic prostate cancer cell lines (PC-3, C42b and MDA-PCa-2b). These cells not only demonstrated an increased ability to adhere to osteoblasts and endothelial cells but were also found to be more resistant to chemotherapeutic drugs when compared to the Tie-2^{Low} population. More importantly, we found that these cells were capable of developing into metastatic tumors *in vivo*, even when a low number of cells were injected. These cells may thus represent the prostate CSC-like population, which contributes to treatment failure and disease metastasis.

Ample evidence has suggested that CSCs isolated from solid tumors share similar characteristics with HSCs [43, 183, 184]. Indeed, several signalling pathways involved in HSC maintenance were also found to be activated in CSCs. For example, CXCL12/CXCR4 was found to be a key regulator of tumor dissemination in cancer cells [185-187], including prostate cancer cells [17, 142] and it also plays an important role in the activation of prostate progenitor cells [188]. Recently, KITLG/c-Kit was found to be involved in CSC maintenance in different cancer types, including prostate cancer [35, 189-191]. Moreover, these studies also showed that the c-Kit receptor was highly expressed in human prostate tumors that metastasized to the bone [29]. It is therefore not surprising that the Ang-1/Tie-2 signaling pathway may also play a key role in regulating the stemness of prostate CSCs, particularly because Tie-2 was found to be expressed by glioma stem cell populations [179]. Indeed, our results suggest that activation of Tie-2 may be required for maintaining both the stemness and quiescent state of prostate cancer cells. The finding that the Tie-2^{High} population was more resistant to Cabazitaxel

further supports this notion. Meanwhile, we also observed a significant increase in HSC factors/markers (KITLG, CXCR4 and FGF1) in Tie-2^{High} PC-3 cells when compared to the Tie-2^{Low} population, further suggesting that Tie-2^{High} prostate cancer cells are similar to HSCs.

Prostate cancer cells are known to actively secrete a large amount of Ang-1, which induces tumor angiogenesis by binding to and activating Tie-2 in endothelial cells [192, 193]. Our finding that prostate cancer cells also express functional Tie-2 suggests that Ang-1 may also function through an autocrine loop. Indeed, knockdown of either Ang-1 or Tie-2 was found to downregulate CSC and quiescence markers in PC-3 and C42B cells. Further support was derived from the finding that C42B cells, which express the highest level of Ang-1, failed to respond to exogenous Ang-1 unless endogenous Ang-1 was knocked down by siRNA transfection (data not shown). It is worth noting that autocrine loops such as KITLG/c-Kit and CXCL12/CXCR4 may also exist, although their underlying functions and therapeutic potential remain to be elucidated.

Apart from regulating the stemness of HSC, the Ang-1/Tie-2 pathway is also known to facilitate the adhesion of HSCs to the osteoblastic niche [30]. Similarly, in our *in vitro* adhesion assays, compared to Tie-2^{Low} cells, Tie-2^{High} PC-3 cells were more adhesive to osteosarcoma MG-63 and SaOS-2 cells. This result suggests that Tie-2 receptor may also play a key role in mediating the adhesion of prostate cancer cells to osteoblasts. This possibility was further confirmed by our finding that Tie-2 overexpression promoted the adhesion of DU145 cells to osteoblast cells. Interestingly, similar effects were also observed in endothelial cells, where Tie-2^{High}

PC-3 cells showed increased adhesion to endothelial cells, where Tie-2^{High} PC-3 showed increased adhesion to endothelial cells. Because both intravasation and extravasation of tumor cells required their active adhesion to endothelial cells [194, 195], it is conceivable that Tie-2 may play roles in both processes during the development of prostate tumor metastasis and that Tie-2^{High} prostate cancer cells are likely to be more metastatic. This was indeed confirmed by the finding that injection of only 3,000 Tie-2^{High} cells could induce the formation of metastatic tumors *in vivo*. Interestingly, kidney metastasis was found in one of the mice, which may reflect the rich blood supply of the kidney tissue. Indeed, sub-renal capsule grafting has been shown to provide the optimum microenvironment for tumor growth, and was most efficient in terms of uptake rate (>90%) for both benign and malignant prostate tissues [196].

In summary, we have demonstrated for the first time that Tie-2 is expressed by a rare population of prostate cancer cells and plays an important role in regulating the stemness and metastatic ability of the cells (summarized in Suppl. Figure A.4). Our results highlight the therapeutic potential of targeting Tie-2 with existing inhibitors for the treatment of metastatic prostate cancer, which warrants further investigation.

3.5 Materials and Methods

qRT-PCR analysis

Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Germantown, MD, USA) following the manufacturer's instructions. Two micrograms of RNA were used to synthesize cDNA using the SuperScript® III First-Strand Synthesis Systems (Invitrogen, Carlsbad, CA, USA). qRT-PCR was carried out with the ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Sense and anti-sense primers targeted against the genes of interest are listed in Suppl. Table A.1. The transcript level of ribosomal protein L32 *(RPL32)* was used as an internal control.

Small interfering RNA

Small interfering RNAs (siRNAs) targeting Tie-2 (J-003178-11 and J-003178-12) and Ang-1 (J-007802-07 and J-007802-08) as well as a scrambled RNA oligo were purchased from Dharmacon, Lafayette, CO, USA. Cells were transfected with the indicated siRNA using Lipofectamine RNAiMax (Invitrogen) following the manufacturer's instructions. Forty-eight hours after transfection, the cells were lysed for Western blotting analysis or for RNA extraction and qRT-PCR analysis.

Flow cytometry analysis and fluorescence-activated cell sorting (FACS)

Cells were collected, washed twice with phosphate-buffered saline (PBS) and resuspended in 50 µl of FACS buffer (0.02% sodium azide and 2% FBS in PBS) before incubating with the fluorescent dye-conjugated antibodies at 4°C in the dark for 30 minutes. After incubation, the cells were washed twice with PBS and subsequently resuspended in 200 µl of FACS buffer. Flow cytometry analysis was

performed using BD™ LSR II as described in the manufacturer's instruction manual, and the results were analyzed using KALUZA software.

For cell sorting, PC-3 cells were stained with Phycoerythrin (PE)-conjugated Tie-2 antibody in 200 μl of FACS buffer (2% FBS in PBS*)* at 4°C in the dark for 2 hours and the corresponding IgG isotype was used as negative control. After incubation, cells were washed twice with PBS and then resuspended in 500 μl of FACS buffer. The Tie-2^{High} population was sorted using the Beckman Coulter MoFloAstrios

Immunohistochemistry (IHC)

Sections rehydrated with standard procedures were incubated with 3% hydrogen peroxide (Sigma-Aldrich) for 10 minutes at room temperature. Antigen retrieval was performed with sodium citrate buffer at pH 6 in a pressure cooker for 10 minutes. Sections were then blocked with normal goat serum diluted in TBS for 1 hour. After the blocking, the sections were incubated with antibody against Tie-2 (1: 5000) (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 hour, followed by a 1 hour incubation with biotinylated rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) and a 30 minute incubation with VECTASTAIN® ABC Reagent complex. Signals were developed with the ImmPACT DAB Peroxidase (HRP) Substrate (Vector Laboratories). Slides were then counterstained with hematoxylin (Biocare Medical, Concord, CA, USA) before being mounted for analysis under the microscope.

Generation of the stable Tie-2 overexpressing line

DU145 cells overexpressing Tie-2 (DU-Tie-2-GFP) were generated using the lentiviral gene delivery system as described in our previous study [198]. Briefly,

pLenti6-Tie-2-GFP or the empty vector control was transfected into 293FT cells for lentiviral packaging. Viruses were collected and used to infect DU145 cells in the presence of polybrene (8 μg/ml). Positive transfectants were selected with blasticidin (10 μg/ml) and were further enriched by FACS based on the GFP signal.

Western blot

Details regarding the experimental procedures have been described in our previous studies [197]. Briefly, whole cell lysates were prepared by lysing cell pellets with lysis buffer (Cell Signaling) containing 100 μM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, St. Louis, MO, USA). The cell lysates were quantitated using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA) before loading onto a SDS-polyacrylamide gel. The resolved proteins were then transferred onto a PVDF membrane (Millipore, Billerica, MA, USA), and the membrane was subsequently probed with the indicated antibody for 1 hour at room temperature prior to being washed with TBS-T buffer. The membrane was then incubated with the corresponding secondary antibodies for another hour at room temperature. After washing with TBS-T buffer, the membrane was incubated with Immobilon Western Chemiluminescent HRP Substrate (Millipore), and the signals were visualized using a Bio-Rad ChemiDoc™ XRS Gel Documentation System.

Hoechst33342/ Pyronin Y (HO/PY) quiescent staining

To determine the percentage of quiescent cells, all cells were first stained with 10 μg/ml HO for 45 minutes in the dark at 37°C. The cells were then incubated with 5 μM PY for another 45 minutes in the dark at 37°C as described in previous studies

[198-200]. After staining, the cells were analyzed using BD™ LSR II, and the results were further analyzed using KALUZA software.

Annexin V staining

To determine the percentage of apoptotic cells, Annexin V staining was performed using BD Pharmingen™ Annexin V-FITC kits following the manufacturer's instructions. Briefly, Tie-2^{High} and Tie-2^{Low} PC-3 cells were washed twice with cold PBS, resuspended in 100µl of 1X binding buffer and then incubated with Annexin V-FITC antibody and propidium iodide in the dark at room temperature for 15 minutes. After incubation, the apoptotic cells were analyzed using BD™ LSR II, and the results were further analyzed using KALUZA software.

Ang-1 ELISA

To quantitate Ang-1 secretion by the prostate cancer cells, the cells were cultured in serum-free medium for 24 hours. The cells were harvested and counted using a Scepter™ Automated Cell Counter (Millipore). Conditioned medium was collected and concentrated using the 10K Amicon Ultra2-ml Centrifugal Filters (Millipore). The medium was analyzed using the Human Angiopoietin-1 DuoSet kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions and the positive signals were determined using a LUMIstar OPTIMA Luminescence Microplate Reader and then, normalized it to the cell numbers.

Cell adhesion assay

Prostate cancer cell lines were first labelled with HO for 45 minutes. Labelled cells (3×10^3) were then overlaid directly onto confluent HUVECs, MG-63 cells or

SaOS-2 cells. The cells were incubated for 30 minutes in the dark at 37°C. Unattached cells were dispersed with PBS, and the adherent cells were quantified using a LUMIstar OPTIMA Luminescence Microplate Reader. This experiment was repeated in the presence of 5 μM Tie-2 inhibitor or an equal volume of DMSO as a control. The data are presented as the fluorescence intensity from triplicate experiments.

Animal studies

All animal studies were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the Animal Ethics Committee at the Queensland University of Technology (Approval No: 1100001393). Tumor metastasis were examined via intra-cardiac tumor cell injection using procedures described previously [180]. Briefly, the Tie-2^{High} population was isolated from PC3-Luc cells, which were stably transfected with a luciferase expression construct [201]. Three thousand Tie-2^{High} or Tie-2^{Low} cells were suspended in 100 μl of PBS before injecting into the left cardiac ventricle of 6-8-week-old male NOD-SCID mice (n = 8). Tumor metastasis were monitored every 2 weeks for a total of 8 weeks by intraperitoneal injection of D-luciferin (150 mg/kg) followed by bioluminescence imaging. The signal was detected using the Xenogen IVIS 100 imaging system. Mice were sacrificed at the end of the experiment, and *ex vivo* bioluminescence imaging was performed to confirm the incidence of metastasis. Statistical analysis was performed with the two-tailed t test, and differences were considered to be significant if $p < 0.05$.

3.6 Supplementary Tables and Figures

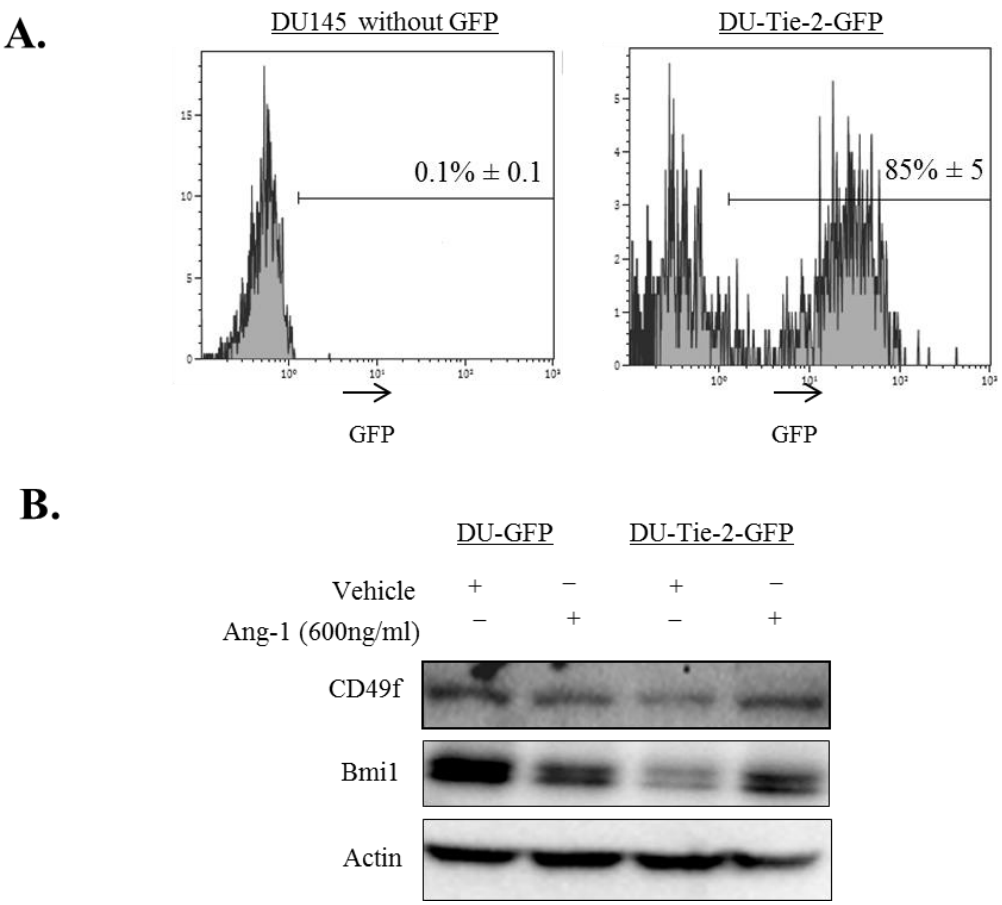
Suppl. Table A.1: List of the primers used in this study.

