ABSTRACT

Title of Document: INFLAMMATION AND PROSTATE CANCER DEVELOPMENT: THE ROLE OF CHEMOKINE (CXC MOTIF) LIGAND 12 AND ITS RECEPTORS CXCR4 AND CXCR7, TUMOR ENVIRONMENTS AND MODULATION BY DIET-DERIVED COMPOUND PHENETHYL ISOTHIOCYANATE

Lu Yu, Doctor of Philosophy, 2018

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Prostate cancer is the most prevalent and the third leading cause of cancer death in men in the United States and effective cure remain elusive. In the etiology of prostate cancer, the mediators and cellular effectors of inflammation are important constituents associated with the regulation of prostate cancer progression but detail mechanisms remain unclear. It would be important to further elucidate the mechanisms to allow for development of effective preventive/therapeutic strategy for prostate cancer. C-X-C motif ligand 12 (CXCL12) is a constitutive and inflammatory chemokine that modulates autoimmune inflammation and homeostasis in the immune system. It is also a pleiotropic chemokine that is expressed in malignant prostate tumors and regulates prostate cancer migration and

invasion via interaction with its two receptors CXCR4 and CXCR7. However, regulations of CXCL12 and its receptors in the immune system and prostate cancer are poorly understood. In addition, phenethyl isothiocyanate (PEITC) is a dietary compound from Cruciferae family with an inhibitory effect on prostate cancer progression. The mechanisms that underlie the anti-cancer effects of PEITC on prostate cancer are not well studied.

This dissertation elucidated 1) the role of CXCL12 and its receptors in the immune system, specifically during monocyte-macrophage differentiation; 2) the transcriptome alterations in androgen-responsive human prostate cancer LNCaP cells and its xenograft; 3) roles of prostate cancer microenvironment in prostate cancer progression; 4) molecular effects of diet-derived cancer-preventive compound phenethyl isothiocyanate (PEITC) on monocytes.

The accomplishment of this dissertation will 1) provide critical mechanistic information of the interaction between inflammation and prostate cancer development and 2) validate the role of CXCL12/CXCR4/CXCR7 chemokine axis and PEITC in the regulation of prostate cancer development and immune response. Ultimately, the study of the mechanisms mentioned above could help identify new strategies for the prevention/treatment of prostate cancer.

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2018

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Dedication

This thesis is dedicated to love and affection from my dear parents, Suogeng Yu and Huiqin Jiao. A lot of thanks also to my husband Hao and my best friends Jing and Jia for persistent support and encouragement in these years. I am truly thankful for having all of you in my life.

Acknowledgements

I would like to gratefully acknowledge countless individuals who have been journeyed with me during my study in graduate school. Without you, I may not have been able to overcome the difficulties and reach the achievements.

First, I owe an enormous debt of gratitude to my advisor Dr. Lucy Yu. She has been an extraordinary mentor to the fullest extent. Her dedication, helpful insights, tremendous knowledge, patience, and motivation have enabled me to navigate through the dissertation with enlightening experience and bring my research to fruition.

Second, I would like to thank Dr. Thomas T.Y. Wang, for his encouragement, sincere support and nurture of my mental growth in my research. I also want to express my gratitude to my dissertation committee members, Drs. Xiaoping Zhu, Seong-Ho Lee, and Abani Pradhan, for their support and guidance throughout my research. Their constructive suggestions and comments have been crucial to my work.

My thanks also go to previous and present labmates who have assisted and helped in my research: Dr. Margaret Slavin, Dr. Zhuohong (Kenny) Xie, Dr. Haiqiu (Jason) Huang, Dr. Yingjian Lu, Dr. Arnetta Fletcher, Dr. Boyan Gao, Dr. Rumana Yasmeen, Quynhchi Pham, Eric Cho, Yinghua Luo, Yanfang Li, Yanbei Wu, and Zhangyi Song.

My family have provided tremendous support for my study. I am particularly thankful to my parents and my husband Hao Dang for their support as well as remarkable abilities to ground me and encourage considerate thought.

Last but not the least, I'd like to thank the entire University of Maryland community for fostering a supportive, academic community.

Table of Content

List of Tables

List of Figures

List of Abbreviation

PCa	Prostate cancer
LPS	Lipopolysaccharide
CXCL12	CXC-motif ligand 12
CXCR4	CXC-motif receptor 4
CXCR7	CXC-motif receptor 7
AR	Androgen receptor
PSA	Prostate-specific antigen
TNF-α	Tumor necrosis factor α
DHT	Dihydrotestosterone
PI3-K	Phosphatidylinositol 3-kinase
VEGF	Vascular endothelial growth factor
TLR-4	Toll-like receptor 4
MSR1	Macrophage scavenger receptor 1
MDSC	Myeloid-derived tumor suppressor cells
MMP9	Matrix metalloproteinase-9
CDS	Charcoal-stripped fetal calf serum
GEO	Gene expression omnibus

FBS	Fetal bovine serum
ARE	Androgen responsive element
PMA	Phorbol 12-myristate 13-acetate
IL-1β	Interleukin 1-β
IL-6	Interleukin-6
u-THP-1	Un-differentiated THP-1
d-THP-1	Differentiated THP-1

Introduction

Prostate cancer (PCa) is reported to be the most frequently diagnosed cancer and the second leading cause of death in men among all cancer types in the US and Europe. Despite the high prevalence, the etiology of prostate cancer development remains unclear. Androgen is a known risk factor for hormone-dependent prostate cancer development. The mortality of PCa is correlated with poor prognosis, PCa bone metastasis and occurrence of secondary metastases. Currently, there is no effective cure for prostate cancer. Therefore, it is critical to understand the cause(s) as well as molecular mechanisms of the development of prostate cancer.

Inflammation has emerged as a critical risk factor in the development of prostate cancer. Existing literature including our previous research suggest an interaction between androgen and inflammatory pathways. However, the molecular mechanisms of the interaction between immune system and prostate cancer development remain unclear and warrant elucidation.

Chemokine (C-X-C Motif) ligand (CXCL) 12 is a pleiotropic chemokine that is highly expressed in malignant prostate tumors, and functions as a pivotal regulator for prostate cancer migration, invasion, metalloproteinase activity and bone metastasis. In addition, CXCL12 has also been proposed to play a crucial role in the regulation of autoimmune inflammation, tissue homeostasis, and angiogenesis via interaction with its two receptors CXCR4 and CXCR7. However, regulations of CXCL12 and its receptor in immune cells and prostate cancer cells are poorly understood, and the role of androgen in these regulations is not known. Furthermore, the precise role of CXCR4 or 7 in prostate cancer development is also not clear.

Phenethyl isothiocyanate (PEITC) is a naturally occurring chemopreventive agent from cruciferous vegetables and has been reported for its ability to inhibit prostate cancer. It has also been reported for its anti-inflammatory effects on mouse macrophages. However, the mechanisms underlying prostate cancer and immune response regulation by PEITC are not fully understood.

In this regard, the study of mechanisms responsible for the regulation of CXCL12/CXCR4/CXCR7 chemokine axis and effects of dietary chemopreventive compound PEITC in prostate carcinogenesis and immune system will provide critical information towards a sound strategy to eliminate prostate cancer.

In this dissertation work, I will investigate 1) how CXCL12 and its receptors work in immune system, specifically during monocyte-macrophage differentiation; 2) the role of CXCL12 and its receptor in prostate xenograft tumor as compared to cultured cells; 3) alterations of global transcriptomes and canonical pathways in prostate xenograft tumor as compared to cultured cells, and the relevance to clinical outcomes; 4) regulation of CXCL12 and its receptors in monocytes by PEITC in response to LPS induced inflammation; 5) genes and pathways regulated by PEITC in monocytes in response to LPS induced inflammation. The accomplishment of this dissertation will 1) provide critical mechanistic information on the interaction between inflammation and prostate cancer development and 2) validate the role of CXCL12/CXCR4/CXCR7 chemokine axis and the effects of PEITC on prostate cancer development and regulation of immune response. Ultimately, the study of the mechanisms mentioned above can help identify new strategies for the prevention/treatment of prostate cancer.

Chapter 1: Literature Review

1.1. Prostate cancer

Prostate cancer (PCa) is the most prevalent diagnosed cancer and the second leading cause of death in men in the United States (Pruthi et al., 2006). In 2013, as many as 238,590 men have been identified to have prostate cancer, which accounts for 14.37% of total cancer incidences. At the same time, the mortality of prostate cancer is estimated to be as high as 12.46% ("AACR Cancer Progress Report 2013," 2013). On a global scale, the incidence of prostate cancer differs from country to country. Specifically, the incidence of prostate cancer is found to be much higher in United States, Canada, Australia/New Zealand and Europe, compared to China and other parts of Asia (Parkin et al., 2005). Despite different distribution globally, the incidence of prostate cancer is increasing worldwide.

The early stage of prostate cancer often exhibits no symptoms, which makes the early diagnosis challenging. Though symptoms including frequent urination, weaker flow of urine, blood in the urine, pain, and weakness in legs and feet could sometimes be observed in more advanced prostate cancer (American Cancer Society, 2015), poor prognosis of prostate cancer, tumor recurrence and cancer metastasis make the disease life-threatening (National Institute of Health, 2015). In terms of financial cost of prostate cancer, in 2010, the average cost for each inpatient and outpatient PCa encounter was as high as $12,286 and $4,364 per visit respectively. The cost for PCa might further increase in the future due to the emerging trend of multimodal therapy (Seal et al., 2015). Given that no cure has been found for advanced prostate cancer, it is of importance to discover effective strategies for

the prevention of prostate cancer, which would increase the life quality of each individual and reduce the burden of the entire society.

1.1.1. Prostate carcinogenesis

In spite of the prevalence of prostate malignancy, the causes of prostate carcinogenesis are fairly complicated and poorly understood (Bosland & Mahmoud, 2011). Epidemiology studies strongly suggest that hormones, genetic risk factors, and environmental factors (particularly nutrition and diet patterns) are the major determinants of risk for prostate cancer (Bosland, 2000). Among all these factors, mounting evidence indicates that prostate carcinomas are closely related to hormone levels. Prostate cancer is often initially androgen-sensitive and responsive to hormonal therapy by temporary hormone remission but is followed by a relapse to an androgen-insensitive state and is not responsive to hormone deprivation (Debes & Tindall, 2004). These well-established features of prostate cancer strongly suggest that sex steroids, particularly androgens, play important roles in the pathogenesis of prostate cancer although the precise mechanisms are not fully understood (Bosland, 2000; Gann et al., 1996). The remaining part of this section will focus on highlighting the current understanding of the mechanisms of androgen-dependent and androgen-independent prostate cancer.

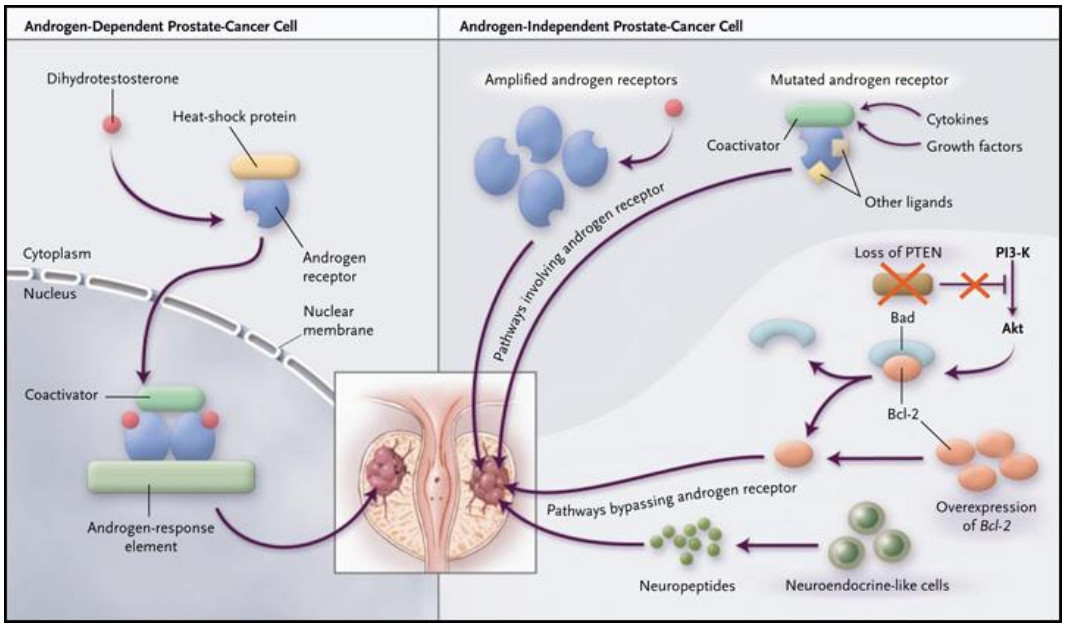

Fig. 1.1. Androgen receptor involved signaling pathway for prostate cancer development (Debes & Tindall, 2004).

1.1.1.1. Hormone-dependent prostate cancer

Hormones, especially androgens, are important not only for the normal function of prostate gland, but also for the maintenance of prostate cancer cells (Huang & Tindall, 2002). One of the most important androgens that associate with prostate cancer development is testosterone (Friedman, 2005). Testosterone could be transformed to a more potent metabolite dihydrotestosterone (DHT) by 5α-reductase. The inactivation of 5α-reductase by finasteride efficiently decreased the progression of prostate cancer by approximately 25% in a 7-year follow-up by inhibiting the conversion of testosterone to DHT (Roddam et al., 2008). It has also been shown that with increased levels of testosterone and its metabolite DHT after many years, a progressive state of prostate cancer could be observed (Bostwick et al., 2004). At the hormone-dependent state, hormone therapy or androgen deprivation therapy (ADT) is efficient for prostate cancer treatment. For example, surgical

castration, chemical castration using luteinizing hormone-releasing hormone (LHRH) analogs, LHRH antagonist, an anti-androgen, are all effective treatments as they can block the production of androgen (American Cancer Society, 2015).

The development and progression of prostate cancer are dependent on the action of androgens through the androgen receptor (AR) (Shafi et al., 2013). AR plays a pivotal role throughout prostate cancer development and progression (Heinlein & Chang, 2004). ARs are widely expressed in many types of cells in the prostate (Prins et al., 1991). Overexpression of ARs in prostate epithelial cells lead to the earliest recognizable stage of prostate cancer, and loss of ARs significantly reduces the risk of prostate carcinogenesis (Eng et al., 1999; Stanbroughet al., 2001). During the androgen-dependent prostate cancer development, AR is located primarily in the cytoplasm where it is associated with heat shock proteins (HSP), cytoskeletal proteins and other chaperones when it is absent from ligands (Smith & Toft, 2008). However, when binding to DHT, AR dissociates from HSP, translocates to the nucleus, and then binds to the androgen-response elements. Since DHT has higher affinity with AR than testosterone does, DHT binds to AR and regulates gene expressions even at a very low concentration (5 nM) (Zhou et al., 1995; Wright et al., 1996). After DHT binds to AR, the complex migrates to the nucleus and activates the androgen response element, which results in prostate cancer cell proliferation (Tso et al., 2000). Also, androgen can inhibit apoptosis induced by both tumor necrosis factor α (TNF-α) and Fas activation thus increased prostate cancer cell survival (Kimura et al., 2001).

1.1.1.2. Hormone-independent prostate cancer

Hormonal therapies such as androgen deprivation or chemical castration are effective in the treatment of advanced PCa. However, most hormone-dependent PCa relapses to

castrate-resistant/hormone-independent state which is not curative after primary androgen ablation therapy (Saeed et al., 2015). In androgen-independent state, androgen is not required for the growth of prostate cancer cell, and markers such as prostate specific antigen (PSA) do not respond to androgen (Craft et al., 1999). The mechanisms of hormone-refractory PCa might involve AR mutation, gene amplification, changes in steroid metabolism, or alternative splicing (Brooke et al., 2015; Park et al., 2015). During androgen-independent prostate cancer development, multiple signaling pathways might be involved. In the AR involved pathways, amplified ARs could be activated by reduced levels of DHT. On the other hand, insulin-like growth factors, cytokines such as Interleukin-6 and other ligands, could activate the mutated AR by assisting AR coactivators (Lonergan & Tindall, 2011). In the pathway bypassing AR, the loss of PTEN activates the phosphatidylinositol 3-kinase (PI3-K)–Akt pathway, which permits the activation of Akt to phosphorylate Bad. The overexpression of Bcl-2 as a result could increase prostate cancer survival bypassing the AR. In addition, prostate-cancer cells may develop neuroendocrine-like behavior. The neuropeptides secreted by neuroendocrine cells could induce the growth of adjacent cells and increase prostate cancer cell survival (Debes & Tindall, 2004).

In the androgen-independent state, the traditional hormonal therapy is not effective for the patients who have the advanced hormone-refractory prostate cancer. Thus, improved understanding of the cellular events during hormone-independent PCa development would help discover novel therapeutic strategies and increase survival rates of PCa patients.

1.1.2. Other factors involved in prostate cancer development and progression

Though the causes of prostate cancer remain largely unclear, the etiology of prostate cancer is a combination of many types of inter-related risk factors. First, other than hormone levels, the risk of prostate cancer is found to be closely linked to age (Ramon & Denis, 2007), and the risk of cancer has been reported to be 15%–30% in men older than 50 years and 60%–70% in men older than 80 (Pienta & Esper, 1993). Second, family history or genetic factor is closely correlated with the incidence of prostate cancer. An analysis of more than 30 epidemiological studies has pointed out that the first-degree relatives of men who have been diagnosed with prostate cancer have 2.53 times greater chance of getting prostate cancer than the general men population (Zeegers et al., 2003). The risk of men with brothers or twin who have prostate cancer is even higher than that of men whose fathers have prostate cancer (Albright et al., 2014). Third, life-style and eating habits might also affect the risks of having prostate cancer (Yang et al., 2015). For example, lifestyle factors, such as smoking, alcohol consumption, physical inactivity, and diet patterns, are all responsible for the risk of PCa and are the main targets for primary prevention (Katzke et al., 2015). Additionally, prostate cancer has also been found to be associated with factors such as UV light, infectious agent, radiation and cadmium consumption (Pruthi et al., 2006).

1.1.3. Prostate cancer metastasis

Prostate cancer metastasis is a process in which prostate cancer cells spread from the primary prostate tumor to other areas of the body such as lymph nodes and the bones through the lymphatic system or the bloodstream (Morrissey et al., 2007). Prostate cancer in most of the patients is present as a localized disease. However, once prostate cancer metastasizes, it becomes life-threatening (Albin & Mason, 2008). In response to hormone ablation therapy, the average time for patients presenting with symptomatic metastases to bone is only 2 years. Once the PCa progresses to the home-independent stage, life expectancy for most patients is only several months (Galasko, 1986; Albin & Mason, 2008). The most common metastatic site for prostate cancer is bone (90%), followed by lung (46%), liver (25%), pleura (21%), and adrenals (13%) (Bubendorf et al., 2000). Despite the low survival rate of bone metastasis in prostate cancer, metastatic bone pain at the metastatic site is also prevalent for patients with advanced prostate cancer, as prostatic metastasis causes fractures (breaks) of bones (Litwin et al., 1998).

Clearly, prostatic metastasis is ominous and with poor prognosis of prostate cancer, identifying patients with more aggressive prostate cancer is currently a great clinical challenge for clinicians and researchers. Therefore, identifying strategies to prevent prostate cancer metastasis or lower the prostatic metastasis rate is very important.

1.2. Inflammation and prostate cancer

1.2.1. Relationship between inflammation and prostate cancer

The word “inflammation” originates from the Latin word “inflammare” and is considered as a protective attempt against harmful stimuli such as bacteria, fungi, viruses, and protozoa (Smith, 1994), by non-specific immune responses.

Inflammation can be classified into acute inflammation and chronic inflammation. Acute inflammation is the initial response of the body to harmful stimuli, which involves the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues (https://en.wikipedia.org/wiki/Inflammation). Chronic inflammation is a process that leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process (Ferrero-Miliani et al., 2009). Chronic inflammation is closely related to harmful degenerative processes, gene mutation, and protein dysfunction and could further increase the risks of cancer (Tracy, 2003; Dandona et al., 2004; Hofseth &Wargovich, 2007). Inflammation has long been associated with the development of cancer. Accumulating evidence has shown that chronic inflammation is an important etiological factor for prostate cancer development, through multiple genes that are involved in the inflammatory pathways. Prostate cancer cells will further interact with the secreted signaling molecules from the surrounding inflammatory cells, which plays a pivotal role in promoting prostate tumor invasion, migration and metastasis (Marzo et al., 2007). Ribonuclease L (RNASEL), macrophage scavenger receptor 1 (MSR1), interleukins (IL-8, IL-10), vascular endothelial growth factor (VEGF), intercellular adhesion molecule (ICAM), and Toll-like receptors (TLR-4, TLR-1-6-10 gene cluster) are all candidates for

genetic determinants of the risk of prostate cancer (Sun et al., 2007). Cytokines, including MIC-1, IL-1 receptor antagonist, IL-8, IL-10, and VEGF, play key roles in prostate tumorigenesis. In addition, cytokines are also able to interact with hormone and modulate monocyte migration towards the tumor site in hormone-responsive prostate cancer development (Karan et al., 2009).

Currently, serum PSA level measurement is the most prevalent method for the diagnosis and prediction of prostate cancer. Evidence has shown that prostatitis, especially when being related to infection by bacteria, is also associated with increased serum PSA levels (Kawakami et al., 2004). The association of prostatitis with prostate cancer has been further confirmed as biopsies from 80% of the patients who had prostate cancer were found to contain a certain degree of inflammation. In another study of a randomized trial of 328 (male) patients with PSA ranging from 2.5 to 10 ng/mL, leucocytes have been observed in expressed prostatic secretions in more than 45% of the patients (Sfanos & Marzo, 2012). Therefore, a relationship between inflammation and prostate cancer exists.

1.2.2. Role of monocytes/macrophages in prostate carcinogenesis

Recruited monocytes and macrophages provide a primary response to pathogens and critical defense mechanism against infectious agents (Serbina et al., 2008; Martinez et al., 2009). In response to infection, monocytes move quickly to the infectious sites. Monocytes not only participate in tissue healing, clearance of pathogens and dead cells and adaptive immunity initiation, but also contribute to the pathogenesis of chronic inflammatory disease (Ingersoll et al., 2011). Monocytes differentiate into macrophages in response to infection. The differentiation of monocytes to macrophages is a key event involved in

pathogen clearance (Murray & Wynn, 2011). In addition, macrophages participate in tissue repair and regeneration (Kuby, 2009).

Besides the important function in inflammation, monocytes and macrophages also play pivotal roles in prostate carcinogenesis (Loberg et al., 2007). In hormone-dependent prostate cancer development, monocytes are recruited by signaling molecules (such as CCL2) secreted from prostate cancer cells through modulation by androgen (Kim et al., 2013). The production of CCL2 indirectly regulates prostate cancer growth and metastasis by regulating monocyte/macrophage infiltration into the tumor microenvironment (Loberg et al., 2007).

1.2.3. Relationship between hormone and inflammation in the prostate carcinogenesis

Androgens are not only capable to mediate prostate cell differentiation, proliferation, and apoptosis, but also immune/inflammatory responses (Asirvatham et al., 2006). In general, immune response could be suppressed by androgens (Cutolo et al., 2004), though the exact mechanism is unclear. In *in vitro* model of epithelial prostate cells, androgens are observed to modulate the inflammatory response. Two androgen-dependent immune/inflammatory pathways, including IFN pathway and IL-6/IL-12 regulated pathway, are upregulated and suppressed respectively (Asirvatham et al., 2006). The activation of IFN regulated pathway by androgens might cause the loss of immunosurveillance and lead to benign prostate hyperplasia and prostate cancer (De Marzo et al., 2003). In the ventral prostate, androgen withdrawal upregulates the IL-15 and IL-18, possibly due to the influx of T cells, macrophages, and mast cells (Desai et al., 2004). In addition, our previous study indicated that CCL2 increased secretion in hormone-responsive prostate cancer cell line by DHT treatment and also promoted human monocyte THP-1 migration. These results suggested

that a relationship between androgen and cytokine regulation exists which promotes inflammatory micro-environments in prostate tumors (Kim et al., 2012).

1.2.4. Chemokines in inflammation and prostate cancer

1.2.4.1. Classification of chemokines

Chemokines are a family of small molecular signaling polypeptides which play a pivotal role in leukocyte trafficking (Kindt et al., 2007). Clinically speaking, the management of immune diseases often requires the recruitment of leukocytes to the target infectious organ or tissue. In this process, chemokines play a key role in leukocyte activation and immune response manifestation (Godessart & Kunkel, 2001). To date, more than 50 chemokine ligands and 20 G protein-coupled receptors have been identified in humans (Gerard & Rollins, 2001). Based on the positions of their cysteine residues in N-terminal, chemokines could be classified into four subgroups including CC, CXC, C and CX3C (Ji et al., 2005). Two main chemokine subfamilies, CXC and CC, are classified by the positions of their first two cysteines, as for whether they are separated by one amino acid (CXC) or adjacent (CC) (Moser et al., 2006). The three-dimensional basic structure of a chemokine comprises two disulfide bonds which link Cys1 to Cys3 and Cys2 to Cys4, contributing to the formation of a rigid core in the central part. The amino-terminal domain usually contains 3–10 amino acids and the carboxyl-terminal helix is comparably longer which consists of 20–60 amino acids (Clark-Lewis et al., 1995). Recently, there are totally 16 documented chemokine (C-X-C motif) ligands, named as CXCL 1 to 14, and 16 to 17. Eight specific cell-surface G protein-coupled chemokine receptors (CXCR1-8) which modulate the transmission of chemokine-encoded messages have also been reported. Some of the CXC ligands share the same receptor (e.g. CXCL1-3, CXCL5, and CXCL7-8 bind to CXCR2;

CXCL4, and CXCL9-11 bind to CXCR3; CXCL11-12 share CXCR7). On the other hand, some ligands have their specific binding receptors (e.g. CXCL6 binds to CXCR1, CXCL13 binds to CXCR5 and CXCL16 binds to CXCR6, CXCL17 binds to CXCR8). Additionally, some of the ligands might have multiple receptors, which might interact with each other and function in the receptor-regulated pathways (e.g. CXCL8 (IL-8) could bind to CXCR1 and CXCR2, and CXCL12 binds to CXCR4 and CXCR7) (Zhang et al., 2002; Rotondi et al., 2007; Lee et al., 2013).

CXC chemokines could also be classified into two main groups including ELR+ group and ELR- group according to the presence/absence of the tripeptide motif glutamic acid-leucine-arginine (ELR) in the sequence. Based on the observation that the presence or the absence of ELR motif is closely linked to the angiogenic or angiostatic functions of CXC motif chemokine, ELR+ CXC chemokines are thought to be correlated to angiogenesis, while ELR- CXC chemokines are thought to be linked to angiostasis (Rotondi et al., 2007). Interestingly, although CXCL12 does not contain ELR motif, it possesses angiogenic property (Liekens et al., 2010).

1.2.4.2. Role of chemokines in inflammation and prostate cancer

Recent clinical studies support that chemokines and chemokine receptors are significantly altered during the evolution of human autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, Sjögren's syndrome, systemic lupus erythematosus and Graves' disease (Godessart & Kunkel, 2001). It has been reported that the reduction of inflammation could contribute to the inhibition of primary and metastatic tumor progression by postponing the accumulation of myeloid-derived tumor suppressor cells (MDSC), which are thought to be involved in angiogenesis, tumor growth and metastasis

(Raman et al., 2011). Chemokines and chemokine receptors not only mediate the chronic autoimmune diseases, but also function as the mediators of MDSCs recruitment, tumor migration and metastasis (Wang et al., 2009; Mantovani et al., 2010).

Among the factors suspected to be involved in prostate cancer development, chemokines and their receptors are now being studied intensively (Vindrieux et al., 2009). CXC chemokines are constitutively produced by human prostate cancer cells and stromal microenvironment, both in the primary tumor site and in distant metastatic locations. In prostate cancer development, prostate cancer cell lines can use distinct CXC chemokines to mediate their tumorigenicity. Chemokines affect not only leukocyte infiltration, proliferation, and angiogenesis, but also hormone-escape (Moore et al., 1999; Vindrieux et al., 2009).