Primer name	Sequence
Tie-2 forward	5′-CTTTCTGGAACTGTGGAAGG-3′
Tie-2 reverse	5′-CTGGTGCTGGTTCATTAAGG-3′
KITLG forward	5′-CTGCTCCTATTTAATCCTCTCGT-3′
KITLG reverse	5′-TTGTACTACCATCTCGCTTATCC-3′
CXCR4 forward	5′- GCAGCAGGTAGCAAAGTGAC -3′
CXCR4 reverse	5′-AGAAGATGATGGAGTAGATGGTGG-3′
FGF1 forward	5′-ACAAGAAGCCCAAACTCCTC-3′
FGF1 reverse	5′-GTTCTCCTCCAGCCTTTCCA-3′
ANGPT1 forward	5′-ACGATGGCAACTGTCGTGAG-3′
ANGPT1 reverse	5′-TCCGACTTCATGTTTTCCACAA-3′

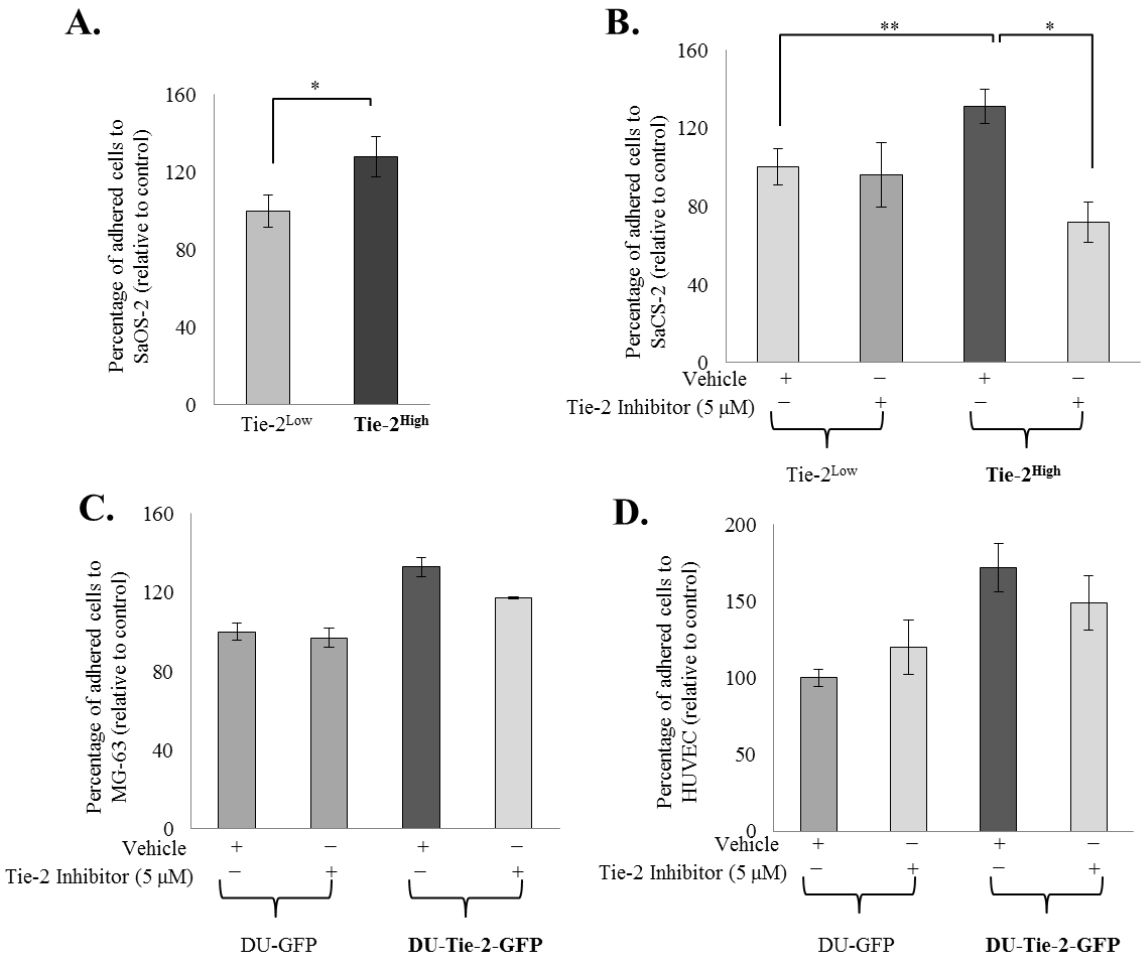
Suppl. Table A.2: Summary of cDNA microarray analysis.

Gene Symbol	Description	RefseqID	Fold Change
FGF1	fibroblast growth factor 1 (acidic) (FGF1), transcript variant 6, mRNA.	NM_001144935	4.9
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A), transcript variant 2, mRNA.	NM_078467	2.8
PLAUR	plasminogen activator, urokinase receptor (PLAUR), transcript variant 2, mRNA.	NM_001005376	2.6
PROM1	prominin 1 (PROM1), transcript variant 4, mRNA.	NM_001145852	2.3
KITLG	KIT ligand (KITLG), transcript variant a, mRNA.	NM_003994	2.3
CD3D	CD3d molecule, delta (CD3-TCR complex) (CD3D), transcript variant 1, mRNA.	NM_000732	2.1
FLOT2	flotillin 2 (FLOT2), mRNA.	NM_004475	2.0
CD38	CD38 molecule (CD38), mRNA.	NM_001775	2.0
TEK	TEK tyrosine kinase, endothelial (TEK), mRNA.	NM_000459	1.9
BMP7	bone morphogenetic protein 7 (BMP7), mRNA.	NM_001719	1.9
CXCR4	chemokine (C-X-C motif) receptor 4 (CXCR4), transcript variant 1, mRNA.	NM_003467	1.9
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2 (ABCG2), mRNA.	NM_004827	1.9
FLT3	fms-related tyrosine kinase 3 (FLT3), mRNA.	NM_004119	1.8
CD24	CD24 molecule (CD24), mRNA.	NM_013230	1.8
IL8	interleukin 8 (IL8), mRNA.	NM_000584	1.7
SOX2	SRY (sex determining region Y)-box 2 (SOX2), mRNA.	NM_003106	1.5

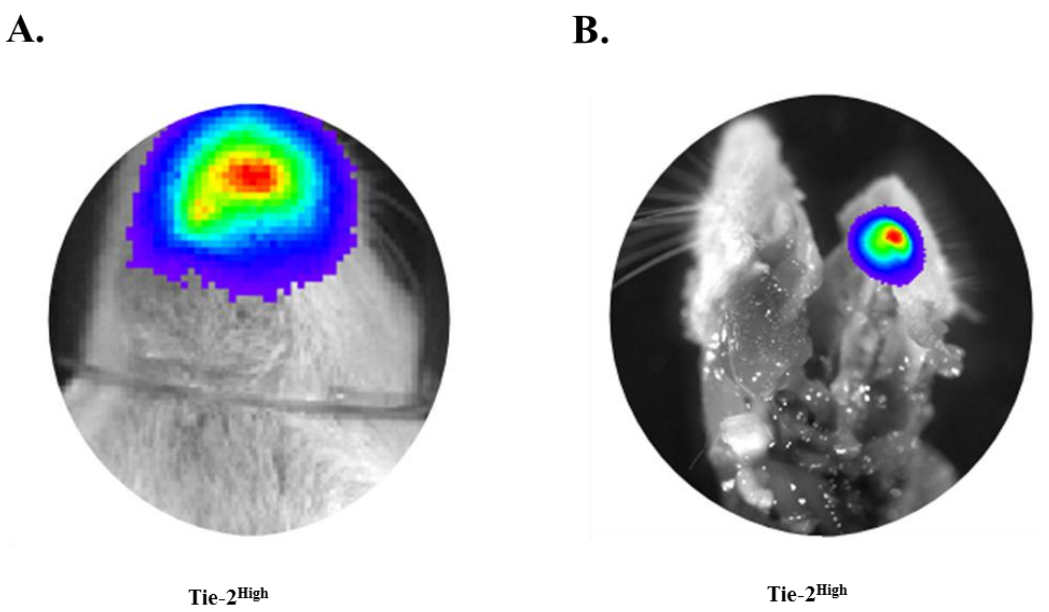
The fold induction of stem cell factors and markers in Tie-2^{High} population when compared to Tie-2^{Low} population as determined by cDNA microarray analysis. Each experiment was repeated at least twice.



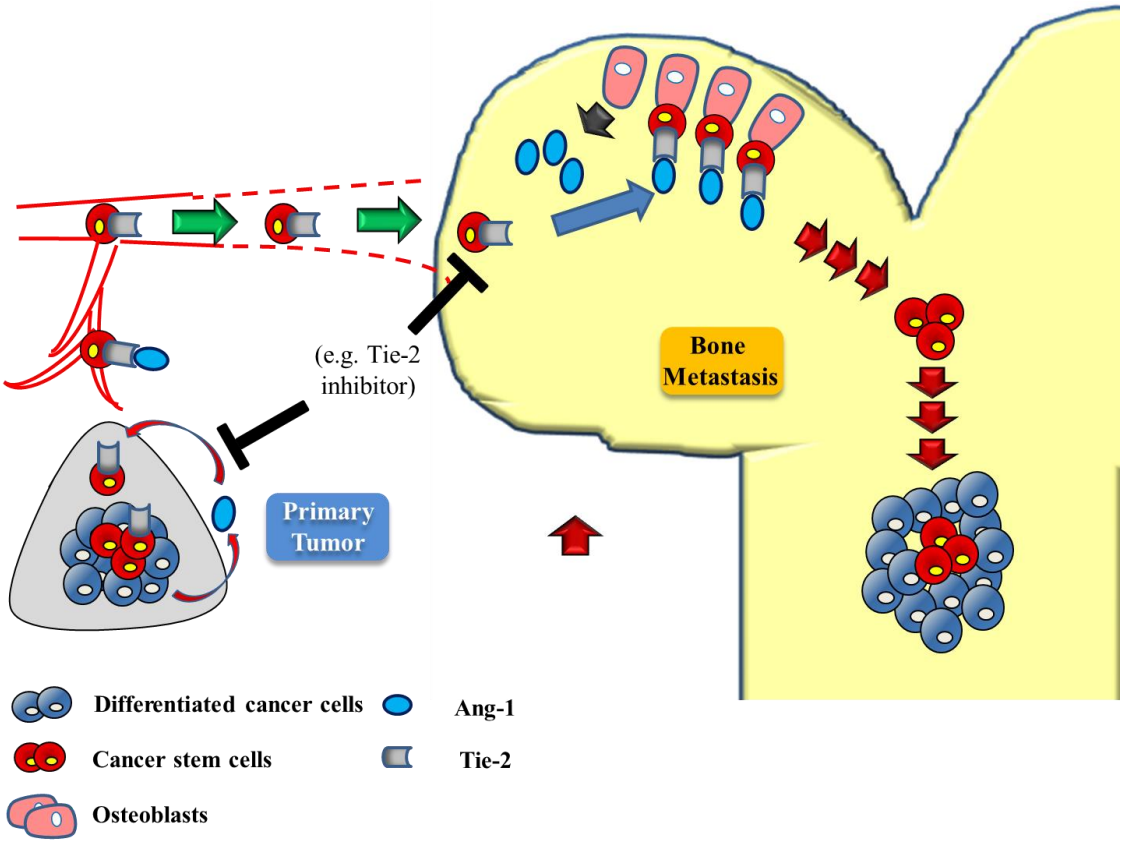
Suppl. Figure A.1: Ang-1 upregulated prostate CSC markers in DU-GFP-Tie-2 cells. (A) DU145 constitutively expressing the Tie-2 protein (DU-Tie-2-GFP) was sorted by FACS using GFP as the marker. (B) Western blotting of prostate CSC markers (CD49f and Bmi-1) after Ang-1 treatment in DU-GFP and DU-Tie-2-GFP.



Suppl. Figure A.2: Tie-2 facilitated the adhesion of prostate cancer cells to bone (MG-63 and SaOS-2) and HUVEC cells. (A) Tie-2^{High} PC-3 cells were more adhesive to SaOS-2 cells when compared to Tie-2^{Low} PC-3 cells. (B) Effect of Tie-2 inactivation on the adhesive ability of Tie-2^{High} PC-3 cells. Treatment with a Tie-2 inhibitor (5 µM) prior to the adhesion assay significantly suppressed the adhesion ability of Tie-2^{High} cells, while the same treatment failed to affect the Tie-2^{Low} population. (C & D) The addition of Tie-2 inhibitor to DU-Tie-2-GFP cells drastically inhibited the adhesion ability of cells to MG-63 and HUVEC cells, but not in DU-GFP cells. Each experiment was repeated at least three times, and the results are presented as the mean ± SD.



Suppl. Figure A.3: Localization of metastatic tumor in the mice with Tie-2^{High} cell implantation. (A) Bioluminescence images of mice with tumor metastasis at 8 weeks after the Tie-2^{High} cell implantation. (B) Ex vivo imaging of the metastatic tumors. Note that, one of the mice exhibited jaw metastasis.



Suppl. Figure A.4: Model for the role of Tie-2 in prostate tumor metastasis. Ang-1/Tie-2 functions as an autocrine loop that regulates the stemness and quiescence of a rare population of Tie-2 positive prostate cancer cells. These cells, which are capable of adhering to endothelial cells and obsteoblasts, are highly metastatic *in vivo* and may be responsible for mediating tumor metastasis *in vivo*. Thus, targeting the Ang-1/Tie-2 autocrine loop with Tie-2 inhibitor may offer opportunities for inhibiting prostate tumor metastasis by eliminating the metastatic cancer cell population.