Thus, chemokines and their receptors are not only novel markers of prostate cancer but also possible targets for future therapies.

1.2.4.3. Importance of CXC-motif chemokine 12 and its receptors

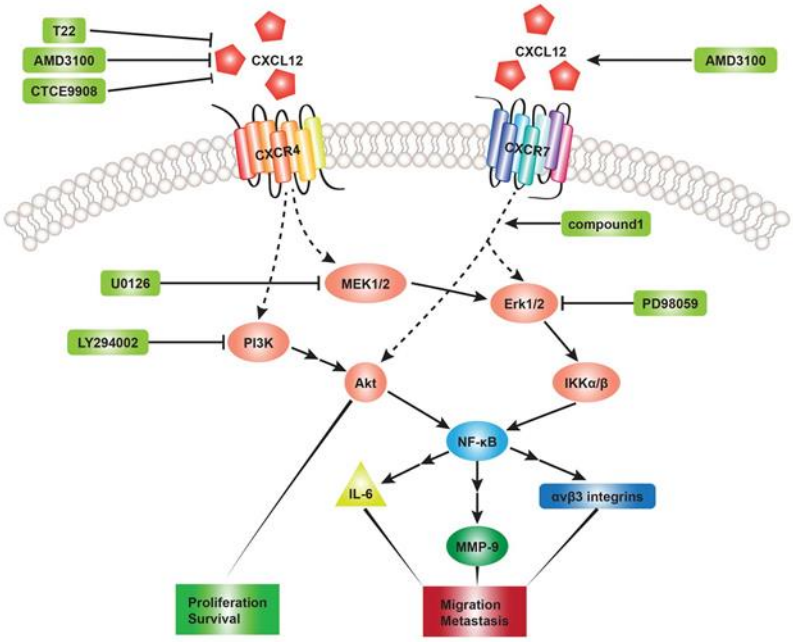

Fig.1.2. Downstream pathways regulated by the CXCL12/CXCR4/CXCR7 axis. The binding of CXCL12 to CXCR4 or CXCR7 can activate the phosphoinositide 3-kinase (PI3K)-Akt-nuclear factor-κB (NF-κB) and MEK-ERK-IκB kinase αβ (IKKαβ)-NF-κB pathways, which regulate both proliferation/survival and migration/metastasis in tumors. Interleukin-6 (IL-6), matrix metalloproteinase-9 (MMP-9) and αvβ3 integrins induced by NF-κB correlate with tumor migration and metastasis. T22, AMD3100, and CTCE9908 block CXCL12-CXCR4 interaction. Nevertheless, AMD3100 is an allosteric agonist of CXCR7. CXCR7 agonist compound 1 could induce Akt and ERK phosphorylation (Liao et al., 2013).

1.2.4.4. CXC-motif ligand 12, CXC-motif receptor 4 and 7

Chemokine (CXC motif) ligand 12, which is also known as Stromal Derived Factor-1, was first discovered in 1993 (Tashiro et al., 1993). As a chemokine from CXC-motif families, CXCL2 first acts as a leukocyte attractant via the activation of G protein-coupled receptors during the immune response (Hartmann et al., 2008). Besides leukocytes such as monocytes, lymphocytes, and neutrophils, CXCL12 could also modulate the trafficking of

various types of immunity-related cells including B cells, dendritic cells and megakaryocytes (Luther et al., 2002; Salcedo & Oppenheim, 2003).

Chemokine CXCL12 signaling is activated by binding to its specific seven-transmembrane G protein-linked receptors (Kelvin et al., 1993; Premack & Schall, 1996). CXCR4 is a guanine nucleotide-binding protein (G-protein) coupled receptor with 7-transmembrane region that specifically binds to CXCL12 (Duda et al., 2011). The structure of CXCR4 and binding of CXCR4 with CXCL12 triggered the downstream signaling pathways involved in the regulation of important biofunctions such as 1) chemotactic of immune cells 2) cancer metastasis and progression 3) hematopoietic stem cell homing and quiescence and 4) neuronal guidance (Guan et al., 2003; Kucia et al., 2004; Liang et al., 2005; Sugiyama et al., 2006).

CXCR7 is also a G-protein coupled receptor, which is newly discovered receptor for ligand CXCL12. CXCR7 deficient mice do not display an obvious brain phenotype and die at birth with ventricular septal defects and semilunar heart valve malformation (Sierro et al., 2007). The bio-function of CXCR7 is controversial. Some studies have documented that CXCR7 regulates pathological processes in prostate carcinogenesis and is regulated by CXCR4 (Wang et al., 2007). But in other reports, CXCR7 has been considered as a decoy signaling receptor interacting with CXCL12 (Maksyma et al., 2009). CXCR7 is an important receptor which is involved in prostate carcinogenesis and plays an essential role in the CXCL12/CXCR4/CXCR7 chemokine axis involved signaling pathway. However, CXCR4/7 and CXCL12 have not been fully understood.

1.2.4.5. Role of CXCL12 and its receptors in inflammation

The accumulation of CXCL12 has often been observed in pathological sites with inflammation. It has been demonstrated that CXCL12 is closely associated with cell trafficking, migration, differentiation, and proliferation. Initially defined as a growth factor of B-lymphocyte precursors and a bone marrow stromal cell-derived factor, the bioactivity of CXCL12 is first known as a chemoattractant for lymphocytes in vitro and in vivo with high efficacy. CXCL12 is postulated to help direct and recruit lymphocytes, which play an essential role in immunological memory, trafficking to the inflamed sites from the bloodstream to lymphoid or non-lymphoid tissues during recirculation by several times in life cycle (Bleul et al., 1996). Besides, CXCL12 could also recruit human CD34+ hematopoietic progenitor cells and cause the cell migration in vitro and in vivo toward the gradient CXCL12 produced by stromal cells (Aiuti et al., 1997). Additionally, CXCL12 has been found to have an proliferative effect on B lineage acute lymphoblastic leukemia cells through the interaction with IL-7 and IL-3 (Juarez et al., 2007).

CXCR4 which specifically binds to CXCL12 ligand also plays a crucial role in inflammatory response and immune system. CXCR4 functions as a coreceptor for T-tropic HIV-1 entry, and the mice lacking CXCR4 exhibit deficiencies in hematopoietic and nervous systems and die perinatally. The B-lymphopoiesis and myelopoiesis are significantly reduced in the fetal liver of CXCR4-deficient mice, together with an absence of myelopoiesis in bone marrow (Ma et al., 1998). CXCL12 was also reported to be continuously secreted by stromal cells and primary blood monocytes (Sánchez-Martín et al., 2011). CXCL12 is a critical regulator of cell trafficking and assists in the directional movement of immune cells responding to inflammatory signals via CXCR4 and CXCR7

(Werner et al., 2013). The activation of CXCR4 by CXCL12 binding to CXCR4 triggers downstream pathways involving matrix metalloproteinase-9 enzyme activity, and regulates lymphocytes and phagocytes migration (Ghosh et al., 2006; Campana et al., 2009). Up-regulation of CXCR7 is involved in the trafficking/ adhesion of selected human leukemic cells (Tarnowski et al., 2010). Overall, CXCL12/CXCR4/7 pathways contribute to critical cellular events such as tumor metastasis, organ development, wound healing and angiogenesis (Ansel & Cyster, 2001; Liekens et al., 2010).

1.2.4.6. Role of CXCL12 and its receptors in prostate cancer development

One in vivo study of human prostate cancer has already revealed significantly high expression of CXCL12 and its receptor CXCR4 in metastatic prostate cancer using high-density microarrays in over 600 clinical prostate cancer samples. Using ELISA kit, approximately 25, 100 and 70 pg/mL of CXCL12 protein secreted were detected in the media of LNCaP, LNCaP C4-28, and PC3, respectively, after 72 h. This study also demonstrated that the treatment of CXCL12 antibody inhibits the proliferation of LNCaP C4-2B and PC3 metastatic tumor cells (Sun et al., 2003). These findings provide important evidence that CXCL12 and its receptors are firmly linked to prostate cancer development. Another study involving mechanisms in prostate cancer metastasis focused on the interactions of CXCL12 and CXCR4. Compared to normal prostate epithelial cells, CXCL12 and CXCR4 are highly expressed in prostate cancer cell lines including LNCaP and PC3. Also, this study screened the correlation between CXCL12 and different types of matrix metalloproteinase, which have been considered as a major regulator of cell proliferation, migration, angiogenesis, and apoptosis. The MMP expression differs in different prostate cancer cell lines. In LNCaP cells, MMP-1, MMP-2, MMP-10, MMP-13

are up-regulated by CXCL12 elevation, while in PC3 cells, MMP-1, MMP-3, MMP-9, MMP-13, MMP-14 show a high level after CXCL12 induction. This study emphasizes the importance roles of CXCL12 and CXCR4 in facilitating the migration, invasion and MMP expression by prostate tumor cells (Singh et al., 2004).

The interaction between CXCL12 and CXCR4 critically regulates prostate cancer migration, invasion, metalloproteinase expression, tumor growth and contributes to the angiogenesis and altered patterns of cytokine secretion (Singh et al., 2005; Darash-Yahana et al., 2004) The expression of CXCR4 is significantly elevated in prostate cancer cell lines LNCaP and PC3 when compared to the normal prostatic epithelial cells (PrEC) (Singh et al., 2004). It has been reported that the CXCL12/CXCR4 signaling may play an important role in the metastasis of prostate cancer to bone (Taichman et al., 2002). The metastasis of prostate cancer is largely regulated by matrix metalloproteinases (MMPs), a group of proteinases that particularly contribute to the proteolysis of extracellular matrix (ECM) components (Butcher et al., 1999).

Despite the important roles CXCL12 and its receptors play in prostate cancer development, a number of key topics are still poorly understood, including the regulation of CXCL12/CXCR4/CXCR7 chemokine axis in the immune system, the correlation between androgen activity and CXCL12/CXCR4/CXCR7 chemokine axis, and the role of CXCR7 in regulation of prostate cancer metastasis.

1.3. Models used for prostate cancer research

1.3.1. *In vitro* cell culture models

Human prostate cancer tissue culture cell lines are commonly used for the *in vitro* studies of androgen responsive and androgen non-responsive prostate cancer progressions. The most frequently used human cell lines in prostate cancer research include LNCaP, PC-3 and DU145 cells (Wu et al., 2013; Kumar et al., 2008).

The report of the first available human prostate cancer cell line DU145 could be dated back to 1977, by Duke University (Mickey et al., 1977). This cell line was described to be able to grow in both tissue culture and xenograft model (Rage et al., 1990; Church et al., 1999). In 1979, the second prostate cancer cell line PC-3 was described by Kaighn's group (Kaighn et al., 1979). In 1983, LNCaP cell model with the presence of high-affinity specific androgen receptors was established by Horoszewicz's group (Horoszewicz et al., 1983). Both PC-3 and DU145 are androgen non-responsive and are representatives for the early androgen depletion independent prostate cancers (Litvinov et al., 2006). The LNCaP cell line is developed from a metastatic lesion of human prostatic adenocarcinoma. It expresses androgen receptor, and its ability to respond to hormones is preserved (Horoszewicz et al., 1983). The LNCaP cell line was usually used as a cell model in the study of hormone involved regulation of prostate cancer progression (Veldscholte et al., 1992; Tan et al., 1997).

1.3.2. *In vivo* mouse models

Numerous mouse models have been established to recapitulate the salient features of prostate carcinogenesis in humans (Abate-Shen & Shen, 2002). The use of

immunodeficient hosts and mouse models including genetically engineered, chemical carcinogenic and xenograft mouse models that mimic the human prostate cancer has been important in studying the genetic pathways and mechanisms involved in prostate cancer development (Ito et al., 1991; Greenberg et al., 1995; Leenders et al., 2008; Mavropoulos et al., 2009; Valkenburg & Williams, 2011).

1.3.2.1. Genetically engineered mouse (GEM) models

The genetically engineered mouse (GEM) models include TRAMP, LADY, c-Myc and PTEN knockout models (Wu et al., 2013; Carnero & Paramio, 2014; Grabowska et al., 2014).

The transgenic adenocarcinoma mouse prostate (TRAMP) and LADY mouse models are two transgenic models that express SV40 oncogenes (Kasper et al., 1998; Huss et al., 2001). The TRAMP model is established by expressing SV40 large T and small t tumor antigens. In this model, the high-grade PIN or prostate cancer can be developed within 12 weeks, and the metastases of prostate cancer could be observed after 30 weeks (Greenberg et al., 1995). The TRAMP is androgen responsive and is usually used for the study of aberrant growth-factor signaling in prostate cancer progression (Foster et al., 1998). On the other hand, the LADY model lacks small t antigen in transgene and expresses less aggressive properties as compared to TRAMP (Hensley & Kyprianou, 2012). The time for prostate tumor development of LADY is longer as compared to TRAMP, which is between 12-20 weeks. It develops high-grade PIN but lacks the ability to generate metastasis of prostate tumor (Kasper et al., 1998). The c-Myc model is a transgenic model not targeting SV40 oncogene (Zhang et al., 2000). The c-Myc is amplified, and the development of a prostate tumor is relatively modest in this model. Only low-grade PIN can be established

using this model, and it does not allow the progression to more advanced tumor status (Abate-Shen & Shen, 2002). Phosphatase and tensin homolog (PTEN) is a tumor suppressor. The PTEN knockout, sometimes with other genes such as p21 or Nkx3.1, will lead to the development of high-grade PIN (Valkenburg et al., 2011). The PTEN knockout model in which PTEN is lost will be of clinical importance for the study of PTEN mutations.

1.3.2.2. Chemical carcinogenic models

The chemical carcinogenic model involves the use of chemical carcinogens, or with the combination of testosterone, to induce prostate carcinomas in hosts (Wang et al., 2002). The application of chemical carcinogens to animals (such as rats) can induce the development of sarcomas or squamous cell carcinomas (Bosland, 1996). The chemical carcinogens used for prostate cancer models include N-nitrosobis (oxopropyl) amine (BOP), 3,2'-dimethyl-4-aminobiphenyl (DMAB), and *N*-methylnitrosourea (Bosland, 1996; Wang et al., 2002). As compared to the xenograft and GEM models, the chemically induced primary malignancies are easily conducted with fruitful tumor generation and high analogy to clinical human primary cancers. However, this model is difficult for assessing noninvasive tumor progression in small animals (Liu et al., 2015). The chemical carcinogenic models are not commonly used in prostate cancer research.