3.7 Supplementary Materials and Methods

Cell lines and culture conditions

Prostate cancer cell lines PC-3, DU145, LNCaP and LAPC4 were obtained from ATCC (Rockville, MD, USA) and were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS, Invitrogen) and 2% (wt/vol) penicillin-streptomycin P/S, Invitrogen); whereas 22Rv1 was a generous gift from Prof. Franky Chan (The Chinese University of Hong Kong) and was maintained in RPMI 1640 medium supplemented with 10% FBS and 2% (wt/vol) P/S. C42B was kindly provided by Prof Leland Chung (Cedars-Sinai Medical Center) and was maintained in T-Medium (Invitrogen) supplemented with 5% FBS and 2% P/S. MDA-PCa-2b was a kind gift from Dr Nora Navone (MD Anderson Cancer Center) and was maintained in BRFF-HPC1 medium (Athena Enzyme Systems) supplemented with 20% FBS and 1% P/S. Osteosarcoma cell lines MG-63 and SaOS-2 were obtained from ATCC and were maintained in Dulbecco's modified Eagle's medium (Invitrogen) (DMEM containing 10% FBS, 1% P/S) and McCoy's 5a medium (Invitrogen) containing 10% FBS, 1% P/S respectively. Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from ScienCell Research Laboratories, Carlsbad, CA, USA and were maintained in endothelial culture medium (ECM) supplemented with 5% FBS, 1% endothelial cell growth factor (ECGS) and 1% P/S (ScienCell Research Laboratories). All cell types were kept at 37^0C in a 5% CO2 environment.

Antibodies and reagents

Tie-2 inhibitor, Hoechst33342 (HO) and Pyronin Y (PY) were purchased from Santa Cruz Biotechnology, Dallas, TX, USA. Recombinant Human Tie-2 Fc Chimera, CF

was purchased from R&D Systems, Minneapolis, MN, USA and Human Ang-1 recombinant protein was purchased from PROSPEC, East Brunswick, NJ, USA. Gamma-tocotrienol (γ-T3) was provided by Davos Life Science Pty Ltd from Singapore, and was dissolved in absolute ethanol (100 mM). Cabazitaxel was purchased from Selleck, Houston, TX, USA and was dissolved in absolute ethanol (100 μM).

The following antibodies were used in this study: Phycoerythrin (PE) conjugated Tie-2 antibody and Mouse IgG1 PE Isotype Control (R&D Systems, Minneapolis, MN, USA); Human CD49f, pAKT and AKT antibodies (Cell Signalling Technology, Danvers, MA, USA); Bmi-1 antibody (Millipore, Billerica, MA, USA); p27 antibody (BD Biosciences); Tie-2, Actin and donkey anti-goat IgG-HRP antibody (Santa Cruz) and HRP conjugated anti-mouse and anti-rabbit secondary antibodies (GE Healthcare, Buckinghamshire, UK).

Microarray analysis

Duplicates of sorted Tie-2^{Low} and Tie-2^{High} populations were prepared for microarray profiling, which was performed on a custom Agilent 4X180k oligo array. The detailed experimental procedures have been described in a previous study [202].

Chapter 4: Research Paper 2: Adipocytes promote prostate cancer stem cell self-renewal through a amplification of the cholecystokinin autocrine loop

While the role of osteoblast in the development of prostate tumor bone metastasis have been well-established, little is known about the role of adipocytes within the bone marrow tumor microenvironment. For this reason, in this chapter, I have focused on the investigating whether adipcoytes contribute to the CSC niche within the bone marrow. Through this study, I have established a novel 3D spheroid co-culture model and subsequently used it to demonstrate the role of adipocytes in regulating the prostate CSC self-renewal and expansion. More importantly, I have shown that inactivation of both CCKBR and CTSB with specific inhibitors resulted in a significant suppression of the CSC-promoting effect of adipocytes. My study therefore demonstrated the underlying mechanism in the crosstalk between the prostate CSC and adipocytes. Meanwhile, further testing the effect of targeting this amplication loop may drive the development of a new treatment for advanced stage prostate cancer patients. This work will be submitted as a research article shortly.

Statement of Contribution of Co-Authors for Thesis by Published Paper

The following is the format for the required declaration provided at the start of any thesis chapter which includes a co-authored publication.

The authors listed below have certified* that:

1 they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
2 they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3 there are no other authors of the publication according to these criteria;
4 potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit, and
5 they agree to the use of the publication in the student's thesis and its publication on the QUT ePrints database consistent with any limitations set by publisher requirements.

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Publication title and date of publication or status:
Adipocytes promote prostate cancer stem cell self-renewal through amplification of the cholecystokinin autocrine loop
Manuscript soon to be submitted

Contributor	Statement of contribution*
Kai Dun Tang	Conceived, designed and performed the experiments. Wrote the manuscript.
	
30.09.2015	
Ji Liu*	Contributed to data analysis and technical support.
Lidija Jovanovic*	Performed the cDNA microarray analysis.
Jiyuan An*	Contributed to the cDNA microarray data analysis.

Pamela J. Russell*	Provided feedback on the manuscript.
Judith A. Clements*	Provided feedback on the manuscript.
Ming-Tat Ling*	Conceived and designed the experiment. Edited the manuscript.

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

Ming Tat Ling		30-09-2015
Name	Signature	Date

4.1 Abstract

Obesity has long been linked with prostate cancer progression, although the underlying mechanism is still largely unknown. Here, we report that adipocytes promote the enrichment of prostate cancer stem cells (CSCs) through a vicious cycle of autocrine amplification. In the presence of adipocytes, prostate cancer cells actively secrete the peptide hormone cholecystokinin (CCK), which not only stimulates prostate CSC self-renewal, but also induces cathepsin B production of the adipocytes. In return, cathepsin B facilitates further CCK secretion by the cancer cells. More importantly, inactivation of CCK receptor not only suppresses cathepsin B secretion by the adipocytes, but also synergizes the inhibitory effect of cathepsin B inhibitor on adipocyte-promoted prostate CSC self-renewal. In summary, we have uncovered a novel mechanism underlying the mutual interplay between adipocytes and prostate CSCs, which may help explaining the role of adipocytes in prostate cancer progression and provide opportunities for effective intervention.

4.2 Introduction

Prostate cancer is the second most common cause of solid tumours in men worldwide [203]. Despite recent advances in the detection of early prostate cancer, there remains no effective therapy for patients with metastatic disease. The majority of patients with advanced disease will respond initially to androgen ablation therapy. However, 75-80% of them will go on to develop bone metastasis and once the tumor established in the bone, the disease is considered as incurable [2, 204, 205].

Tumor metastasis develops when cancer cells disseminate into the circulation, colonize secondary tissues and redevelop into bulk tumors [4]. Recent evidence supports the idea that tumor metastasis originates from a rare population of cancer cells known as cancer stem cells (CSCs). CSCs are characterized by their highly invasive characteristics and by their ability to self-renew and differentiate into heterogeneous lineages of cancer cells [16]. The stemness of CSCs is highly dependent on the presence of a stem cell niche. Recent studies suggest that CSCs from breast cancer are capable of creating their own niche by recruiting mesenchymal stem cells derived from bone marrow, resulting in expansion of the CSC population within the primary tumor [206]. The same process is suggested to occur during the development of bone metastasis, whereby disseminated prostate and breast tumor cells with CSC properties have been found to occupy and manipulate the hematopoietic stem cell niche in bone marrow [207, 208]. Therefore, identifying the key components of the CSC niche that support prostate cancer metastasis may offer opportunities for new treatment strategies.

Emerging data from recent studies support that adipocytes play a key role in prostate tumor metastasis. For example, obesity, which is associated with abnormal growth and functions of adipocytes, has been shown to correlate strongly with tumor metastasis in prostate cancer patients. Meanwhile, high-fat diet has also been consistently shown to promote the development of prostate tumor metastasis [209]. Furthermore, adipocytes isolated from periprostatic adipose tissues were found to induce invasiveness of prostate cancer cells [28]. Recently, bone marrow adipocytes have also been reported to stimulate prostate tumor growth in bone via a fatty acids binding protein 4 (FABP4)-dependent mechanism [26]. Considering that adipocyte lineage cells were found to stimulate follicular stem cell expansion [154], it is possible that adipocytes may promote prostate tumor metastasis, possibly by contributing to the formation of a CSC niche within the tumor microenvironment.

Here, we demonstrated for the first time the role of adipocytes in supporting self-renewal of prostate CSCs. We found that co-culturing of prostate cancer cells with adipocytes resulted in CSC enrichment, which was associated with upregulation of cholecystokinin (CCK), a peptide hormone regulating fat digestion and satiety. CCK not only functions as an autocrine factor to promote CSC self-renewal, but also acts as a paracrine factor on adipocyte to stimulate the secretion of the cysteine protease cathepsin B (CTSB). Surprisingly, CCK secretion by the cancer cells was found to be induced by CTSB, suggesting that CCK and CTSB contribute to an autocrine/paracrine amplification loop that mediates the mutual interplay between prostate CSCs and adipocytes.

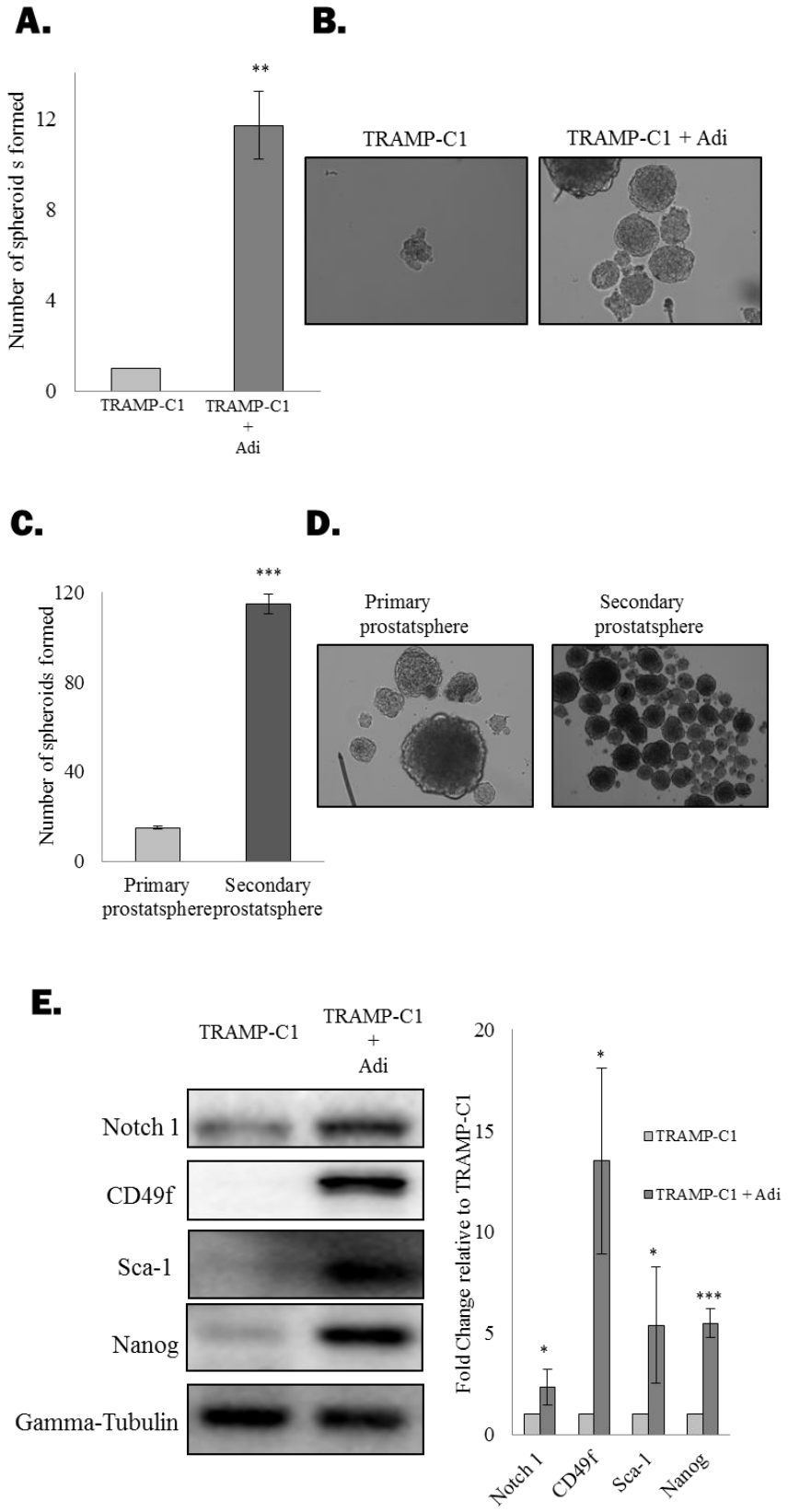
4.3 Results

4.3.1 Adipocytes promote prostate CSC self-renewal

In order to understand the mutual interplay between adipocytes and prostate CSCs population, mouse prostate cancer cell line (TRAMP-C1) was allowed to grow in a non-adherent condition in the presence or absence of adipocytes (derived from the 3T3-L1 pre-adipocyte cell line). Co-culturing with fully differentiated adipocytes strongly induced the self-renewal ability of the TRAMP-C1 cells, as evidenced by the drastic induction of spheroid formation (1 vs 12) under co-culture conditions (Fig. 4.1A&B). Moreover, secondary spheroid formation was also induced in the presence of adipocytes (Fig. 4.1C). Similarly, adipocytes also promoted spheroid formation of three other mouse cell lines TC1-T5, RM1 and RM1-BM (Suppl. Figure B.1). Apart from increasing the number of prostaspheres formed, the size of individual prostaspheres was also significantly increased by co-culturing with adipocytes (Figure 4.1B). Meanwhile, following dissociation of primary prostaspheres and subsequent reseeding in the presence of adipocytes resulted in significantly induced the formation of secondary prostaspheres (Figure 4.1C&D), further confirming the effect of adipocytes on prostate CSC self-renewal ability. Consistently, bone marrow derived adipocytes (OP9) also promoted the prostatsphere formation of TRAMP-C1 cells to a similar extent (Suppl. Figure B.2), suggesting that the CSC promoting effect may be common among adipocytes derived from different origins.

To validate our findings, western blotting and flow cytometry analysis of common CSC markers were performed. As shown in Figure 4.1E, protein expression of Notch1, CD49f, Sca-1 and Nanog in TRAMP-C1 cells was significantly upregulated after co-culturing with adipocytes. Furthermore, the percentage of cells expressing

the two putative CSC markers CD49f and Sca-1 was significantly upregulated after co-culturing with adipocytes (Figure 4.1F). These results confirm that adipocytes actively promote self-renewal of prostate CSCs, leading to subsequent expansion of CSC population.


A.
B.
**
Number of spheroid s formed
12
8
4
0
TRAMP-C1
TRAMP-C1 + Adi
TRAMP-C1
TRAMP-C1 + Adi
C.
D.
***
Number of spheroids formed
120
80
40
0
Primary prostatsphere
Secondary prostatsphere
Primary prostatsphere
Secondary prostatsphere
E.
TRAMP-C1
TRAMP-C1 + Adi
Notch 1
CD49f
Sca-1
Nanog
Gamma-Tubulin
Fold Change relative to TRAMP-C1
20
15
10
5
0
TRAMP-C1
TRAMP-C1 + Adi
*
*
*
***
Notch 1
CD49f
Sca-1
Nanog

F.

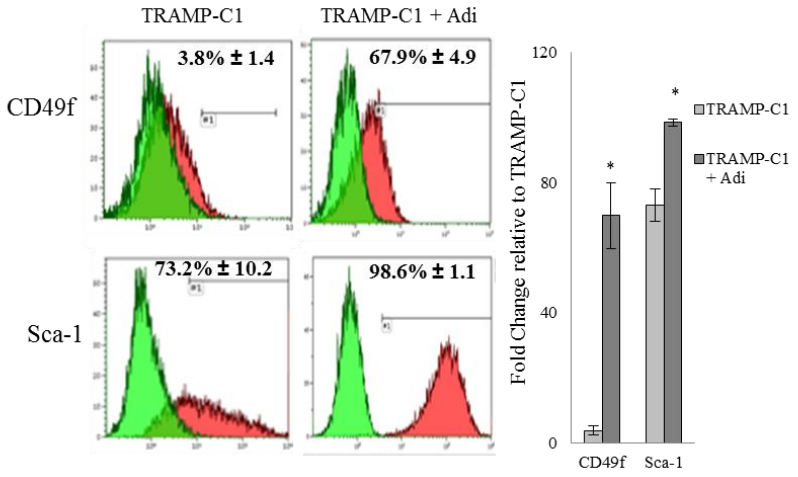
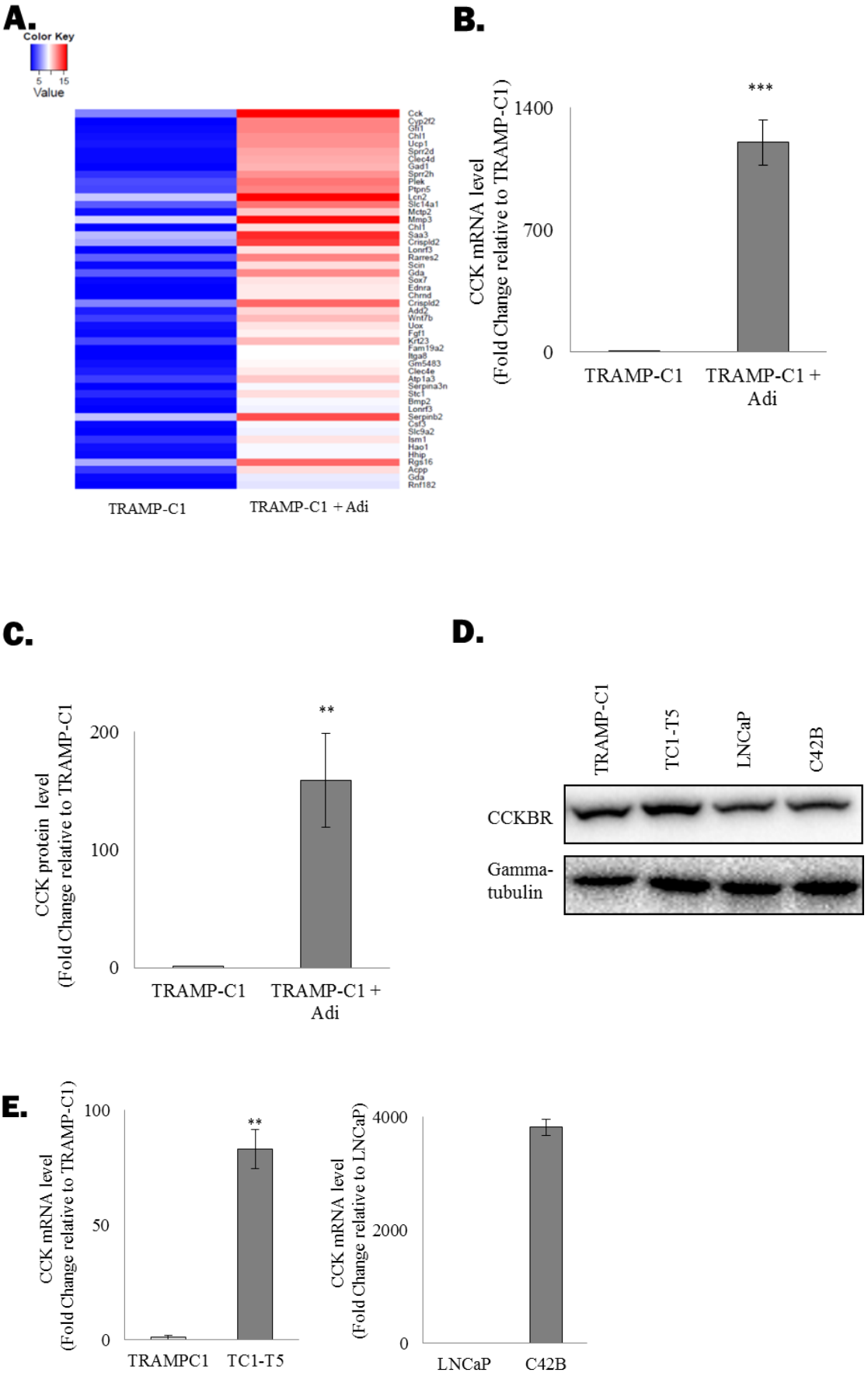


Figure 4.1: Adipocytes promote prostate CSC self-renewal. (A) Adipocytes stimulate formation of prostaspheres. TRAMP-C1 cells were seeded in ultra-low attachment plates in the presence or absence of 3T3-L1-derived adipocytes. After 7 days, prostaspheres formed were counted under the microscope. (B) Representative images of the prostaspheres. (C&D) Primary TRAMP-C1 prostasphere were dissociated and were allowed to grow as secondary prostaspheres in the presence of adipocytes. (E&F) Expression of CSC markers by TRAMP-C1 cells that grow alone or co-culturing with the adipcoytes were examined by western blotting (Notch 1, CD49f, Sca-1 and Nanog) and flow cytometry (CD49f and Sca-1) respectively. The results are presented as the mean ± SD from triplicate experiments in the bar chart (right panels). (p values: * < 0.05, ** < 0.005, *** < 0.0005).

4.3.2 Adipocytes induce the CCK autocrine loop in prostate CSCs

To understand the underlying mechanism that drive the active self-renewal of prostate CSCs under the co-culture conditions, cDNA microarray analysis was performed to compare the gene expression profile of TRAMP-C1 cells that grow alone or in the presence of adipocytes. As expected, expression of a number of known stemness factors such as ALDH1A1, GFI-1 or ITGA2 were highly induced in TRAMP-C1 cells in the presence of adipocytes (Figure 4.2A and Suppl. Figure B.3). However, the gene that showed the highest level of induction was found to encode the protein CCK (Suppl. Table B.2), a peptide hormone expresses mainly by the mucosal epithelium in response to consumption of high-fat diet. Subsequent analysis with RT-PCR and ELISA confirmed that both CCK mRNA and protein secretion were upregulated in prostate cancer cells in the presence of adipocytes (Figure 4.2B&C and Suppl. Figure B.4). Surprisingly, prostate cancer cell lines were found to actively expressing the CCK receptor CCKBR (Figure 4.2D). Since activation of CCKBR has recently been shown to promote the stemness of colon cancer cells [210], it is possible that the induction of CCK expression by adipocytes may lead to the activation of an autocrine loop and as a result promote CSC self-renewal. Indeed, while the level of CCK mRNA in both TRAMP-C1 and LNCaP cells was undetectable, the corresponding bone metastatic sublines (i.e. TC1-T5 and C42B), which are enriched with CSC population (Suppl. Figure B.5), were both found to actively express CCK transcript (Figure 4.2E). Meanwhile, treatment with human recombinant CCK not only induced the expression of CSC marker in TRAMP-C1 cells (Figure 4.2F), but also promotes the formation of prostasphere by the cells (Figure 4.2G), indicating the importance of CCK in regulating prostate CSC self-renewal. To confirm our findings, TRAMP-C1 cells were treated with a CCKBR

specific inhibitor (i.e. YM022). As shown in Figure 2H, inhibition of CCKBR was found to suppress CSC marker expression in TRAMP-C1 cells. More importantly, CCKBR inhibition was found to suppress the promoting effect of adipocytes on spheroid formation of the cancer cells (Figure 4.2I), supporting that adipocytes stimulate prostate CSC self-renewal by facilitating the activation of the CCK/CCKBR autocrine loop.


A.
Color Key
5 15
Value
Cck
Cyp2f2
Gfi1
Chil1
Ucp1
Sprr2d
Clec4d
Gad1
Sprr2h
Plek
Ptpn5
Lcn2
Slc14a1
Mctp2
Mmp3
Chil1
Saa3
Crispld2
Lonrf3
Rarres2
Scin
Gda
Sox7
Ednra
Chrnd
Crispld2
Add2
Wnt7b
Uox
Fgf1
Krt23
Fam19a2
Itga8
Gm5483
Clec4e
Atp1a3
Serpina3n
Stc1
Bmp2
Lonrf3
Serpinb2
Csf3
Slc9a2
Ism1
Hao1
Hhip
Rgs16
Acpp
Gda
Rnf182
TRAMP-C1
TRAMP-C1 + Adi
B.
***
1400
700
0
CCK mRNA level
(Fold Change relative to TRAMP-C1)
TRAMP-C1
TRAMP-C1 + Adi
C.
**
200
100
0
CCK protein level
(Fold Change relative to TRAMP-C1
TRAMP-C1
TRAMP-C1 + Adi
D.
TRAMP-C1
TC1-T5
LNCaP
C42B
CCKBR
Gamma-tubulin
E.
100
50
0
CCK mRNA level
(Fold Change relative to TRAMP-C1)
**
TRAMPC1
TC1-T5
4000
2000
0
CCK mRNA level
(Fold Change relative to LNCaP)
LNCaP
C42B

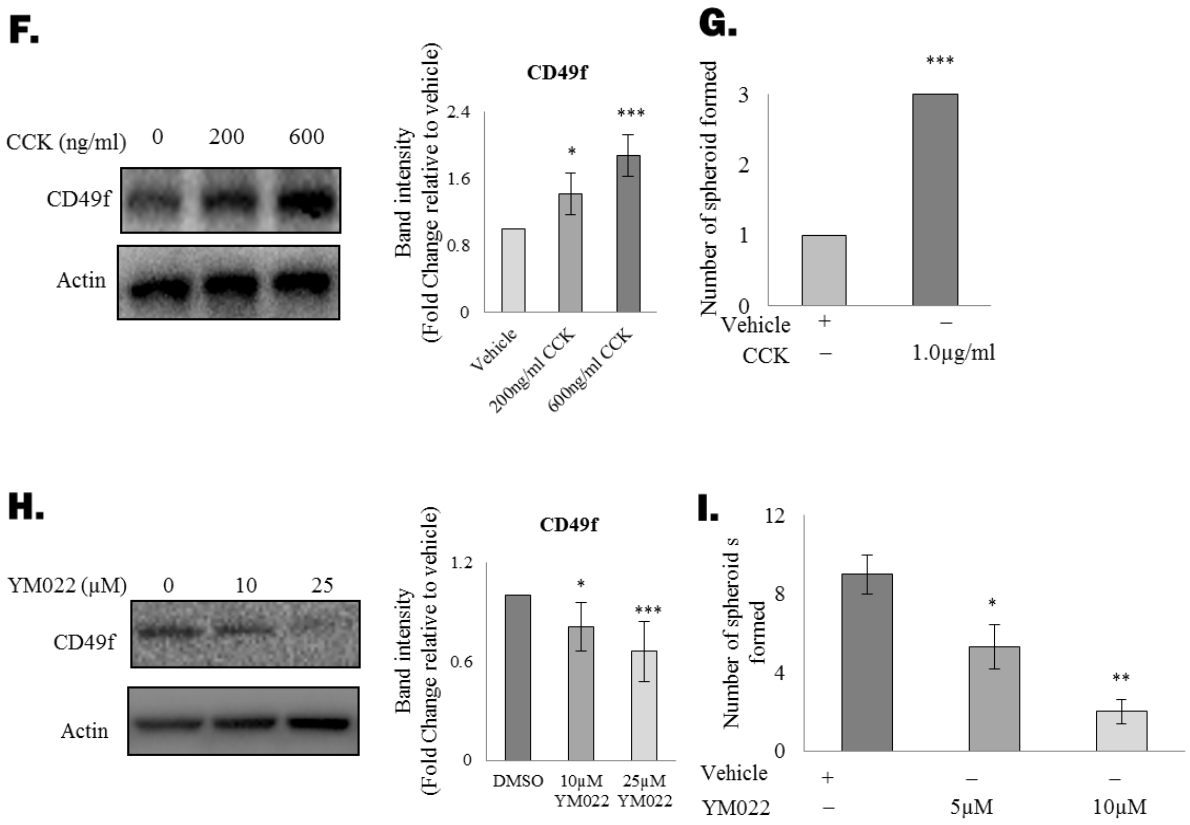


Figure 4.2: Adipocytes induce the cholecystokinin autocrine loop in prostate CSCs. (A) Genes upregulated in TRAMP-C1 cells after co-culturing with adipocytes were deteremined by cDNA microarray analysis and the top 50 genes were displayed as a heat map. (B) Upregulation of CCK mRNA in TRAMP-C1 cells under co-culture condition was validated with RT-PCR. (C) ELISA was performed to confirm the induction of CCK production in TRAMP-C1 cells after co-culturing with adipocytes. (D) CCKBR expression was analysed in mouse and human prostate cancer cell lines (TRAMP-C1, TC1-T5, LNCaP and C42B) using western blotting. (E) CCK is upregulated in metastatic prostate cancer cells. mRNA level of CCK in TRAMP-C1, LNCaP and their corresponding metastatic sublines (TC1-T5 and C42B) were analysed by RT-PCR. (F) Effect of CCK treatment on CSC marker expression in prostate cancer cells. TRAMP-C1 cells were treated with recombinant CCK for 48 hours and the expression of CD49f was examined using Western blotting