1.3.2.3. Xenograft models

The consideration of using human origin in experimental animal models is important to mimic the clinical progression of prostate cancer in patients (Weerden & Romijn, 2000). Among the extensively used prostate cancer animal models, xenograft models showed advantages of using human prostate cancer cells. Moreover, as compared to GEM and

chemical carcinogenic models, the xenograft models are thought to be more relevant to human prostate pathophysiology (Grabowska et al., 2014).

The commonly used mice hosts of prostate cancer xenograft models include Balb/C Nu/Nu, NMRI/Nu, Severely Combined Immune Deficient (SCID), and RAG and NOD/SCID (Chung et al., 2007).

Among the four mice host models used for xenografts, the Balb/C Nu/Nu is the most commonly used in the studies of prostate cancer. The Balb/C Nu/Nu model lacks a functional thymus, thus, it has a deficient cell-mediated immune response (Pandelouris, 1968). As compared to SCID, RAG and NOD/SCID, Balb/C Nu/Nu allows the measurement of tumor size during the prostate tumor growth, and the introduction of human prostate cancer cell xenotransplantation in this mouse model mimics the progression of human prostate cancer development (Fleshner et al., 1999).

The xenotransplantation methods include implantation of patient-derived tumor tissue (Morton & Houghton, 2007), orthotopic injection of cultured human cell line into mouse prostate (Weerden et al., 2009), and subcutaneous injection (Zhuang et al., 2005). The requirement of patient-derived tumor from patient subjects makes the model less accessible, and the individual variance (Stranger et al., 2007) would increase the difficulties of replicating. The orthotopic model allows the transplantation in a specific prostate organism, but the disadvantages of this model include the requirement of expertise and the difficulties in measuring tumor growth in the mouse prostate (Weerden et al, 2009). Together, subcutaneous xenograft model using Balb/C Nu/Nu mouse introduces the use of human cells, allows the measurement of tumor sizes, and increases the feasibility of replication of the experiment.

1.4. Factors for regulation of xenograft tumor progression

As compared to the *in vitro* cell culture model, the in vivo xenograft tumor involves more complicated environment for prostate tumor growth, such as exposure to androgen (Hamilton et al., 1984), hypoxia (Chan et al., 2007), secretions from microenvironment (Polyak et al., 2009), and subcellular matrix (Pickup et al., 2014). These factors might have an influence on gene expressions and xenograft prostate tumor growth.

1.4.1. Androgen

Androgen level is related to the incidence of hormone-related cancers including prostate cancer (Henderson et al., 1982). One of the most important androgens that is associated with prostate cancer development is testosterone (Friedman, 2005). Testosterone could be transformed to a more potent metabolite dihydrotestosterone (DHT) by 5α-reductase. The inactivation of 5α-reductase by finasteride efficiently decreased the progression of prostate cancer by approximately 25% in a 7-year follow-up by inhibiting the conversion of testosterone to DHT (Roddam et al., 2008). It has also been shown that with increased levels of testosterone and its metabolite DHT after many years, a progressive state of prostate cancer could be observed (Bostwick et al., 2004). In addition, epidemiologic data indicate that elevated androgen levels might be associated with the pathogenesis of prostate cancer (Ghanadian et al., 1979). Also, higher mean levels of serum testosterone were found in prostate cancer patients compared to the healthy control (Glantz et al., 1964).

1.4.2. Hypoxia

The tumor microenvironment could be characterized by hypoxia fluctuation and nutrient deprivation (Kimbro & Simons, 2006). Hypoxia is described as a reduction of normal

oxygen level in tissue. Based on the observations of immunohistochemical studies, 30-90% clinically relevant levels of hypoxia were detected in prostate cancers (Chan et al., 2007). Hypoxia-inducible factor-1α (HIF-1α) is a key transcription factor induced by hypoxia and regulating hypoxia-responsive genes (Salceda & Caro, 1997). An increased level of hypoxia in tumor tissue is correlated with cell survival, proliferation, angiogenesis, metastasis and genetic instability (Harris, 2002). In the hypoxia involved prostate tumor progression, the cell-cycle checkpoints and DNA repair will be changed within the prostate epithelium (Chan et al., 2007). Liu et al reported that cobalt chloride ($CoCl_2$) stimulated hypoxia significantly induced vascular endothelial growth factor (VEGF) in prostate cancer cell lines LNCaP, PC3 and PC3ML (Liu et al., 1999). The exposure to hypoxia and up-regulation of HIF-1α is also found to be associated with the down-regualtion of prostate specific antigen, androgen receptor and proliferative regulatory proteins (Ghafar et al., 2003). In addition, it increases the tumor resistance to treatments or therapies (Kimbro & Simons, 2006).

1.4.3. Interaction between immune cells and tumor cells

The prostate tumor microenvironment is composed of cancer cells, immune cells, vascular endothelial cells and fibroblasts (Gleave et al., 1992; Cho, 2013). The development of prostate cancer includes the association between cancer cells and immune cells from tumor microenvironments (Josson et al., 2010). In diverse immune cells, monocytes, macrophages, and stromal fibroblasts, are functional key players in the regulation of cell clearance, wound healing or prostate prostate tumor promotions (Disis, 2010; Josson et al., 2010). Stimulated by TNF, IFNγ or lipopolysaccharide (LPS), M1 and M2 phase macrophages secrete cytokines/chemokines IL-12, IL-23, IL-1, CXCL10, and IL-10, IL-

13, IL-4, CCL2, respectively (Cho, 2013). Besides, cytokines and chemokines have a complex network in prostate cancer progression. DHT time-dependently increases CCL2 protein in androgen-responsive human prostate cancer cell line LNCaP, and the CCL2 regulates the human monocyte THP-1 migration (Kim et al., 2013). In addition, IL-6 secretion from immune reaction could also regulate prostate cancer cell survival, growth arrest and proliferation (Culig et al., 2005). Cytokines and chemokines are produced by immune cells, primary tumor cells and stromal microenvironment during the interaction between host-tumor and immune cells (Vindrieux et al., 2009). The increases of cytokines and chemokines are associated with the promotion of tumor growth, inhibition of anti-tumor immune response and increased migration of immune cells (Colombo & Trinchieri, 2002; Kim et al., 2003; Raman et al., 2007). Together, the interaction between tumor cells and immune cells from surrounded microenvironment is essential for the regulation of cell survival, progression, and migration, and would affect cellular phenotypes (Condon, 2005; Sun et al., 2012). Crosstalk between tumor and immune cells might be a promising target for the prevention of prostate cancer.

1.4.4. Subcellular matrix

In *in vivo* natural systems, prostate tumor involves the interaction with subcellular matrix and grows three-dimensionally (3D) (Ravi et al., 2015). Subcellualr matrix not only serves as the scaffold, but also provides biochemical and biomechanical cues for the direction of cell growth conditions, population, migration, and immune function (Pickup et al., 2014; Rasheena et al., 2014). Besides, subcellular matrix allows interaction in all directions, and regulates cell morphogenesis, adhesion, survival, proliferation, as well as gene and protein expression profiles (Cukierman et al., 2001; Tibbitt & Anseth, 2009; Rasheena et al., 2014).

The monolayer culture of prostate cancer cells is commonly used in *in vitro* experiments for the study of prostate cancer progression (Nilsson et al., 1999). But recently, 3D cell culture has gained increasing interest for its advantages in establishing more physiologically relevant information in cancer study (Rasheena et al., 2014). 3D models are necessary to mimic hallmarks related to physiological carcinogenesis to address the question about the role that subcellular matrix might play in the regulation of prostate cancer progression (Lamhamedi-Cherradi et al., 2014).

1.5. Phenethyl isothiocyanate (PEITC)

Prostate cancer is the most commonly diagnosed invasive cancer, and the second leading cause of cancer death in men in the United States (Steele et al., 2017). Many risk factors associated with prostate cancer progressions are not easily controlled, such as race/ethnicity, age, and family history (Vertosick et al., 2014). Recent studies have established that 30% of cancer mortality and morbidity could be prevented with the proper adjustment in diets (Hsieh & Wu, 1999). Also, dietary compounds and phytochemicals from fruits or vegetables usually play important roles in the regulation of prostate carcinogenesis (Melchini et al., 2013). Thus, consumption of fruits or vegetables containing dietary agents with chemopreventive potentials might be a necessary strategy to reduce the morbidity and mortality of prostate cancer (Anna et al., 2011).

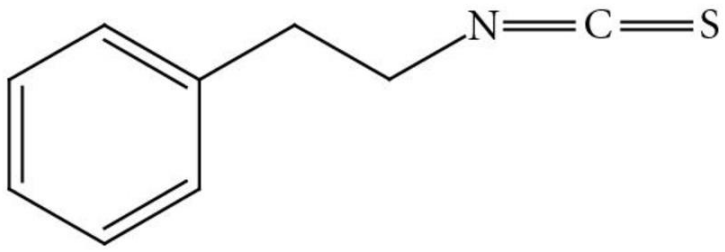

Fig. 3. Chemical structure of PEITC (Moon et al., 2011)

The phenethyl isothiocyanate (PEITC) is a bioactive compound with antitumor effects on human cancer cells, including prostate cancer (Christina et al., 2017). It is a dietary compound found in *Brassica* and other vegetables such as watercress, broccoli, kale, brussels sprouts and cauliflower of Cruciferae family (Moon et al., 2011). PEITC contains both phenethyl and isothiocyanate (R-N=C=S) groups (Fig.3). It is stable at pH 7.4 in biological samples with a half-life of 56.1 h at room temperature (Ji et al., 2005). The study of the pharmacokinetics of PEITC also indicated that PEITC has high oral bioavailability and high protein binding capacities with a low clearance in the rat (Ji et al., 2005). In plants, PEITC is usually present in a glucosinolate form and requires myrosinase enzyme to be hydrolyzed into the bioactive phenethyl isothiocyanate form (Weymarn et al., 2006).

1.5.1. PEITC and prostate cancer

Phenethyl isothiocyanate (PEITC) is a chemopreventive agent naturally occurring in cruciferous vegetables (Xiao et al., 2010), and it has been studied for its potential ability to inhibit prostate cancer (Bommareddy et al., 2009).

In *in vitro* studies, PEITC was found to be involved in the induction of autophagic and apoptotic cell death in prostate cancer LNCaP and PC-3 cells (Bommareddy et al., 2009). The study of the mechanisms of apoptotic effect by PEITC has shown that 1) PEITC is able to induce G_2-M-phase cell cycle arrest in PC3 cells and 2) p53 is not essential for the apoptosis effect by PEITC on PC-3 cell line (Xiao et al., 2002; Xiao & Singh, 2002). A subsequent study by Xiao's group demonstrated that reactive oxygen species-mediated apoptosis could be induced by PEITC in LNCaP and PC-3 cells (Xiao et al., 2006; Xiao et al., 2010). In androgen non-responsive DU145 cells, PEITC could significantly inhibit cell proliferation through cell arrest of G_2-M phase and could decrease the IL-6 induced STAT3

activity (Gong et al., 2009). In androgen-responsive LNCaP cells, PEITC suppressed AR expression and inhibited the prostate cancer cell growth (Wang et al., 2006).

In *in vivo* studies, PEITC inhibited prostate cancer progression by induction of autophagic cell death in Transgenic Adenocarcinoma of Mouse Prostate mice (Powolny et al., 2011). The study of PEITC on androgen-responsive prostate cancer suggested that PEITC exhibited little effect on the regulation of cell cycle, proliferation or androgen-dependent pathways. Instead, PEITC significantly suppressed androgen-responsive prostate tumor growth (Hudson et al., 2012). The suppressive effect of PEITC on human prostate cancer cell LNCaP tumor xenograft was not related to apoptosis markers. Indeed, changes of genes associated with inflammation, such as IL-6, and extracellular matrix were identified (Li et al., 2013).

Both *in vitro* and *in vivo* studies of PEITC on prostate cancer confirmed the promising anti-cancer properties of PEITC, but the molecular mechanisms and regulation of pathways were not fully understood. The comparison of results from *in vitro* and *in vivo* studies of PEITC on prostate cancer suggested that the mechanisms of prostate cancer regulation by PEITC differ between *in vitro* and *in vivo* models. The findings from *in vivo* studies on PEITC indicated that a possible association between the regulation of androgen-responsive prostate tumor growth and immune response by PEITC treatment might exist.

1.5.2. PEITC and inflammation

The studies of the correlation between PEITC and regulation of inflammation indicated that PEITC has anti-inflammatory effects on mouse macrophages (Park et al., 2013). Furthermore, the anti-inflammatory effects of PEITC on mouse macrophage RAW264.7

cells might be mediated through Toll-interleukin-1 receptor domain-containing adapter inducing interferon-β (TRIF)-dependent signaling pathway (Park et al., 2013). In addition, PEITC was able to repress LPS induced IL-1β, IL-6, IL-10, nitric oxide, COX-2 and TNF-α in mouse macrophage RAW264.7 cells (Tsai et al., 2010; Lee et al., 2011).

The anti-inflammatory effects of PEITC on human immune cell lines have been less studied, and the mechanisms are not fully understood. In *in vitro* study, the effect of PEITC was only reported on human mast cell line HMC-1. It was found that the inhibition of IL-1β and IL-6 expression by PEITC might be caused by caspase-1 pathway (Moon & Kim, 2012). For *in vivo* study, a promotion of immune response by PEITC in normal and WEHI-3 leukemia BALB/c mice were found (Tsou et al., 2013). During the progression of androgen-responsive prostate cancer, hormone-responsive prostate cancer cells such as LNCaP are able to secrete C-C chemokine ligand 2 (CCL2). CCL2 is an inflammatory chemokine (Soria & Ben-Baruch, 2008), and the secretion of CCL2 from prostate cancer LNCaP cells are able to recruit inflammatory monocytes through binding to its receptor CCR2 (Kim et al., 2013).

Together, the observations implicate that there may be an association between the regulation of prostate tumor growth and inflammation by PEITC.