(left panel) and the band intensity was presented as in the bar chart (right panel). (G) CCK promotes prostasphere formation by TRAMP-C1 cells. TRAMP-C1 cells were seeded in an ultra-low attachment plate in the presence or absence of human recombinant CCK. After 7 days, prostaspheres formed were counted under the microscope. (H) Inhibition of CCKBR downregulated CSC marker expression (CD49f) in TRAMP-C1 cells. Cells treated with either the vehicle or YM022 were lysed and subjected to Western blot analysis antibodies against the indicated proteins (left panel) and the band intensity was presented as in the bar chart (right panel). (I) Inhibition of CCKBR abolishes the effect of adipocytes on prostasphere formation. TRAMP-C1 cells co-cultured with adipocytes were treated with different doses of the YM022 (5 and 10 μM) and the prostaspheres formed under each condition were counted under the microscope. All experiments were repeated at least three times, and the results are presented as the mean ± SD. (p values: * < 0.05, ** < 0.005, *** < 0.0005).

4.3.3 CCK stimulates CTSB secretion of the adipocytes

Since CCK has been shown to regulate the function of adipocytes through activation of the CCKBR, we therefore speculate that by actively secreting CCK, prostate CSCs may also influence the co-cultured adipocytes, possibly in a way that favour their self-renewal ability. Indeed, mass spectrometry analysis of the conditioned medium (CM) from adipocytes before and after co-cultured with prostate CSCs revealed that the cysteine protease CTSB, which plays an important role in the development and progression of prostate cancer, was among the proteins induced in adipocytes under the co-culture condition (Figure 4.3A). Subsequent analysis of the conditioned medium with ELISA further confirmed that CTSB secretion was upregulated in adipocytes that have been co-cultured with prostate CSCs (Figure 4.3B), although the mRNA level of CTSB was not induced under the same condition (Figure 4.3C). Similarly, CTSB secretion, but not the mRNA level (data not shown), was induced in adipocytes that were treated with recombinant CCK (Figure 4.3D). However, the effect of the recombinant CCK was abolished in the presence of the YM022 (Figure 4.3E), confirming that CCK induced CTSB through activation of the CCKBR.

A.

Accession No.	Name
P11499	Heat shock protein HSP 90-beta
P20029	78 kDa glucose-regulated protein
P08249	Malate dehydrogenase, mitochondrial
P09103	Protein disulfide-isomerase
P27773	Protein disulfide-isomerase A3
P16858	Glyceraldehyde-3-phosphate dehydrogenase
P10126	Elongation factor 1-alpha 1
Q9DBJ1	Phosphoglycerate mutase 1
P58252	Elongation factor 2
P06745	Glucose-6-phosphate isomerase
P10605	**Cathepsin B**
P14152	Malate dehydrogenase, cytoplasmic
P05202	Aspartate aminotransferase, mitochondrial
P22777	Plasminogen activator inhibitor 1
Q00493	Carboxypeptidase E
Q03350	Thrombospondin-2

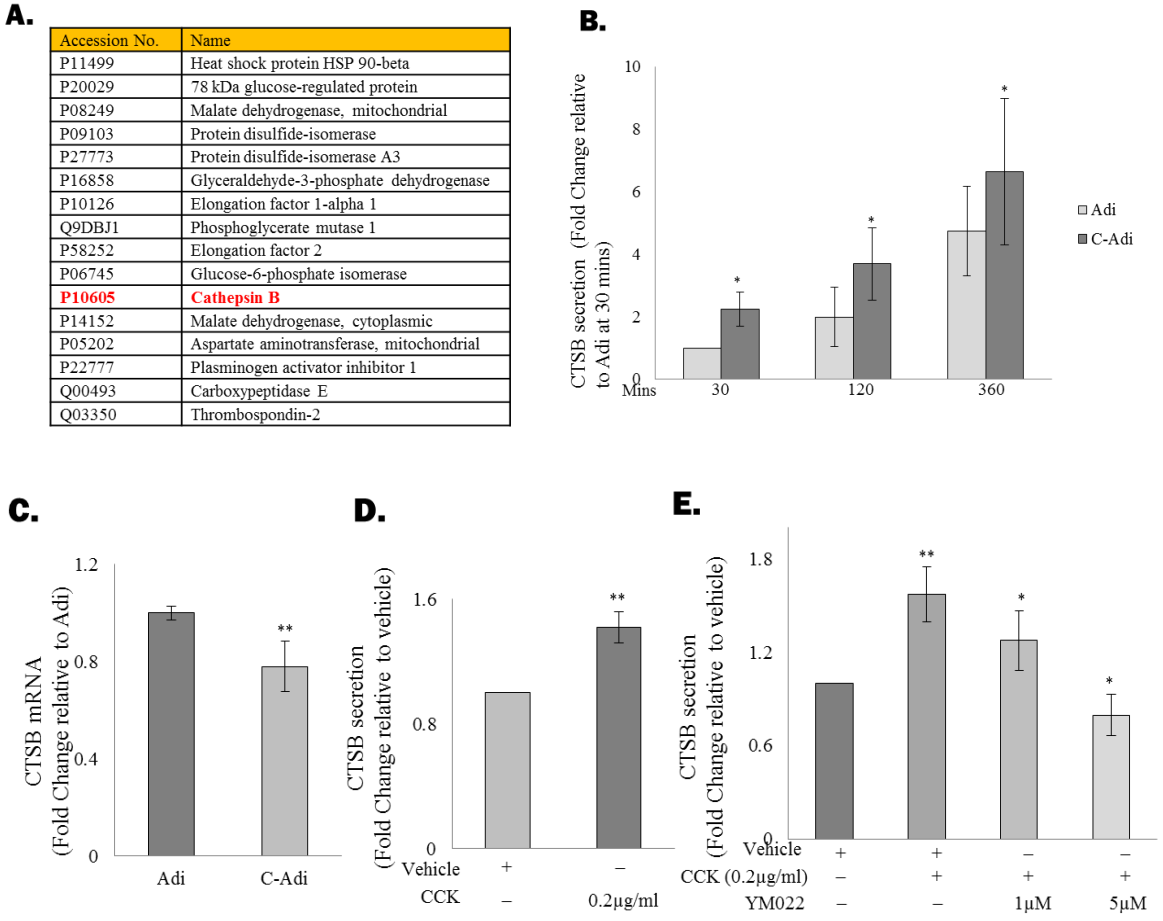


Figure 4.3: CCK stimulates CTSB secretion by the adipocytes. (A) Protein secretion profiles of 3T3-L1-derived adipocytes before or after being co-cultured with TRAMP-C1 cells was determined by mass spectrometry. Proteins upregulated under the co-culture condition were listed in Table A. (B&C) ELISA and RT-PCR were performed to validate the induction of CTSB expression in adipocytes after co-culturing with TRAMP-C1 cells. (D&E) Secretion of CTSB in adipocytes is regulated by CCK. (D) 3T3-L1-derived adipocytes were treated with CCK recombinant protein (0.2µg/ml) for 4 hours and the level of CTSB in the conditioned medium was analysed by ELISA. (E) Addition of YM022 (1 and 5 µM) completely abolished the effects of CCK on CTSB induction. Each experiment was repeated at

least three times, and the results are presented as the mean ± SD. (p values: * < 0.05, ** < 0.005).

4.3.4 CCK and CTSB contribute to an autocrine/paracrine amplication loop

One of the key functions of cathepsin family proteins is the regulation of peptide hormone release and activation [211]. For example, CSTB has been shown to promote the liberation of thorexine at the apical membrane [212]. Meanwhile, cathepsin L1 was also found to induce the processing of pro-CCK, leading to an increase in the secretion of the active CCK [213]. Although CTSB has not been reported to regulate CCK secretion, we found that treatment of TRAMP-C1 cells with recombinant CTSB resulted in significant upregulation of CCK level in the conditioned medium without affecting CCK mRNA levels (Figure 4.4A). Meanwhile, inhibition of CTSB significantly suppressed CCK production by the cancer cells, further supporting that CTSB regulates CCK secretion (Figure 4.4B). Since CCK is also capable of inducing CTSB production of adipocytes, we anticipated that this autocrine/paracrine amplification loop may play a key role in sustaining the adipocyte-promoted prostate CSC self-renewal. Consistent with our hypothesis, the promoting effect of adipocytes on prostasphere formation was significantly suppressed in the presence of CA-074ME (Figure 4.4C). Meanwhile, CA-074ME was found to significantly enhance the effect of YM022 against prostasphere formation (Figure 4.4D). These results clearly indicate the critical role of this novel autocrine/paracrine loop in mediating the effect of adipocytes on prostate CSC self-renewal as summarized in Figure 4.4E.

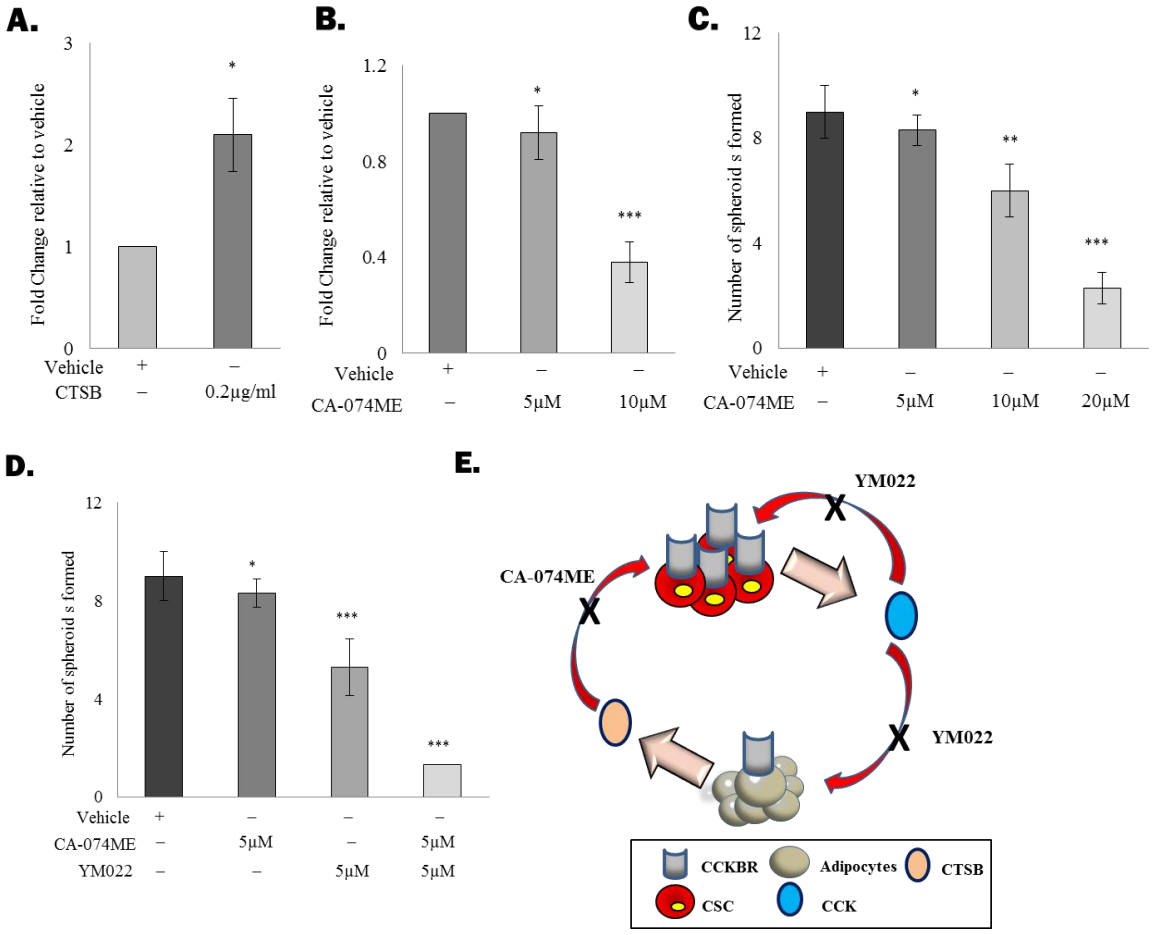


Figure 4.4: CCK and CTSB contributes to an autocrine/paracrine amplification loop. (A) CTSB regulates the secretion of CCK by prostate cancer cells. TC1-T5 cells were treated with CTSB recombinant protein (0.2µg/ml) for 4 hours and the level of CCK in the conditioned medium was determined by ELISA. (B) Inhibition of CTSB with CA-074ME (10µM) suppresses CCK secretion in TC1-T5 cells. (C) CTSB inhibition suppressed the promoting effect of adipocytes on prostasphere formation in a dose dependent manner. TRAMP-C1 cells co-cultured with 3T3-L1-derived adipocytes were treated with different dosage of CA0-74ME (5, 10 and 20µM). Prostaspheres were counted and imaged at day 7. (D) CTSB inactivation sensitizes prostate CSCs to YM022 treatment. TRAMP-C1 cells co-cultured with 3T3-L1-derived adipocytes were treated with the YM022 alone or in combination with CA-074ME. After 7 days, prostaspheres formed were counted and imaged

under the microscope. (E) Summary of the proposed role of adipocytes on prostate CSC maintenance. Each experiment was repeated at least three times, and the results are presented as the mean ± SD. (p values: * < 0.05, ** < 0.005, *** < 0.0005).