Chapter 2: Transcriptional and translational uncoupling in regulation of the CXCL12 and its receptors CXCR4, 7 in THP-1 monocytes and macrophages

2.1. Abstract

The chemokine CXCL12 and its receptors CXCR4 and 7 play crucial roles in the immune system. In the present study, regulation of this pathway was further examined using the *in-vitro* model of undifferentiated human THP-1 monocytes (u-THP-1) and phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 macrophages (d-THP-1), to assess the effects of differentiation and the TLR4 ligand lipopolysaccharide (LPS) on the pathway. Differentiation did not affect the CXCR4, 7 mRNA levels. Interestingly, the CXCL12 and CXCR7 proteins but not CXCR4 were found to be up-regulated during differentiation. LPS, through the CD14-dependent pathway, induced CXCL12 and CXCR4, 7 mRNA levels to a greater magnitude in d- than u-THP-1. The LPS-mediated induction of CXCL12 release was confirmed using ELISA. Increased migration of monocytes was observed using conditioned medium from LPS-treated macrophages. Additionally, d-THP-1, although expressed higher CXCR7 protein levels, failed to migrate towards CXCL12. In contrast, LPS did not affect CXCR4, 7 protein levels. Hence, this study indicated that CXCL12, CXCR4, and CXCR7 were differentially expressed and regulated in u-THP-1 and d-THP-1 cells in response to external stimuli. Importantly, we reported here a novel observation that uncoupling exists between transcriptional and translational regulation of CXCR4, 7 expressions by differentiation and TLR stimuli.

2.2. Introduction

Monocytes and macrophages respond to pathogens and provide a primary defense that ultimately leads to pathogen clearance (Serbina et al., 2008; Martinez et al., 2009). During the pathogenic clearance process, differentiation of monocytes to macrophages is essential (Murray et al., 2011). Moreover, mounting immune responses in these cells often involve cross-talk between cellular signaling pathways such as the pattern recognition and chemokine receptors-mediated pathways (Lee & Kim, 2007). These cross-talks are critical for the immune system to efficiently rid the body of the pathogen, yet remain to be fully delineated. Chemokines are important signaling regulators of monocytes and macrophages trafficking, migration and thus, play an important role in immune responses (Kindt et al., 2006). Chemokines are classified into four subgroups including CC, CXC, C, and CX_3C, based on the position of cysteine residues (Ji et al., 2005). The chemokine CXC motif ligand 12 (CXCL12, or also known as stromal-derived factor-1, SDF-1) is a member of CXC subgroup. CXCL12 not only modulates immune cell migration but may also be a critical contributor in tumor metastasis, organ development, wound healing and angiogenesis (Liekens et al., 2010; Ansel & Gyster, 2001). CXCL12 is widely distributed in many types of tissues, organs and is constitutively secreted by primary blood monocytes (Liekens et al., 2010; Ansel & Cyster, 2001; Sánchez-Martín, 2011). CXCL12 acts via its guanine nucleotide-binding protein-coupled CXC motif receptors (CXCR) 4 and 7 (Duda et al., 2011). Binding of CXCL12 to CXCR4 is known to trigger the activation of CXCR4-mediated downstream events that include, activation of matrix metalloproteinase-9 enzyme, ERK signaling and the regulation of lymphocyte and phagocyte migration (Ghosh et al., 2006). CXCR7, on the other hand, is a newly discovered receptor for CXCL12 and

was reported to serve as a decoy receptor for CXCL12 in some systems (Naumann et al., 2010). The bio-function and regulation of CXCR7 in monocytes and macrophages are not as well defined compared to those of CXCR4. Hence, further elucidation of the regulation and function of CXCR7 in monocytes and macrophages are warranted.

Recent studies have reported that exposure to lipopolysaccharides (LPS), a major outer membrane component of Gram-negative bacteria and a Toll-like receptor (TLR) ligand, can induce CXCL12 expression in THP-1 monocytes (u-THP-1) at a pharmacological concentration (0.2 μg/mL) (Hung et al., 2007; Huang et al., 2012). The Toll-like receptors (TLRs) are a family of well-known pattern recognition receptors in both monocytes and macrophages (Paul-Clark et al., 2006). The TLRs are known to induce inflammatory responses that converge at NF-κB to stimulate the release of cytokines such as interleukin-1 (IL-1) and interleukin-6 (IL-6) (Acosta-Rodriguez et al., 2007; Mege et al., 1993). Also, CXCR7 but not CXCR4 has been reported to be induced by LPS at high concentration (1 μg/mL) in combination with IFN-γ, M-CSF or GM-CSF (Ma et al., 2013). It is not known whether LPS alone, at physiological concentration (<100 ng/mL), can also elicit similar responses (Huang et al., 2012). However, these results suggest that a cross-talk between TLR4 and the CXCL12/CXCR4, 7 pathway might exist. Moreover, LPS stimulation of inflammation through TLR4 involves interaction with two other proteins, CD14 and MD-2 (Gioannini & Weiss, 2007; Miyake, 2007). LPS can act through both CD14-dependent and independent pathways (Lu et al., 2008). The role of CD14 in the LPS-mediated regulation of the CXCL12/CXCR4, 7 pathways has not been reported.

In addition to the inflammatory stimuli, monocyte differentiation has also been reported to regulate CXCL12/CXCR4, 7 pathways. Gupta et al. observed up-regulation of CXCR4

mRNA level within 1 h of PMA induction of HL-60 cell differentiation (Gupta et al., 1999), but the mRNA levels declined to baseline level after 3 h. Although mRNA expression of CXCR4 has been compared among human myeloid leukemia cell lines at different differentiation states in HL-60, U-937, THP-1 and K-562 cells, the expression of CXCL12 or CXCR7 was not reported in the study (Gupta et al., 1999). Moreover, inconsistency in CXCR4 and 7 regulation exists in the literature. Contrary to Gupta et al., Ma et al. reported that, in differentiated THP-1 (d-THP-1), CXCR7 but not CXCR4 was up-regulated at mRNA and protein levels (Gupta et al., 1999; Ma et al., 2013). Hence, it is unclear whether coordinated changes in the CXCL12/CXCR4, 7 axes occurred during differentiation.

The critical nature of the CXCL12/CXCR4, 7 axes in immune responses, carcinogenesis, as well as a lack of full understanding of regulatory mechanism(s) for this pathway, prompted us to ask the following questions: 1) how do CXCL12/4, 7 respond to external stimuli such as LPS, differentiation, and whether CXCL12/CXCR4, 7 pathway in monocyte/macrophage is regulated in a coordinated manner; 2) at what concentration can LPS elicit a response in this pathway; and 3) what is the role of CD14 in LPS stimulated CXCL12/CXCR4, 7 response in monocyte/macrophage. We hypothesized that inflammatory and differentiation stimuli elicit coordinated regulation of CXCL12, CXCR4, and CXCR7 in monocyte and macrophage. The current study used the human THP-1 cell monocyte/macrophage culture model to elucidate the effects of LPS and differentiation on the CXCL12/CXCR4, 7 pathways (Auwex, 1991). Importantly, we reported here a novel observation that an uncoupling of transcriptional and translational responses of the CXCL12/CXCR4, 7 pathway occurred in monocyte and macrophage exposed to different stimuli.

2.3. Materials and Methods

2.3.1. Materials and reagents

Human monocytic leukemia cell line THP-1 was purchased from American Type Culture Collection (Manassas, VA). Phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharides from *Escherichia coli* 0111: B4 were obtained from Sigma-Aldrich (St Louis, MO). Penicillin and streptomycin (pen-strep), RPMI medium with phenol red, fetal bovine serum, TRIzol reagent, TaqMan Fast Universal PCR master mix, primers for Tbp (Hs00427620_m1), CXCL12 (Hs00171022_m1), CXCR4 (Hs00237052_m1), CXCR7 (Hs00604567_m1), TLR4 (Hs00152939_m1), TNF-α (Hs00174128_m1), CCL2 (Hs00234140_m1), IL-1β (Hs01555410_m1), IL-6 (Hs00985639_m1), NuPAGE antioxidant, NuPAGE LDS sample buffer, NuPAGE reducing agent, iBlot gel transfer stacks, and NuPAGE MES SDS running buffer were all purchased from Life Technologies (Grand Island, NY). The 8 μm pore polycarbonate membrane inserts 24-well plates, Pierce RIPA buffer, Halt protease inhibitor cocktail, 20X TBS buffer, 20X TBS-tween-20 buffer were from Thermo Fisher Scientific (Waltham, MA). Mouse IgG1 isotype control, human CD14 blocking antibody (Cat #MAB3832), human CXCL12 neutralizing antibody (Cat #AF-310-NA) and human CXCL12/SDF-1α Immunoassay ELISA kit (Cat #DSA00) were purchased from R&D Systems Inc (Minneapolis, MN). Mouse monoclonal β-actin antibody (Cat #sc-47778) and rabbit polyclonal anti-CXCR4 antibody (Cat #sc-9046) were from Santa Cruz Biotechnology (Dallas, TX). Rabbit monoclonal anti-GPCR RDC1 (CXCR7) (Cat #ab138509) antibody was purchased from Abcam (Cambridge, MA). IRDye 800 CW goat anti-mouse (Cat #925-32210) and goat anti-rabbit (Cat #925-32211) secondary antibody were obtained from LI-COR Biosciences (Lincoln, NE).

2.3.2. Cell culture

Human u-THP-1 monocytes were maintained in RPMI medium (RPMI 1640 with glutamine and phenol red, 10% FBS, 1% pen-strep). The d-THP-1 were obtained after 48 h exposure in the presence of the differentiation agent PMA (25 ng/mL) in RPMI medium.

2.3.3 Effect of LPS and differentiation on gene expression

To compare CXCL12, CXCR4, and CXCR7 mRNA levels in u-THP-1, cells were plated in RPMI medium at the density of 2.5×10^5 cells/mL in 6-well plates at 37°C in 5% CO_2. Treatment initiated after 24 h. For d-THP-1, 5×10^5 cells/mL of human THP-1 monocytes were seeded in 6-well plate in RPMI medium in the presence of differentiation agent PMA (25 ng/mL). After 48 h-treatment with PMA~~,~~ cells attached to the bottom of the plate and treatments were initiated. For u-THP-1 and d-THP-1 comparison studies, LPS treatment was for 4 h at 10 ng/mL then the cells were collected for RNA isolation and gene expression determination. For concentration and time course experiment with d-THP-1, LPS at 0, 1, 10, 25, 100 ng/mL were added to the cells after 48 h incubation in PMA. Concentrations of LPS were chosen based on effective ranges described in previous work (Huang et al., 2012). D-THP-1 cells were harvested at 0, 2, 4, 8, 10, 12, 16, 20, 24 h respectively for RNA isolation and gene expression determination.

2.3.3. Effect of CD14 blocking antibody on d-THP-1 cell's responses to LPS

The effects of CD14 blocking antibody on CXCL12 expression was studied using the anti-human CD14 mAb (Clone # 134620). D-THP-1 cells on 6-well plate were obtained as described above. Mouse IgG control and CD14 antibody (10 µg/mL) were added to the cells for 1 h. The medium was then replaced with or without 10 ng/mL of LPS and

incubated for 4 h before the cells were collected for RNA isolation and gene expression analysis.

2.3.4. RNA isolation, cDNA synthesis and Real-time PCR analysis of gene expression

RNA isolation, cDNA synthesis and Real-time PCR analysis of gene expression were performed as described previously (Hudson et al., 2012). Briefly, total RNA was isolated using the TRIzol reagent (Life Technologies, Carlsbad, CA) and Affinity Script Multiple Temperature cDNA Synthesis kit (Agilent Technologies, Santa Clara, CA) was used to reverse transcribe mRNA to cDNA. Real-time PCR was performed on ViiA7 Real-Time PCR Detection System using the TaqMan Universal Fast Master Mix and TaqMan gene expression assay (Life Technologies, Carlsbad, CA) to quantify gene expression levels of human CXCL12, CXCR4, 7, IL-1β, and IL-6, and CCL2. Human TATA box binding protein (TBP) was used as a housekeeping gene for calculation of relative expression levels using the ddCt method as previously described (Hudson et al., 2012).

2.3.5. Determination of CXCL12 production using ELISA

The d-THP-1 were obtained by culturing in RPMI medium in the presence of PMA for 48 h in 6-well plates as described above. LPS (10 ng/mL) was added and media (100 µl) were collected every 4 h during the 24 h treatment. For u-THP-1 and d-THP-1 comparison study, cells were cultured in 6-well plate as described above and media (100 µl) were collected from both u-THP-1 and d-THP-1 treated with/without 10 ng/mL of LPS for 24 h. CXCL12 protein levels in the media were determined using a commercially available human CXCL12/SDF-1α ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

2.3.6. Western blot analysis of CXCR4, 7 protein levels

The protein levels of CXCR4 and CXCR7 in u-THP-1 and d-THP-1 with/without LPS treatments were assessed using Western blot analysis. The u-THP-1 and d-THP-1 cells (5×10^5 cells/mL) were treated with/without 10 ng/mL of LPS for 2, 4, and 24 h in T-175 cm^2 flasks and were harvested. For u-THP-1, cells were washed once with 15 mL of phosphate-buffered saline (PBS) and centrifuged at 1500 rpm for 5 minutes. After centrifugation, PBS was removed and cells were lysed in 200 µL RIPA buffer containing EDTA and protease inhibitors. The lysates were homogenized on ice three times (10s each) using a Branson digital sonifier (Branson, CT), samples were then centrifuged at 10000 rpm for 15 min at 4 °C. The supernatant was collected and protein concentrations were tested using BCA assay following manufacturer's protocol (Thermo Scientific, Rockford, IL). For d-THP-1, cells were washed with 15 mL of PBS and scraped off using cell Falcon scraper (Corning Life Sciences, Acton, MA). The harvested cells were then centrifuged at 1500 rpm for 5 minutes and 200 µL RIPA lysis buffer containing EDTA and protease inhibitors was added to cells after PBS removal. The lysates were homogenized three times on ice (10 s each), using a Branson digital sonifier (Branson, CT), samples were then centrifuged at 10000 rpm for 15 min at 4 °C. The supernatant was collected and protein concentration was determined using the Pierce BCA assay following manufacturer's protocol (Thermo Scientific, Rockford, IL). Routinely, 5 µg per sample was used for electrophoresis separation on SDS-PAGE using 10% Bis-Tris gel following manufacturer's protocol (NUPAGE, Invitrogen, Carlsbad, CA). After electrophoresis, the protein was transferred from the gel onto a nitrocellulose membrane using iBlot apparatus according to manufacturer's procedure (Invitrogen, Carlsbad, CA). After the transfer, nitrocellulose

membrane was then blocked in 1X TBS buffer with 5% milk for 1 h at room temperature while shaking, followed by washing with SuperBlock T20 (TBS) blocking buffer (3 times, 5 min each). After washing, the membrane was then incubated with loading control mouse monoclonal β-actin antibody (1:1000), primary antibody rabbit polyclonal anti-CXCR4 (1:2000) or rabbit monoclonal anti-GPCR RDC1/CXCR7 (1:10000) in blocking solution overnight at 4 °C. After overnight incubation, the membrane was washed with 1X TBS-Tween 20 buffer (3 times, 5 min each) and incubated with IRDye 800 CW goat anti-mouse secondary antibody (1:20000 for β-actin), and IRDye 800 CW goat anti-rabbit secondary antibody (1:10000 for CXCR4 and 1:20000 for CXCR7) in SuperBlock T20 for 2 h, in the dark, at room temperature. After secondary antibody incubation, the membrane was washed using 1X TBS-Tween 20 buffer and preserved in 1X TBS buffer before imaging and quantitation. Specific CXCR4 and CXCR7 proteins were detected and quantitated using the LICOR ODYSSEY® CLx Infrared Imager according to manufacturer's procedure (LiCOR, Lincoln, NE).