4.4 Discussion

The frequent invasion of local tumor into periprostatic adipose tissues and the metastasis of advanced tumors into the adipocyte-rich bone marrow clearly suggest the importance of adipose tissues during prostate cancer progression [20, 214]. What is not yet clear is how prostate cancer cells interact with the adipocytes to support their eventual expansion into metastatic tumor. Here, we report for the first time that adipocytes actively interplay with prostate CSCs through amplification of a novel CCK autocrine loop, leading to rapid enrichment of the CSC population.

Similar to normal stem cells, the stemness of prostate CSCs is highly dependent on the presence of a stem cell niche [17]. The stromal cells within the primary prostate tumor appear to play a key role in promoting the tumorigenicity of the prostate cancer cells, as evidenced by the ability of cancer associated fibroblasts in transforming normal prostate epithelial cells [215]. Similarly, our finding that adipocytes actively stimulate prostate CSC self-renewal support the notion that adipose tissues may contribute to a metastatic niche necessary for the maintenance of prostate CSCs. Since obesity is associated with adipocyte dysfunction and abnormal adiposity, the resulting enrichment of the metastatic niches (i.e. the periprostatic adipose tissues and bone marrow fat) may in turn contribute to the frequent tumor metastasis and poor prognosis observed in obese prostate cancer patients [209].

The significant upregulation of CCK mRNA in prostate cancer cells upon co-cultured with adipocytes suggests that CCK is playing an important role in mediating the effect of adipocytes on CSC self-renewal. Expression of CCK was believed to be restricted to the enteroendocrine cells and to specialized neurons within the brain

[216, 217]. It is therefore surprising that CCK and its receptor were both found to be expressed by prostate cancer cells. Our study therefore represents the first to discover the autocrine function of CCK in the maintenance of prostate CSCs. Exactly how CCK promotes the stemness of prostate CSCs is still unclear, although the binding and activation of CCKBR by gastrin, a hormone peptide structurally related to CCK, was found to regulate the proliferation of normal and malignant colon stem cells through suppression of both BMP-2 and Id4 expression [210]. Since CCK also binds to and activates CCKBR, it is possible that CCK may act through the same downstream m signalling pathway.

Dietary fat is the major risk factor not only for obesity, but also for prostate cancer progression [218, 219]. Intake of dietary fat is known to induce a rapid upregulation of CCK expression by the enteroendocrine cells within the intestine [220]. Therefore, long term high-fat diet consumption is expected to result in chronic elevation of serum CCK level. Indeed, in a recent study, mice that consumed a high-fat diet were found to have serum CCK level ten folds higher than that of the control group [221]. In pancreatic cancer, CCK expression was also found to be associated with larger tumors and a higher incidence of metastasis [221, 222]. Considering that prostate cancer cells also express CCKBR and respond to CCK stimulation, the effects of a high-fat diet on prostate tumor progression reported in a previous study [223] may well be a consequence of CCK elevation. Therefore, exposing the cancer cells to high lipid content (i.e. high-fat diet or co-culturing with adipocytes) may promote the CCK-autocrine loop and further driven the expansion of prostate CSCs.

It is worth noting that CCK not only regulates fat and protein digestion, but also controls the level of satiety. In an animal study, systemic administration of CCK was found to induce anorexia [224]. Meanwhile, serum CCK level was found to be significantly higher in older individuals [225], and is suggested to contribute to aging-associated anorexia. Interestingly, according to previous studies, CCK and leptin worked synergistically in reducing the short-term food intake [226] through activation of Src/PI3K and JAK2/PI3K/STAT3 signaling pathway respectively [227]. Since leptin is an adipokine that actively secreted by adipocytes [152], it is possible that CCK secreted by cancer cells may work synergistically with leptin in the development of anorexia commonly found in advanced stage prostate cancer patients, although further examination of serum CCK and leptin levels in prostate cancer patients are necessary to confirm this hypothesis.

Our findings suggest that the secretion of CCK by prostate CSCs may also play roles in modulating the function of adipocytes, possibly in a way that further facilitates CSC expansion. One of the key pieces of evidence is the stimulation of CTSB secretion by the adipocytes, which in turn further promotes the secretion of CCK by the cancer cells. Our discovery of this seemingly paracrine/autocrine amplification loop may help in explaining the mutual interplay between prostate cancer cells and adipocytes. More importantly, despite the presence of adipocytes, disruption of this loop by means of inactivating either CCK receptor or CTSB can significantly inhibit prostate CSC expansion, clearly demonstrating the therapeutic potential of this paracrine/autocrine amplification loop for targeting CSC population.

In summary, we have identified a novel mechanism that mediates the interaction between adipocytes and prostate cancer cells, which may offer opportunities for the development of new treatments against metastatic prostate cancer.

4.5 Materials and Methods

Spheroid formation assay

The spheroid formation assay was modified from a previously reported protocol [201, 228]. Briefly, cells were harvested and counted using a Scepter™ Automated Cell Counter (Millipore, Billerica, MA, USA). Four hundred cells were added to each well of a 24-well ultra-low attachment plate (Sigma-Aldrich, St. Louis, MO, USA). Adipocytes differentiated from the 3T3-L1 cells were then added into a cell culture insert (0.4 μM) (Millipore) placed inside the well. Cells were grown in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10 μg/mL insulin (Sigma-Aldrich), B27 (Invitrogen), 80 ng/mL EGF (Sigma-Aldrich) and 40 ng/mL basic FGF (Invitrogen). The number of spheroid formed was counted at Day 7 or at the end of the treatment. Each experiment was performed in triplicate and was repeated at least 3 times, with each data point represents the mean and SD. Statistical difference was determined by Student's t-test and was considered as significant if $p < 0.05$.

Western blotting

Experimental procedures have been described in our previous studies [197]. Firstly, cell pellets were collected and lysed with lysis buffer (Cell Signalling Technology, Danvers, MA, USA) containing 100 μM phenylmethylsulfonyl fluoride

(PMSF; Sigma-Aldrich). The cell lysates were quantified using the Pierce™ BCAProtein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA) before loading onto a SDS-polyacrylamide gel. The resolved proteins were then transferred onto a PVDF membrane (Millipore), subsequently blocked the membrane with 10% skim milk in TBST-T buffet at room temperature. After the blocking, the membrane was probed with the indicated antibody for 1 hour at room temperature prior to being washed with TBS-T buffer. The membrane was then incubated with the corresponding secondary antibodies for another hour at room temperature. After washing with TBS-T buffer, the membrane was incubated with Immobilon Western Chemiluminescent HRP Substrate (Millipore), and the signals were visualized and quantified using a Bio-Rad ChemiDoc™ XRS Gel Documentation System.

Flow cytometry analysis

Cells were collected, washed twice with phosphate-buffered saline (PBS) and subsequently resuspended in 50 μl of fluorescence-activated cell sorting (FACS) buffer (0.02% sodium azide and 2% FBS in PBS) before incubating with the fluorescent dye-conjugated antibodies at 4°C in the dark for 30 minutes. After incubation, the cells were washed twice with PBS and resuspended in 200 μl of FACS buffer. Fluorescent signal was determined with the BD™ LSR II and the results were analysed using the KALUZA software.

Microarray analysis

TRAMP-C1 cells that were grown alone or co-cultured with adipocyte were lysed for RNA extraction with the RNeasy Mini Kit (Qiagen, Germantown, MD, USA). The resulting RNA was used for the generation of labeled cDNA based on the protocol

described earlier [202]. cDNA was probed against the Mouse GE 44K v2 Microarray G2519F-026655 (Agilent Technologies, Santa Clara, CA, USA) with the standard procedures described by the manufacturer, and the signals were read by the Agilent scanner and analysed with the Genespring software.

Real Time- Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA) and 2 μg of the resulting RNA was used to synthesize cDNA using the SuperScript® III First-Strand Synthesis Systems (Invitrogen). RT-PCR was carried out with the ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Sense and anti-sense primers specific for the genes of interest are listed in Suppl. Table B.1. The transcript level of ribosomal protein L32 (RPL32) was used as an internal control. To quantify CCK transcript level in human prostate cancer cell lines, copy number of CCK mRNA was calculated with a standard curve.

Liquid chromatography–Mass Spectrometry (LC-MS/MS)

Concentrated conditioned media (from adipocytes before and after co-culturing with TRAMP-C1 prostasphere) was quantitated by Pierce™ BCA Protein Assay Kit before being separated on a SDS-PAGE gel and visualized by colloidal coomassie. Protein gel slices (1 mm) were excised using a clean gel cutter (The Gel Company San Francisco, CA,USA), transferred to 96-well U bottom plate containing a solution of 50% acetonitrile: 25mM NH4HCO3 for destaining. Samples were reduced with 20mM DTT followed by alkylation with 50mM IAA. The samples were equilibrated to pH [229].

4.6 Supplementary Tables and Figures

Suppl. Table B.1: List of the primers used in this study.

Primers	Sequences
MANG-1F	TGCCTACACTTTCATTCTTCCA
MANG-1R	GACTGGTTCCTATCTCAAGCA
MALDH1A1F	CAGCTAGCAGGTACTTCTGG
MALDH1A1R	CCGATTACTGCAATCTTCATGG
MCCKF	TTTAAGAGCAGTCACCCTCCC
MCCKR	CTAGGACTGCCATCACCACG
HCCKF	GGTACTCATACTCCTCGGCA
HCCKR	TGGCAAGATACATCCAGCAG
MDKK2F	AAACTCAACTCCATCAAGTCCT
MDKK2R	CTTCACATTCCTTATCACTGCTG
MGFI1F	ATCAAATGCAGCAAGGTCTTCTC
MGFI1R	TCCGAGTGAATGAGCAGATGTG
MITGA2F	CCCAGAGCACTTTAGATTCCC
MITGA2R	GTGAACCAACCAGTAGCCAG
MITGA6F	TACAGCCTTCAACCTGGACAC
MITGA6R	CATCCACTGGTCTTCCTTGCT
MPTHIHF	GGTATTCCTGCTCAGCTACTC
MPTHIHR	GTATCTGCCCTCATCGTCTG
MSTC1F	AAAGCCACAACTTAGCGGA
MSTC1R	ACAAATGTCGTACATCCCATCTG

Suppl. Table B.2: Top-Ten most upregulated genes in TRAMP-C1 after co-culture with inserts containing adipocytes

Primary Accession	Gene Symbol	Description	Fold Change increased
NM_031161	Cck	Mus musculus cholecystokinin (Cck), mRNA	1604
NM_007817	Cyp2f2	Mus musculus cytochrome P450, family 2, subfamily f, polypeptide 2 (Cyp2f2), mRNA	1551
NM_010278	Gfi1	Mus musculus growth factor independent 1 (Gfi1), mRNA	1304
NM_007697	Chl1	Mus musculus cell adhesion molecule with homology to L1CAM (Chl1), mRNA	907
NM_009463	Ucp1	Mus musculus uncoupling protein 1 (mitochondrial, proton carrier) (Ucp1), nuclear gene encoding mitochondrial protein, mRNA	803
NM_011470	Sprr2d	Mus musculus small proline-rich protein 2D (Sprr2d), mRNA	689
NM_010819	Clec4d	Mus musculus C-type lectin domain family 4, member d (Clec4d), transcript variant 1, mRNA	610
NM_008077	Gad1	Mus musculus glutamic acid decarboxylase 1 (Gad1), mRNA	566
NM_011474	Sprr2h	Mus musculus small proline-rich protein 2H (Sprr2h), mRNA	458
NM_019549	Plek	Mus musculus pleckstrin (Plek), mRNA	445

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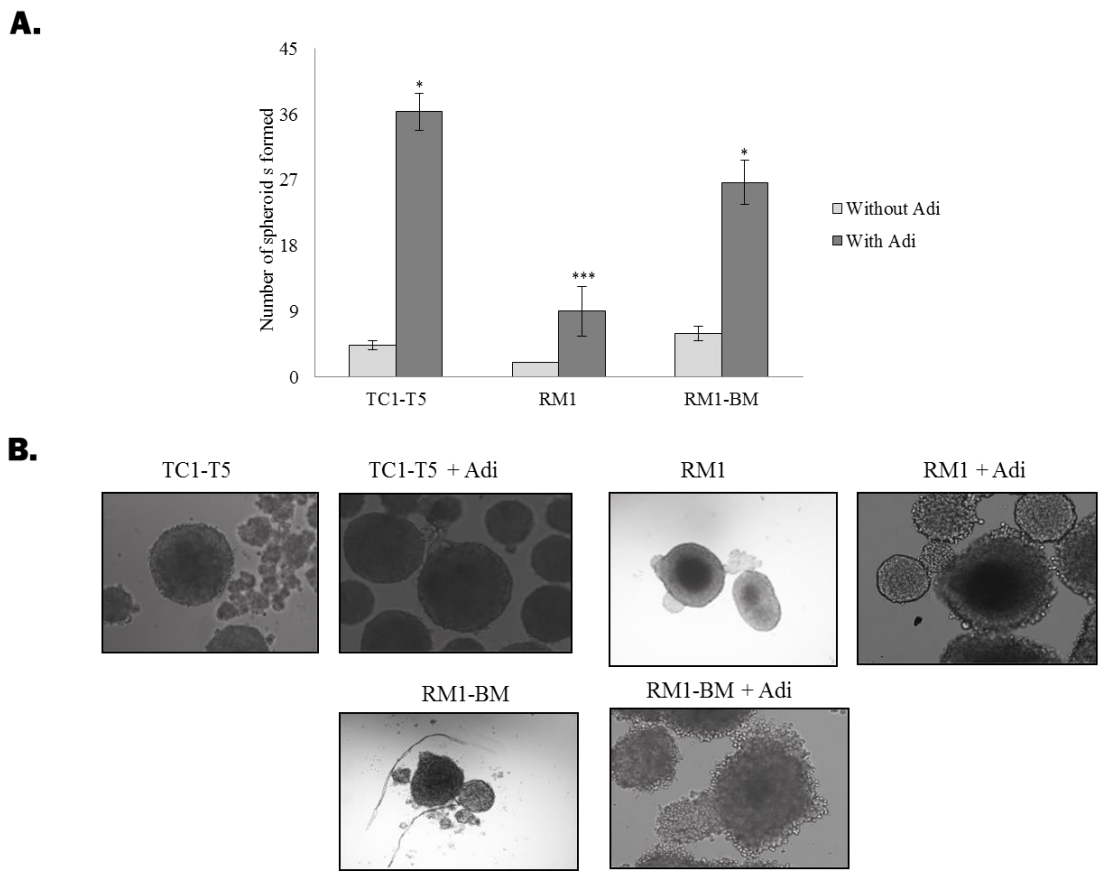
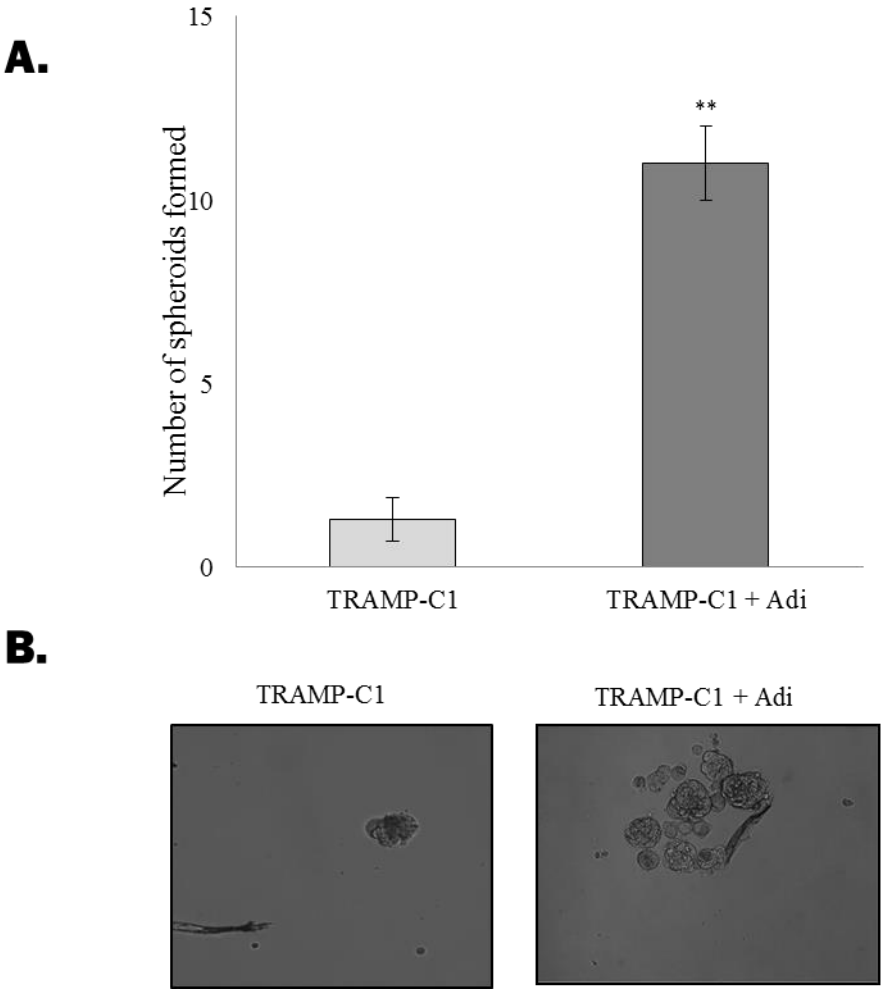
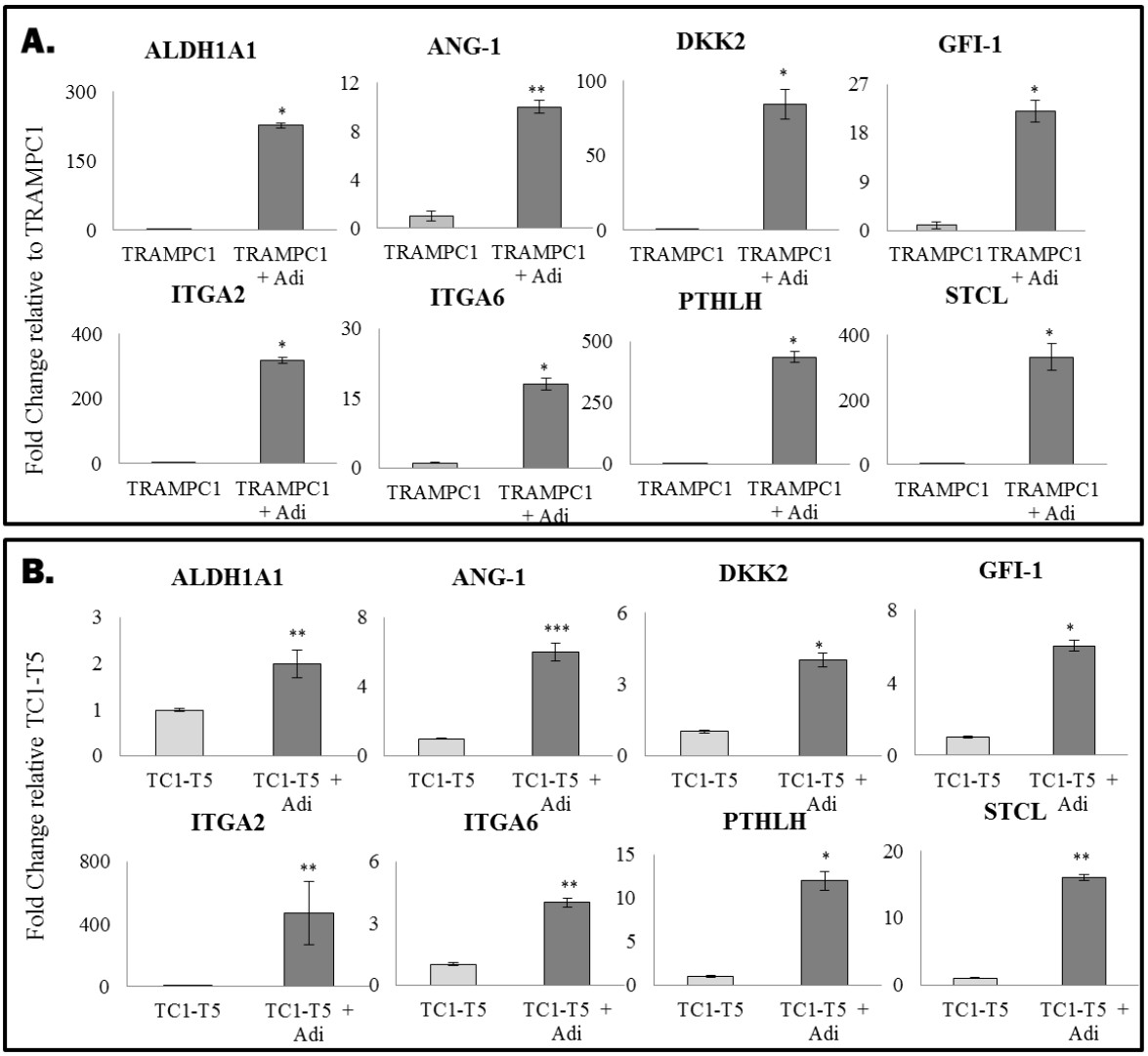


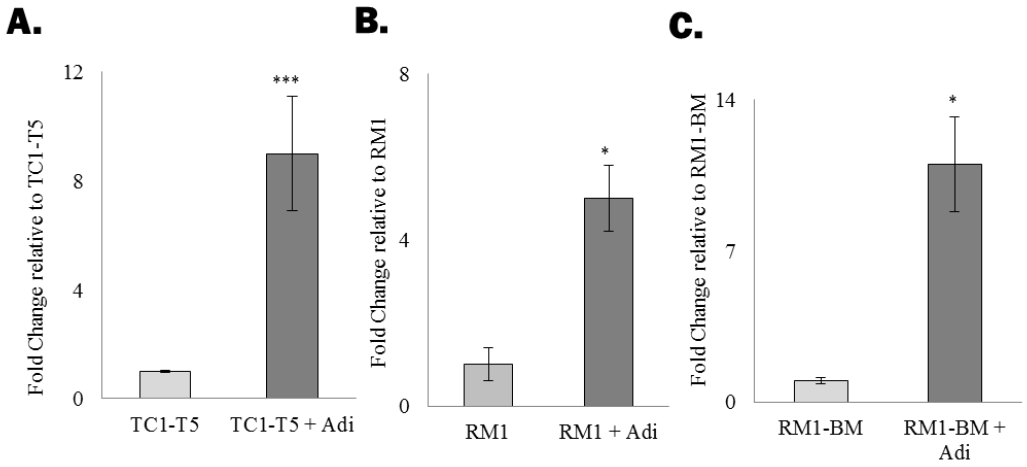
Suppl. Figure B.1: Effect of adipocytes on self-renewal of additional mouse prostate cancer cell lines. (A&B) Prostasphere formation assay was performed with different mouse prostate cancer cell lines (TC1-T5, RM1 and RM1BM) in the presence or absence of 3T3-L1-derived adipocytes, grown in inserts. Each experiment was repeated at least three times, and the results are presented as the mean ± SD. (p values: * < 0.05, *** < 0.0005).



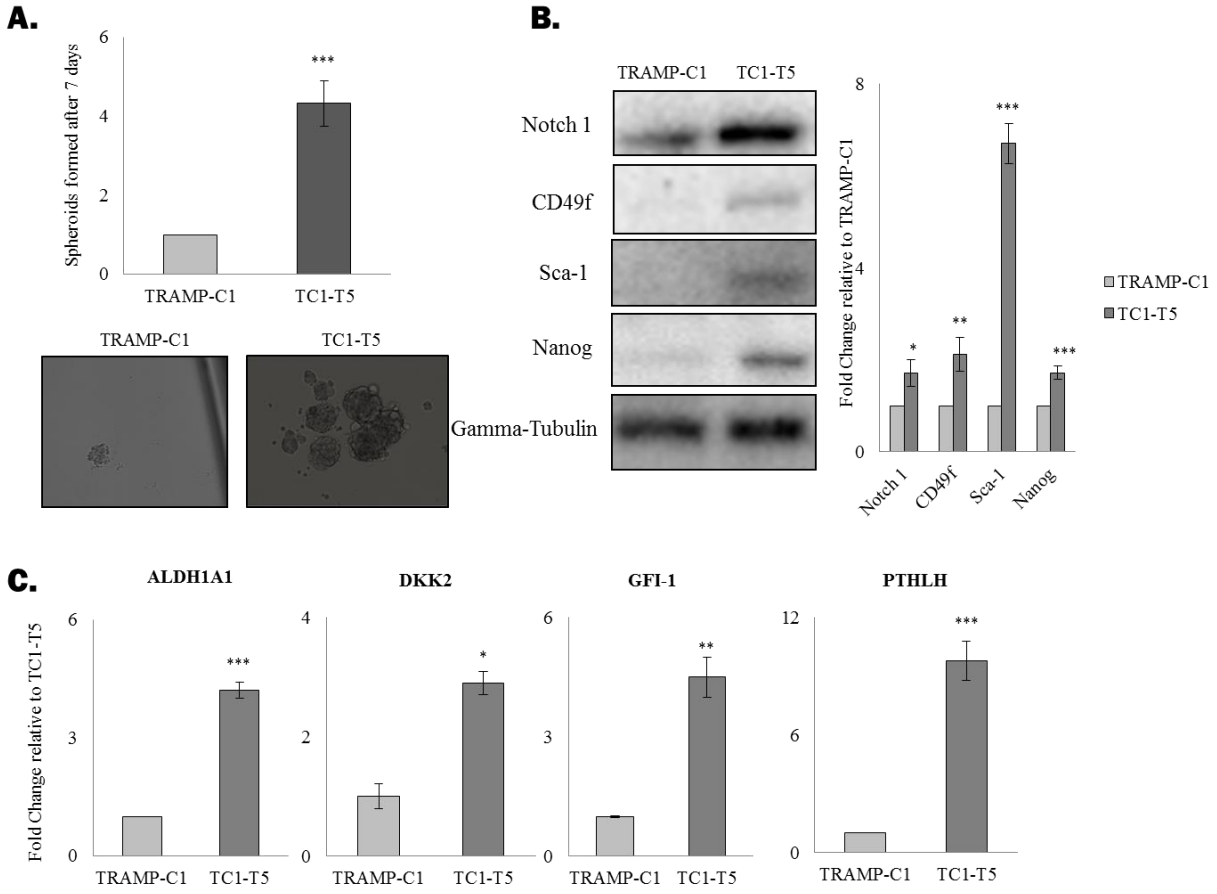
Suppl. Figure B.2: Bone marrow-derived adipocytes promote prostate CSC self-renewal. (A&B) TRAMP-C1 cells were seeded in ultra-low attachment plate in the presence or absence of adipocytes derived from the bone marrow stromal cell line OP9. After 7 days, prostaspheres formed were counted and imaged under the microscope. The results are presented as the mean ± SD from triplicate experiments. (p values: ** < 0.005).