2.3.7. Cell migration assay

The migration of THP-1 cells towards CXCL12 as a functional assay was assessed according to a published method (Wong et al., 2001). An 8 μm pore polycarbonate membrane insert (Thermo Fisher Scientific Inc, Pittsburgh, PA) in 24-well plates was used for the chemotaxis assay. For conditioned media experiment, d-THP-1 cell medium collected after 24 h treatment of LPS induction (10 ng/mL) was used as conditioned medium. Conditioned media (400 μL) were added to the bottom wells of the 24-well chemotaxis chambers. The u-THP-1 (200 μL, 1×10^6 cells/mL) were added to the upper chamber and incubated at 37°C in 5% CO_2. After 5 h, the insert was removed, stained with

Trypan Blue and the cells migrated to the bottom wells were counted under microscopy (10X magnification) using hemocytometer. Experiments where CXCL12 neutralizing antibody was used to assess the specific effect of CXCL12 on migration (Wong et al., 2001), conditioned media collected were treated with/without a neutralizing CXCL12 antibody for 1 h before being added to the lower bottom wells, and numbers of cells migrated after 5 h were counted as described above. To compare u-THP-1 and d-THP-1 migration ability, u-THP-1 (200 μL, 1×10^6 cells/mL) were added to the upper chamber, CXCL12 containing media (10 ng/mL) was placed at the bottom well and incubated at 37°C/5% CO_2. After 5 h, the insert was removed, stained with Trypan Blue and the cells migrated to the bottom wells were counted under the microscope (10X magnification) using hemocytometer. For d-THP-1 cell migration/chemotaxis assay, 24-well plates with 8 μm pore polycarbonate membrane inserts (Thermo Fisher Scientific Inc, Pittsburgh, PA) were used. THP-1 monocytes (200 μL of 1×10^6 cells/mL) with 25 ng/mL PMA in RPMI medium were added to the upper inserts and 400 μL of RPMI medium were added to the bottom wells of the 24-well chemotaxis chambers. After 48 h differentiation at 37°C/5% CO_2, media in the upper inserts were changed to fresh media and media at the bottom were replaced with CXCL12 containing media (10 ng/mL). After 5 h, cells in the upper inserts were removed using cotton swabs for the counting of migrated cells. For the total cell number, cells in upper inserts were not removed by swabbing. Cells were stained with crystal violet (0.2% in 20% ethanol). After 20 min of staining, inserts were rinsed 3 times using DI water, and the plates were placed in the hood to dry in the air. Finally, crystal violet was dissolved in 10% acetic acid (500 μL) and the absorbance of the solution was read at 560 nm using the Molecular Devices SPECTRAMAX 384Plus (Sunnyvale, CA).

2.3.8. Statistical analysis

All experiments were performed in triplicate, and data were reported as the mean ± standard deviation (SD). GraphPad Prism for Windows (Prism 4, GraphPad Software Inc., La Jolla, CA) was used for statistical analysis. Depending on the experimental design, multiple group experiments were analyzed using one- or two-way ANOVA followed by posthoc test. P values ≤ 0.05 were considered significant.

2.4. Results

2.4.1. Comparison of CXCL12, CXCR4, 7 expression pattern in u-THP-1 and d-THP-1 cells

CXCL12 mRNA expressions in THP-1 were significantly induced (1.68-fold) after PMA-induced differentiation (Fig. 2.1). In contrast, there was no difference in CXCR4 or CXCR7 mRNA levels between u-THP-1 and d-THP-1. In comparison, the differentiation marker CD14, cytokines IL-1β and IL-6 mRNA were up-regulated in d-THP-1 by 62.48-fold, 149.44-fold, and 174.56-fold, respectively (Fig. 2.1). LPS treatment of u-THP-1 or d-THP-1 significantly elevated CXCL12, IL-1β, and IL-6 mRNA levels (Fig. 2.1). The magnitude of inductions on CXCL12 and IL-6 mRNA expressions by LPS (10 ng/mL) were much greater in d-THP-1 than those in u-THP-1. In u-THP-1, CXCL12 and IL-6 were increased by 2.97-fold and 48.32-fold, respectively. In d-THP-1, CXCL12 and IL-6 were up-regulated by 11.71-fold and 150.91-fold, respectively. Additionally, LPS induced CXCR7 mRNA level in both u-THP-1 and d-THP-1 cells by 1.81-fold and 15.06-fold, respectively. However, we only detected an increase in CXCR4 mRNA level in d-THP-1 but not in u-THP-1 upon LPS induction (Fig. 2.2).

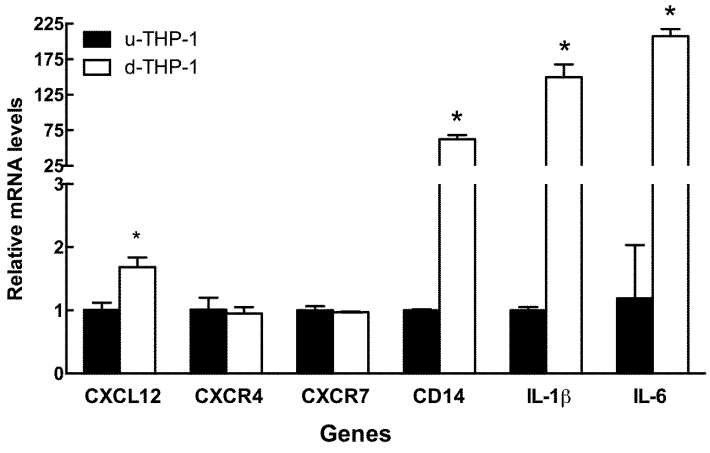

Fig. 2. 1. Comparison of CXCL12, CXCR4, CXCR7, CD14, IL-1β, and IL-6 mRNA levels in u-THP-1 and d-THP-1 without LPS induction. Undifferentiated (u-THP-1) and differentiated (d-THP-1) were cultured, total RNA isolated and gene expression of CXCL12, CXCR4, CXCR7, CD14, IL-1β, and IL-6 were determined using RT-PCR as described in Materials and Methods. Results were normalized to u-THP-1 and expressed as relative mRNA levels (mean ± SD, n = 3). P values ≤ 0.05 were considered significant. * indicates significantly different from u-THP-1. Black bar: u-THP-1, White bar: d-THP-1.

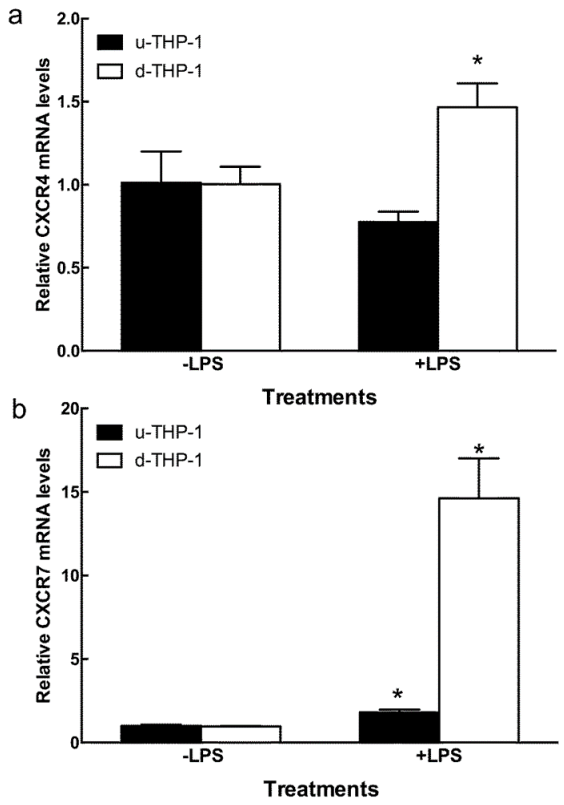

Fig. 2.2. Comparison of LPS induction of CXCR4, 7 expressions in u-THP-1 and d-THP-1. (a) CXCR4. (b) CXCR7. u-THP-1 (2.5 × 105 cells/mL) and d-THP-1 (5 × 105 cells/mL) treated with 10 ng/mL of LPS for 2 h, total RNA isolated and mRNA levels of CXCL4 and 7 were determined using RT-PCR as described in Materials and Methods. Results were normalized to vehicle control and expressed as relative mRNA levels (mean ± SD, n = 3). P values ≤ 0.05 were considered significant and * indicates significantly different from control. (a) CXCR4. (b) CXCR7. Black bar: u-THP-1, White bar: d-THP-1.

2.4.2. Characterization of LPS-induced CXCL12/CXCR4, 7 in d-THP-1 cells

Given that LPS elicits a more robust response in d-THP-1, the time and concentration-dependent effects of LPS on CXCL12, and CXCR4, 7 mRNAs were further characterized in d-THP-1 cells. LPS induced CXCL12 mRNA expression in a dose- and time-dependent manner. CXCL12 mRNA level peaked at 4, 4, 10 and 12 h with 1, 10, 25 and 100 ng/mL of LPS, respectively (Fig 2.3a). CXCL12 mRNA declined almost to the baseline 24 h after addition of LPS. Interestingly, CXCL12 showed distinct LPS induction patterns compared to CXCR4, 7 (Fig. 2.3b-c). CXCR4, 7 mRNA levels both peaked at 2 h after LPS induction, and the magnitude of CXCR7 induction was much higher compared to CXCR4. CXCR7 mRNA increased by as much as 12.4-fold following 2 h of LPS induction (25 ng/mL), while CXCR4 mRNA only elevated by 2.6-fold in the same condition. Additionally, unlike CXCL12, the expression of CXCR4, 7 mRNA leveled off at 25 ng/mL LPS.

As a comparison, LPS significantly up-regulated relative mRNA levels of IL-1β and IL-6, two well documented LPS-responsive cytokines in d-THP-1 (Mullen et al., 2010). The expressions peaked at 4 h (Fig. 2.4a-b). Both IL-1β and IL-6 showed time- and concentration-dependent up-regulation by LPS.

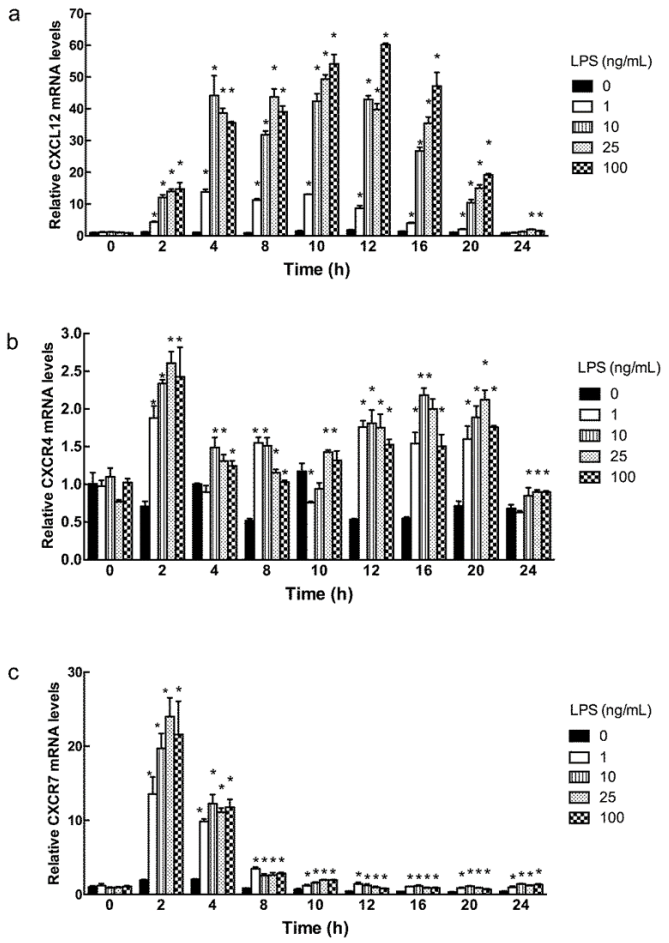

Fig. 2.3. Concentration- and time-dependent effects of LPS on CXCL12 and CXCR4, 7 expression in d-THP-1 cells. (a) CXCL12. (b) CXCR4. (c) CXCR7. d-THP-1 were treated with 0, 1, 10, 25 and 100 ng/mL of LPS and harvested at 0, 2, 4, 8, 10, 12, 16, 20 and 24 h. Total RNA isolated and mRNA levels for CXCL12, CXCR4, CXCR7 were determined using RT-PCR as described in Materials and Methods. Results were normalized to 0 h and expressed as relative mRNA levels (mean ± SD, n = 3). P values ≤ 0.05 were considered significant and * indicates significantly different from 0 ng/mL of LPS treatment. (a) CXCL12. (b) CXCR4. (c) CXCR7.