Suppl. Figure B.3: CSC markers are upregulated in mouse prostate cancer cells that co-cultured with adipocytes. RT-PCR analysis of CSC markers (ALDH1A1, ANG-1, DKK2, GFI-1, ITGA2, ITGA6, PTHLH and STCL) mRNA level in (A) TRAMP-C1 and (B) TC1-T5 cells that grown alone or co-cultured with adipocytes. The results are presented as the mean ± SD from triplicate experiments. (p values: * < 0.05, ** < 0.005, *** < 0.0005).



Suppl. Figure B.4: CCK mRNA level is upregulated in mouse prostate cancer cells that co-cultured with adipocytes. RT-PCR analysis of CCK mRNA level in (A) TC1-T5, (B) RM1 and (C) RM1-BM cells that grow alone or co-cultured with adipocytes. The results are presented as the mean ± SD from triplicate experiments. (p values: ** < 0.005).



Suppl. Figure B.5: Enrichment of CSCs in the bone metastatic cell lines TC1-T5. (A) Self-renewal ability of TRAMP-C1 and its bone metastatic derivative TC1-T5 were examined by prostasphere formation assay. Prostaspheres formed at day 7 were counted and imaged under a microscope. (B) Western blotting was performed to examine the level of CSC markers expressed in the two cell lines. (C) mRNA level of stem cell transcription factors (ALDH1A1, DKK2, GFI-1 and PTHLH) were analyzed with RT-PCR. Each experiment was repeated at least three times, and the results are presented as the mean ± SD. (p values: * < 0.05, ** < 0.005, *** < 0.0005).

4.7 Supplementary Materials and Methods

Cell lines and culture conditions

Mouse prostate cancer cell line TRAMP-C1 was obtained from ATCC (Rockville, MD, USA) and was maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% (wt/vol) penicillin-streptomycin P/S, Invitrogen); whereas TC1-T5 was established as described in a previous study [230]. RM1 and RM1-BM were generous gifts from Dr Carl Power (University of New South Wales) and these cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) (DMEM containing 10% FBS, 1% P/S). Mouse pre-adipocyte (3T3-L1) and bone marrow stromal cell lines (OP9) were obtained from ATCC and were maintained in DMEM medium containing 10% FBS and 1% P/S and Minimum Essential Medium (MEM) α (Invitrogen) containing20% FBS, 1% P/S respectively. Human prostate cancer cell line LNCaP was obtained from ATCC and was maintained in RPMI 1640 medium supplemented with 5% FBS and 1% P/S. C42B was kindly provided by Prof Leland Chung (Cedars-Sinai Medical Center) and was maintained in T-Medium (Invitrogen) supplemented with 5% FBS and 2% P/S. All cell lines were kept at 37^0C in a 5% CO2 environment.

Antibodies and reagents

Human recombinant CCK protein was purchased from Tocris Biosciences (Bristol, United Kingdom). Human and mouse recombinant CTSB were purchased from R&D Systems (Minneapolis, MN, USA). CCKBR (YM022) and CTSB inhibitor (CA-074ME) were purchased from Tocris Biosciences and were dissolved in DMSO.

The antibodies against Notch 1, CD49f (Cell Signalling Technology, Danvers, MA, USA), Sca-1 (R&D Systems), CCKBR (for Immunohistochemistry, Atlas Antibody, Stockholm, Sweden), Gama-tubulin (Sigma-Aldrich, St. Louis, MO, USA), Nanog, CCKBR and actin (Santa Cruz Biotechnology, Dallas, TX, USA) were used in this study. Phycoerythrin (PE) conjugated Sca-1 antibody and 647 conjugated human CD49f were both purchased from BD Biosciences (San Jose, CA, USA).

Adipocyte differentiation

To obtain fully differentiated adipocytes, 3T3-L1 and OP9 cells were seeded into a 6-well plate at a confluency of 80%. Differentiation was induced with StemPro® Adipogenesis Differentiation Kit (Invirtrogen) by following the manufacturer's instructions. Adipocyte differentiation medium was changed every 3 days, and the fully differentiated adipocytes were collected 9 days after the induction.

Chapter 5: Conclusions and Future Work

Prostate cancer preferentially metastasizes to bone, which accounts for its high mortality. Considerable effort has been expended in attempting to understand the mechanisms underlying prostate tumor bone metastasis; however, little is known about how these cells disseminate into the circulation and colonize within bone. There is ample evidence to suggest that CSCs, which share many similar characteristics with HSCs, may play an important role in the development of prostate tumor bone metastasis and treatment resistance. Apart from being capable of self-renewing, CSCs can also enter a quiescent state, thereby protecting themselves from conventional therapies like chemotherapy and radiotherapy. Therefore, targeting CSCs may offer new opportunities for improving the treatment outcome of patient with advanced stage prostate cancer.

Previously, CSCs were found to hijack the bone marrow HSC niche to support their survival and growth. There are various cell types that reside within the bone marrow, with osteoblasts and adipocytes comprising the two major cellular components. Although both cell types have been suggested to play roles in prostate cancer progression, the underlying mechanisms remain largely unknown.

Since osteoblasts actively secrete the cytokine Ang-1, the discovery in the first part of my project that prostate CSCs express the Tie-2 protein, a membrane receptor tyrosine kinase activated by Ang-1, represents a novel finding which may help in understanding how osteoblasts regulate prostate CSC maintenance. Moreover, I found that Tie-2 was highly expressed in more metastatic and aggressive human

prostate cancer cell lines. Apart from that, I found that Tie-2 receptors not only were expressed in PC-3 xenograft tumor tissue that grew within a humanized bone micro-environment in NOD-SCID mice, but also in human prostate tumors that metastasized to the bone, indicating the importance of Tie-2 in the development of prostate tumor bone metastasis.

Next, I have further identified the role of Tie-2 in prostate CSCs maintenance. According to previous studies, Ang-1/Tie-2 was found to regulate the stemness and quiescence state of HSCs. Consistent with these findings, I discovered that Tie-2^{High} prostate cancer cells not only express higher level of quiescent and stem cell markers, but are also more resistant to the chemodrug, cabazitaxel. Meanwhile, my finding suggests that the Ang-1/Tie-2 signalling pathway regulates prostate CSC maintenance through activation of the downstream pathway, Akt. Since PI3K/Akt is one of the well-known signalling pathways that takes part in regulating both HSC and CSC maintanence, my findings further support the importance of Ang-1/Tie-2 in regulating the stemness and quiescence of prostate CSCs.

Over the past decades, the Ang-1 protein secreted by cancer cells was regarded as a paracrine factor that mediates tumor angiogenesis. My work suggests that Ang-1 also functions as an autocrine factor and thus, targeting this loop has the potential to eliminate the CSC population. My discovery is expected to open a new research paradigm on the functional role and therapeutic potential of the Ang-1/Tie-2 autocrine loop.

Consistent with previous studies, Tie-2^{High} prostate cancer cells were more adherent to osteoblast and endothelial cells, suggesting that Ang-1/Tie-2 signalling pathway may play roles in intravasation or extravasation of tumor cells during the development of prostate tumor bone metastasis. Indeed, I have demonstrated that Tie-2^{High} prostate cancer cells, but not the Tie-2^{Low} population, were able to form metastatic tumors (2 bone metastasis and 1 kidney metastasis). In this study, therefore I provide solid data to support the critical role of Tie-2 in prostate tumor metastasis. I believe that the present study not only describes a major breakthrough in understanding the mechanism underlying tumor metastasis, but also highlights the therapeutic potential of inactivating the Ang-1/Tie-2 pathway in the treatment of prostate cancer.

To further confirm the function of Tie-2 in bone metastasis, an ideal approach is to inject the Tie-2 overexpressing DU145 cells into the NOD-SCID mice and investigate whether the ectopic Tie-2 expression promote bone metastasis of the DU145 cells. Meanwhile, since a specific inhibitor against Tie-2 is currently undergoing Phase II clinical trial, it will be timely to validate whether a Tie-2 inhibitor can suppress prostate tumor bone metastasis in an *in vivo* animal model that have been developed as mentioned in Chapter 3. Furthermore, since, Tie-2^{High} prostate cells were found to be more metastatic, examination of Tie-2 expression in circulating tumor cells (CTCs), which disseminate into the blood stream and subsequently developed as metastatic tumors, may help to predict the development of bone metastasis.

Adipocytes within the periprostatic tissue or inside the bone marrow have both been suggested to play roles in prostate cancer development and progression; however, it is not clear if adipocytes regulate prostate CSC self-renewal and homing. In the second part of my project, I have confirmed the effect of adipocytes on prostate CSC self-renewal, as evidenced by induction of prostasphere formation after co-culturing with adipocytes using a 3D spheroid co-culture model.

Most importantly, I have identified a novel CCK/CCKBR autocrine loop in prostate CSCs that can be triggered by adipocytes. I found that CCK expression was induced in prostaspheres after co-culturing with adipocytes. Meanwhile, both mouse and human bone metastatic prostate cell lines were found to express higher level of CCK when compared to their non-metastatic parental lines, further supporting the association between CCK and tumor metastasis. Moreover, the CCK receptor, CCKBR was found to be expressed in human prostate cancer bone metastasis tissue sections. Besides that, there are already have the data to support the role of CCK/CCKBR in CSC self-renewal in gastric and pancreatic cancer. In this study, I have provided solid data to confirm the role of this pathway in regulating prostate CSCs. Since targeting this loop with a specific inhibitor can eliminate the effect of adipocytes on CSC expansion, my finding is expected to drive the development of a new treatment against metastatic prostate cancer.

Cell-cell comunication is one of the hottest topics over the past decades. In this study, I have demonstrated for the very first time how adipocytes promote prostate CSC self-renewal through a vicious cycle of CCK and CSTB secretion. Our results suggest that CSTB, which is a well known CSC factor, can be secreted by adipocytes

and its secretion from adipocytes can be triggered by CCK from the CSC population. In return, CSTB was found to induce CCK secretion by the CSC population, supporting that both CCK and CTSB contribute to an autocrine/paracrine amplification loop and may explain how the crosstalk occurred between adipocytes and CSC population. Furthermore, the combination of CCKBR and CTSB inhibitors contributed to a synergistic effect on elimination of the CSC population, further confirming the importance of both CCK and CTSB in regulating CSC self-renewal.

Although I have identified crosstalk between adipocytes and cancer cells using a 3D co-culture model, further investigation with both CCK and CTSB inhibitors in a mouse xenograft model is crucial to demonstrate the therapeutic potential of these inhibitors against metastatic prostate cancer. Thus, future work can focus on testing the combined effect of CCK and CTSB inactivation against tumor metastasis with in vivo prostate xenograft models. Apart from the therapeutic prospective, CCK and CTSB are secretory proteins that can be found in human serum and thus can serve as prognostic markers for predicting the development of bone metastasis in patients with advanced prostate cancer.

Overall, this project has successfully uncovered new roles and mechanisms of action of both osteoblasts and adipocytes in prostate CSC self-renewal and the development of bone metastasis as summarized in Figure 5.1. Therefore, further preclinical studies that target these key signalling pathways (i.e. Ang-1/Tie-2 and CCK/CCKBR) identified in my study may result in the development of effective treatments against this most deadly form of prostate cancer.

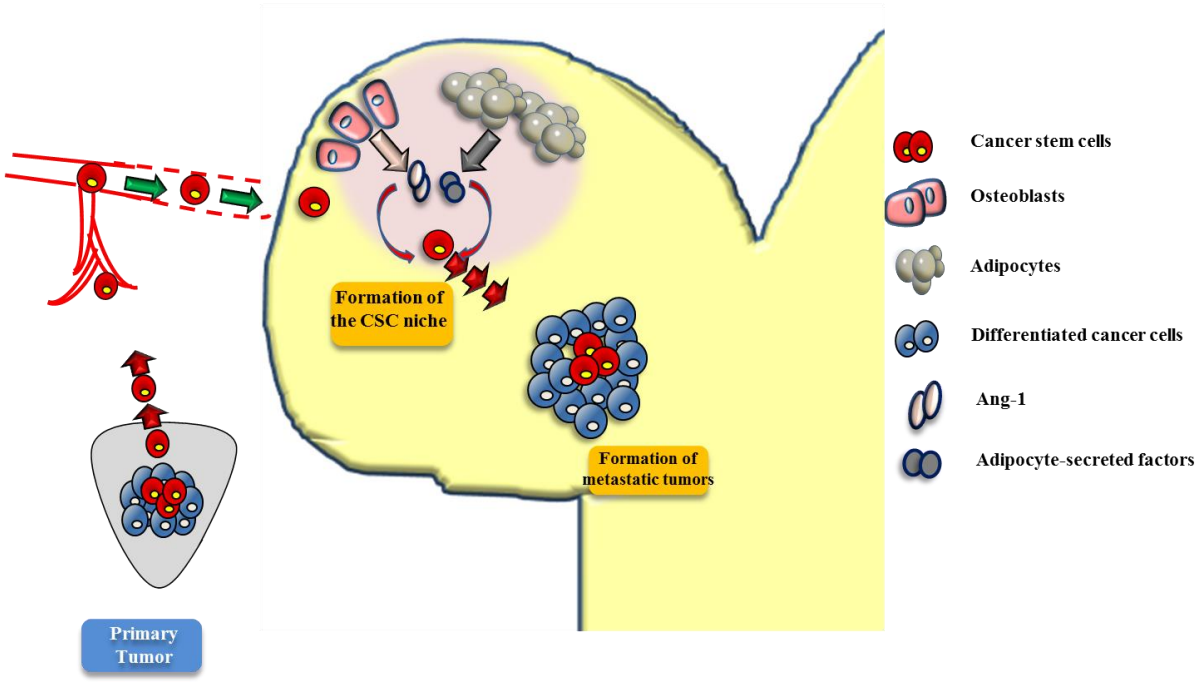


Figure 5.1: Model for the role of osteoblasts and adipocytes in prostate tumor metastasis. Osteoblasts and adipocytes are the two major cellular components in the bone marrow. Both cell types were found to regulate quiescence and self-renewal of prostate CSCs via two specific signalling pathways: Ang-1/Tie-2 and CCK/CCKBR. I believe that further testing the effect of Tie-2 and CCKBR inhibitors in pre-clinical tumor metastatic models may help in the design of an effective treatment regimen against prostate cancer.

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