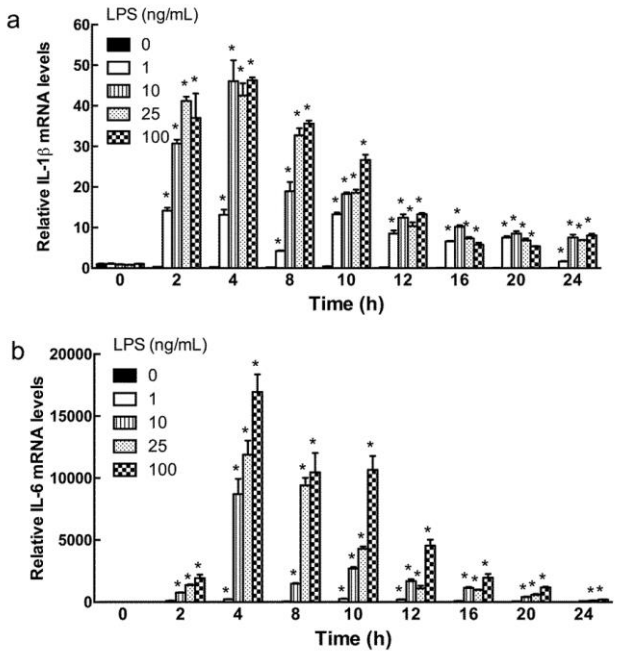

Fig. 2.4. Effects of LPS on IL-1β and IL-6 expression in d-THP-1 cells. d-THP-1 were treated with 0, 1, 10, 25 and 100 ng/mL of LPS and harvested at 0, 2, 4, 8, 10, 12, 16, 20 and 24 h. Total RNA isolated and mRNA levels of IL-1β and IL-6 were determined using RT-PCR as described in Materials and Methods. Results were normalized to 0 h and expressed as relative mRNA levels (mean ± SD, n = 3). P values ≤ 0.05 were considered significant and * indicates significantly different from 0 ng/mL of LPS treatment. (a) IL-1β. (b) IL-6.

2.4.3. Blocking antibody against CD14 inhibited CXCL12-related gene expression in LPS induced d-THP-1

LPS acts through both CD14-dependent and CD14-independent pathways (Lu et al., 2008). The role of CD14 in response to LPS in the regulation of CXCL12 and its receptors were tested. After 1 h pre-treatment using human CD14 blocking antibody, LPS induction of CXCL12 mRNA level was effectively reduced by 78.7% when compared to the IgG control (Fig. 2.5a). In contrast, the effect of CD14 blocking antibody on CXCR7 inhibition was relatively lower than that on CXCL12. LPS induction of CXCR7 was inhibited by 26.4%,

compared to the IgG control (Fig. 2.5b-c). CD14-independent gene, TNF-α, was not affected by CD14 blocking antibody (Fig. 2.5d). At 2 h treatment with 10 ng/mL LPS, TLR4 mRNA level was reduced by 49.8% compared to IgG control (Fig. 2.5e). Consistently, mRNA expressions of the known downstream genes of TLR4 including IL-1β, IL-6, and CCL2 were all attenuated by CD14 blocking antibody. IL-1β mRNA was reduced by 47.0% while IL-6 and CCL2 mRNA reduced by 97.5% and 97.0% respectively as compared to the IgG control (Fig. 2.5f-h).

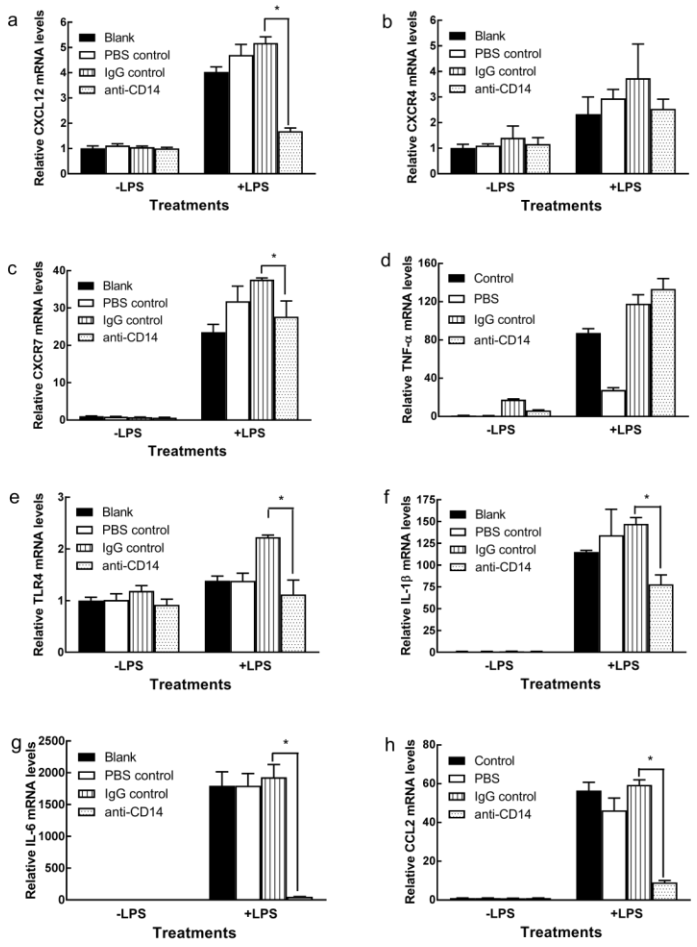

Fig. 2.5 a-g. Effects of anti-CD14 antibody on LPS induction of gene expression in d-THP-1 cells. d-THP-1 were treated with mouse IgG isotype control or anti-CD14 antibody (10 μg/mL) for 1 h then medium was

replaced with fresh medium with or without 10 ng/mL of LPS for an additional 4 h. After LPS treatment, cells were harvested, total RNA isolated and mRNA levels of CXCL12, CXCR4, CXCR7, TNF-α, TLR4, IL-1β, IL-6, and CCL2 were determined using RT-PCR as described in Materials and Methods. Results were normalized to vehicle control and expressed as relative mRNA levels (mean ± SD, $n = 3$). P values ≤ 0.05 were considered significant and * indicates significantly different from control. (a) CXCR12. (b) CXCR4. (c) CXCR7. (d) TNF-α. (e) TLR4 (f) IL-1β. (g) IL-6. (h) CCL2.

2.4.4. LPS induced CXCL12 protein secretion in THP-1 cells

Human CXCL12 protein level was determined by ELISA using media collected from d-THP-1 treated with LPS (10 ng/mL) for 24 h. CXCL12 levels remained low in media for the first 12 h, but its secretion significantly increased (6.73-fold, $p<0.001$) following 24 h of LPS treatment (Fig. 2.6a). Comparing u-THP-1 and d-THP-1, significantly elevated CXCL12 protein level was observed in media from d-THP-1 ($p < 0.001$) after 24 h exposure to LPS, but not from u-THP-1 (Fig. 2.6b).

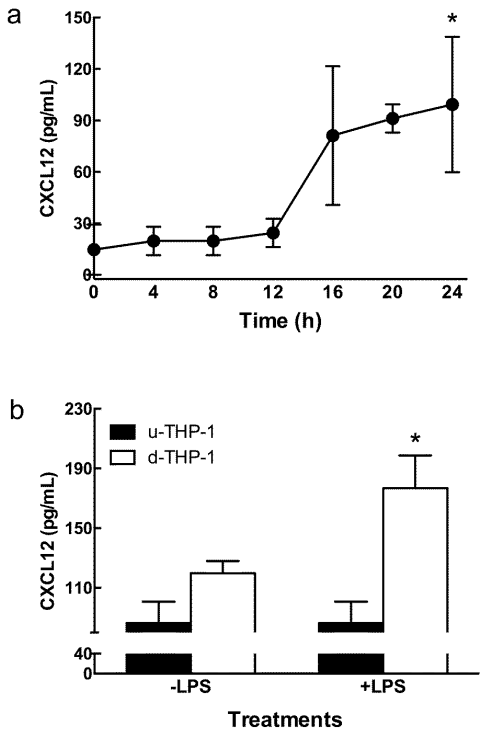

Fig. 2.6. Effects of LPS on CXCL12 protein in THP-1 cells. (a) Time-dependent effect of LPS on CXCL12 protein in d-THP-1. Data were reported as mean CXCL12 protein (pg/mL) ± SEM, n = 3. P values ≤ 0.05 were considered significant and * indicates significantly different from 0 h. (b) Comparison of LPS effect on CXCL12 protein in u-THP-1 and d-THP-1. U-THP-1 or d-THP-1 cells were cultured and treated with LPS (10 ng/mL). Medium harvested after 24 h and CXCL12 protein were determined using ELISA as described in the Materials and Methods. Data were reported as mean CXCL12 protein (pg/mL) ± SD, n = 3. P values ≤ 0.05 were considered significant and * indicates significantly different from vehicle control.

2.4.5. Effect of differentiation and LPS on CXCR4, 7 protein levels in THP-1 cells

Effect of differentiation and LPS on CXCR4, 7 proteins were examined using western blot. Differentiation significantly increased CXCR7 protein (Fig. 2.7a) level, but not CXCR4 protein (Fig. 2.7b) level. On the other hand, unlike the results at the mRNA level, no difference was observed in CXCR4, 7 protein levels when cells were treated with LPS (Fig. 2.7a-b).

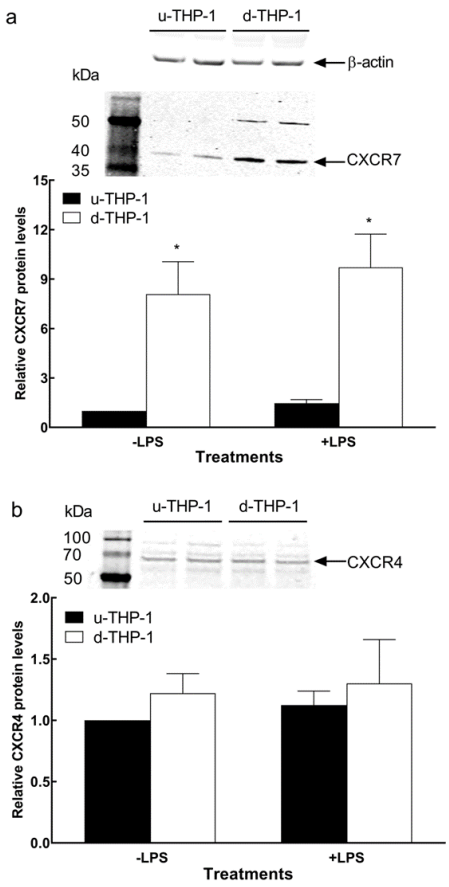

Fig. 2.7. Effects of LPS on CXCR4, 7 protein levels in u-THP-1 and d-THP-1. (a) β-actin (used as loading control protein) and CXCR7, (b) CXCR4. Cells were cultured and treated with or without LPS. Cells were harvested for protein determination and western blot as described in Materials and Methods.

Immunoreactive bands were quantitated using ODYSSEY® CLx Infrared Imaging System as described in Materials and Methods. Results were normalized to vehicle control and expressed as relative protein levels (mean ± SD, n = 3). P values ≤ 0.05 were considered significant and * indicates significantly different from control.

1. Effect of differentiation on gene expressions

Genes	CXCL12	CXCR4	CXCR 7
mRNA	↑	-	-
protein	↑	-	↑

2. Effect of LPS on gene expressions

a. u-THP-1

Genes	CXCL12	CXCR4	CXCR 7
mRNA	↑	-	↑
protein	-	-	-

b. d-THP-1

Genes	CXCL12	CXCR4	CXCR 7
mRNA	↑	↑	↑
protein	↑	-	-

Table 2.1. The effects of 1) THP-1 differentiation and 2) LPS stimulation on expressions of CXCL12 and CXCR4, 7 in THP-1 cells at mRNA and protein level.

2.4.6. CXCL12 neutralizing antibody blocked u-THP-1 migration towards d-THP-1-conditioned media

Migration of u-THP-1 towards d-THP-1-conditioned media in the presence or absence of a neutralizing antibody to CXCL12 was used as a functional assay to validate the presence of CXCL12 (Fig. 2.8). The media collected from 2 h LPS (10 ng/mL) treated d-THP-1 without CXCL12 antibody accelerated u-THP-1 cell migration by 11.3 to 12.9-fold compared to that without LPS treatment. Treatment of conditioned media with CXCL12

neutralizing antibody but not IgG control significant inhibited LPS-induced migratory effect (Fig. 2.8).

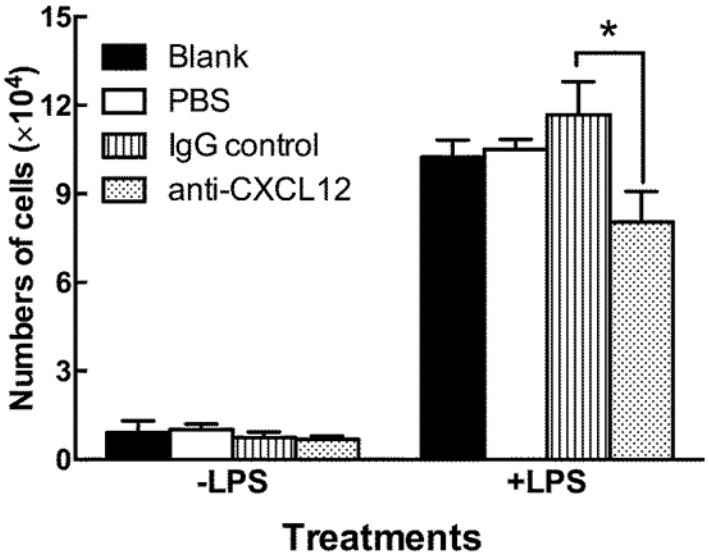

Fig.2.8. Effect of LPS and anti-CXCL12 antibody on u-THP-1 migration towards conditioned media. THP-1 migration towards conditioned media harvested from d-THP-1 treated with or without LPS (10 ng/mL) was conducted as described in Materials and Methods. Cells migrated to the bottom wells were stained with Trypan Blue and counted under the microscope. For experiment using the anti-CXCL12 antibody, the conditioned media were pre-treated with IgG isotype control or anti-CXCL12 antibody for 1 h prior to initiation of migration. Results were normalized to vehicle control and expressed as mean ± SD (n = 3). P values ≤ 0.05 were considered significant and * indicates significantly different from control.

2.4.7. Comparison of migration of u-and d-THP-1 towards CXCL12

The ability of cells to migrate toward CXCL12 was compared between u-THP-1 and d-THP-1. In the absence of CXCL12, a chemoattractant, the percentage of d-THP-1 cells migrated to the bottom well was about half of that of u-THP-1 (Fig. 2.9). In the presence of CXCL12 (10 ng/mL), the percentage of u-THP-1 migrated to the bottom well was

significantly increased (2.05-fold, $p<0.001$). In contrast, no difference in migration was detected in d-THP-1 with or without CXCL12 treatment (Fig. 2.9).

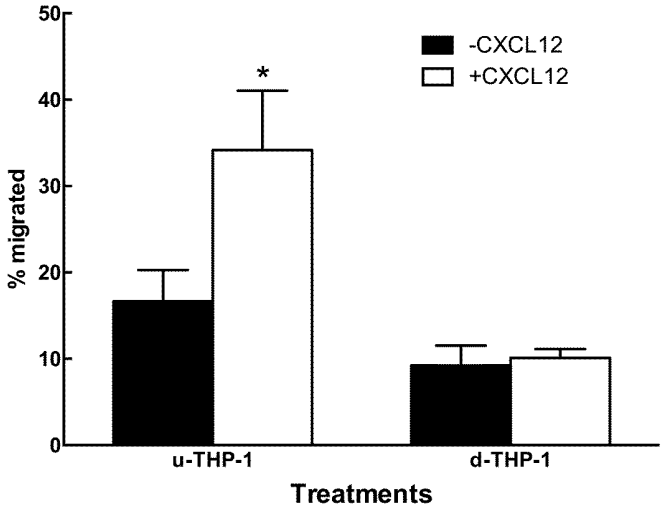

Fig. 2.9. Migration of u-THP-1 and d-THP-1 towards CXCL12 (10 ng/mL). uTHP-1 and d-THP-1 migration towards CXCL12 (10 ng/mL) were conducted as described in Materials and Methods. u-THP-1 cells migrated to the bottom wells (with 10 ng/mL of CXCL12) were stained with Trypan Blue after 5 h of migration and counted under the microscope. For d-THP-1, monocytes were treated with PMA (25 ng/mL) for 48 h. After cell differentiation, migration of d-THP-1 towards 10 ng/mL of CXCL12 (5 h) was tested. For migrated cell counting, cells in the upper inserts were removed using cotton swabs. For total cell counting, cells in upper inserts were not removed by swabbing. Crystal violet-stained cells were rinsed, dried and re-dissolved in 10% acetic acid. Absorbance was read at 560 nm. Results were normalized to vehicle control and expressed as mean ± SD (n = 3). P values ≤ 0.05 were considered significant and * indicates significantly different from control.

2.5. Discussion

The current study addresses several deficiencies in the literature regarding the regulation of CXCL12 and its receptors in monocyte/macrophages. We provide mechanistic insights into the regulation of the CXCL12/CXCR4, 7-pathway through detailed analyses of responses of the CXCL12/CXCR4, 7 pathway toward differentiation and a TLR4 stimulus, LPS. We also provide data supporting the role of CD14 in LPS-induced response through the pathway. More importantly, as summarized in Table 1, we report here a novel observation that an uncoupling between transcriptional and translational machinery exists in the regulation of CXCL12/CXCR4, 7 pathway by different stimuli.

2.5.1 Comparison of CXCL12, CXCR4, 7, IL-1β and IL-6 expression patterns in u-THP-1 and d-THP-1 cells

In this study, we found differentiation of monocyte to macrophage had no effect on CXCR4 and CXCR7 at mRNA level. In contrast, the cytokines CXCL12, IL-1β and IL-6 mRNA were significantly increased upon differentiation. Interestingly, despite the lack of difference in CXCR7 mRNA level, at the protein level, both CXCL12 and CXCR7 proteins were significantly increased in d-THP-1 compared to u-THP-1. Protein levels sometimes might not be correlated with mRNA levels, especially in higher organisms (Anderson & Seilhamer, 1997; Tian et al., 2004). Post-transcriptional regulation of gene expression, mRNA decay, translation, protein half-lives and protein degradation play crucial roles in the determination of steady-state protein concentrations (Anderson & Seilhamer, 1997; Greenbaum et al., 2003; Vogel et al., 2010). The observation of this study supports that, during differentiation, uncoupling exists between mRNA and protein expressions of CXCR7. The lack of CXCR4 response to PMA at the mRNA level, observed in this study,

is different from a previous report in which Gupta et al. reported an increase of CXCR4 mRNA expression during HL-60 monocyte differentiation within 1 h after the stimulation of GM-CSF (Gupta et al., 1999). This may be partially explained by the different cell types, inducing agent, and length of treatment used in the two studies, which warrant further elucidation.

2.5.2 Comparison of transcriptional to translational expressions of CXCL12, CXCR4/7

The transcriptional and translational uncoupling was also observed in the responses of THP-1 cells to the TLR4 stimulus, LPS. At mRNA level, induction with LPS for 4 h significantly increased both CXCR4, 7 in d-THP-1 when compared to that in u-THP-1. However, neither CXCR4 nor 7 proteins changed upon exposure to LPS. In contrast, CXCL12 mRNA, as well as protein, was induced by LPS, which is consistent with a recent report (Hung et al., 2007), supporting inflammatory effect elicited by bacteria is in part mediated through regulation of CXCL12 but not CXCR4, 7. Moreover, we observed that LPS at a physiologically relevant 20-fold lower concentration (10 ng/mL) (Hung et la., 2007; Huang et al., 2012) than that reported by Hung et al., elicited a response through CXCL12. These data further support a physiological role for CXCL12 in immune response towards bacteria in monocytes/macrophages. The responses of the CXCL12/CXCR4, 7 pathway toward inflammatory stimuli also differ between monocytes and macrophages. Treatment with LPS elicits stronger responses in d-THP-1 cells than those in u-THP-1 cells for CXCL12, CXCR4, 7 at mRNA level, similar to the effect of LPS on other well documented LPS-responsive genes, such as IL-1β and IL-6.

2.5.3 Blocking antibody against CD14 inhibited CXCL12-related gene expression in LPS induced d-THP-1

The results show that LPS induction of CXCL12 and CXCR4, 7 mRNAs is CD14 dependent, as pre-treatment of CD14 blocking antibody inhibited LPS induced increase of CXCL12, CXCR7 mRNA expressions. Also, CD14 was up-regulated during differentiation. Hence, this enhanced transcriptional response may be in part due to the increase in CD14 (Schwende et al., 1996). Interestingly, CD14 blocking antibody appeared to differentially block LPS induction of downstream genes. CXCL12, IL-6, and CCL2 expressions appeared to be inhibited to a greater extent than those of CXCR4, 7 or IL-1β. These results suggest that CXCR4, 7 and IL-1β mRNA may be also regulated by CD14 independent pathway. Additional studies are warranted to dissect these differences.

2.5.4 Effects of CXCL12 neutralizing antibody on u-THP-1 migration towards d-THP-1-conditioned media

Conditioned media from the LPS induction of d-THP-1 significantly increased the migratory effect of u-THP-1 (Fig. 4), which is consistent with a previous observation by Gouwy et al (Gouwy et al., 2011). However, in our study the protein level of CXCL12 after LPS stimulation reached around 0.1 ng/mL in the media, this concentration is much lower than 1 ng/mL reported by Gouwy et al. It was also observed in this study that neutralizing CXCL12 in conditioned media using CXCL12 antibody partially inhibited u-THP-1 migration by 31.0% when compared to the IgG control. This is consistent with LPS induction of other cytokines such as CCL5, CCL3, and CCL8 that synergize with CXCL12

to affect monocyte migration but support the fact that CXCL12 also plays a crucial role in promoting monocyte migration (Gouwy et al., 2011).

2.5.5 Comparison of migration of u-and d-THP-1 towards CXCL12

CXCL12 is a relatively ubiquitously expressed chemokine (Moyer et al., 2007). As shown in our conditioned media migration assay, production of CXCL12 by macrophage will further attract monocytes. Monocyte, which has lower expression level of CXCR7, will migrate to the inflamed site and enhance the immune response. Given the proposed role of CXCR7 as a decoy receptor (Naumann et al., 2010), the higher CXCR7 protein levels in combination with no change in CXCR4 protein levels in differentiated macrophage may help the macrophage reside at the inflamed local site instead of migrating toward CXCL12 produced by other tissues. Our comparison of u-THP-1 and d-THP-1 migration supported this notion. We observed that u-THP-1 and not d-THP-1 migrated toward CXCL12 (Fig. 6S, Supplements). Hence, we consider that CXCR7 serves as a decoy receptor in the macrophage which binds to CXCL12 and short circuit the CXCL12/CXCR4 interaction.

Activation of CXCL12/CXCR4 not only is involved in the migration of monocyte but also the migration of T cell (Zou et al., 2004), dendritic cell (Kabashima et al., 2007), neuronal cell as well as a cancer cell (Tiveron & Cremer, 2008). For example, migration of prostate cancer cell could be induced by CXCL12/CXCR4 activation through Akt-1 and MMP-9 signaling pathway and migration of pancreatic cancer cells is modulated through MMP-2 and MMP-9 pathway (Chinni et al., 2006; Shen et al., 2013). The chemotactic effect on multiple cell lines by CXCL12 and its receptors might indicate the global importance of CXCL12/CXCR4, 7 chemokine axis in the modulation of cell migration and mediates cross-talk as well as the interaction between immune and cancer cells. More importantly,

the role of CXCR7 needs to be further defined. Based on our results as well as others', we reason that CXCR7 serves as a decoy to CXCR4 and can potentially nullify CXCL12's biological effects including migration. Human peripheral blood monocytes are known to contain multiple cell sub-types (Ziegler-Heitbrock et al., 2010; Hofer et al., 2015) and differentiation signal such as those induced by PMA also can lead to the generation of multiple cell types (Zhao et al., 2003). It remains unclear how the CXCL12/CXCR4,7 pathways are regulated among the cells. Understanding the precise role of CXCR7 in other cell types would be critical in future studies.

2.6. Conclusion

In summary, the CXCL12/CXCR4, 7-axis in THP-1 cells is subject to multiple regulations including differentiation and TLR4 ligand. The CXCL12/CXCR4, 7 chemokine axis is differentially regulated when exposed to differentiation agent and TLR-mediated inflammatory stimuli. Additionally, we observed the uncoupling of CXCR4, 7 expressions at the transcriptional and translational level. Regulation at post-transcriptional level appeared to contribute to changes in CXCR7. Also, CXCL12 appeared to work in concert with other chemokines in the regulation of monocyte migration. Finally, our results also support a decoy role for CXCR7 in monocyte/macrophage. Overall these changes may facilitate and direct cell migration, localization during inflammation.

Chapter 3: Transcriptomic Analysis of Androgen-dependent Human Prostate Cancer LNCaP Cells and its Tumor Xenograft to Elucidate the Roles of Tumor Environment

3.1. Abstract

LNCaP athymic xenograft model has been widely used in pre-clinical research allowing researchers to study human diseases. However, the biological characteristics of human LNCaP cells before/after implanting in athymic mouse and its relevance to clinical human prostate outcomes are not known. In this study, transcriptome profiles and pathways of human prostate LNCaP cells before (*in vitro*) and after (*in vivo*) implanting into xenograft mouse were compared using RNA-sequencing technology (RNA-seq). Gene expressions were also compared with the findings of *in silico* clinical prostate tumors studies. The results of this research indicated that CXCL12 (26.703-fold) and CXCR7 (-4.12-fold) were the only chemokine/chemokine receptors changed in LNCaP xenograft tumor as compared to cultured cells. Genes modulating cell proliferation, such as F3 and CREB3L, were increased by 210.04 and 182.71-fold, respectively. A shift from androgen-responsive to androgen non-response status was observed in cultured cells as compared to LNCaP xenograft tumor. Rho family GTPase signaling was found to be the canonical pathway that was changed in LNCaP xenograft tumor. Compared to clinical outcomes, the LNCaP xenograft tumor consistently regulated genes associated with RhoB GTPase signaling, such as RhoB, ITGA1, and ACTA2. The androgen receptor and its determinants, such as UGT2B15 and FLNA, were oppositely modulated. The data indicated that the alterations of genes in LNCaP xenograft tumor were not all the same as compared to the clinical outcomes of prostate cancer progression. The regulation of CXCR7, AR, UGT2B15, F3, FLNA and RhoB were also affected by tumor microenvironment factors such as androgen,

hypoxia, tumor cell-immune cell interaction and subcellular matrix. These results suggest that the tumor environments might be associated with the regulation of genes in xenograft tumor.

3.2. Introduction

Understanding the biology of prostate cancer is important for the prevention of this disease and/or development of therapeutic strategies. Animal models are the most commonly used methods for the study of prostate cancer initiation, development and treatment modalities (Wu et al., 2013). Among numerous animal prostate cancer models, genetically engineered mouse (GEM) (Parisotto & Metzger, 2013), chemical carcinogenic (Ito et al., 1991) and xenograft models (Mavropoulos et al., 2009) are extensively used to investigate the molecular and cellular mechanisms underlying prostate cancer progression (Greenberg et al., 1995; Leenders et al., 2008). The GEM model includes TRAMP mice model, LADY, PTEN knockout and c-Myc overexpression (Grabowska et al., 2014; Wu et al., 2013; Carnero & Paramio, 2014). The chemical carcinogenic model involves the use of chemical carcinogens, or in combination with testosterone, to induce prostate carcinomas in the hosts. The available prostate cancer models are not perfect and do not allow one to address specific questions (Bibby, 2004; Ittmann et al., 2013). Compared to GEM and chemical carcinogenic models, xenograft model has an advantage in allowing researchers to introduce known human prostate cancer cells into the hosts, which is thought to be more relevant to the study of human prostate pathophysiology than the other two models (Grabowska et al., 2014). Balb/C Nu/Nu, NMRI/Nu, Severely Combined Immune Deficient (SCID), and RAG and NOD/SCID are the four major hosts used in prostate xenograft study (Chung et al., 2007). The Balb/C Nu/Nu model has a deficient cell-

mediated immune response by lacking a functional thymus due to Foxn1 mutation (Pandelouris, 1968). Balb/C Nu/Nu mouse is one of the most common hosts for xenotransplantation of human tumors allowing tumor size measurement (Fleshner et al., 1999). There is more than one way to do xenografting, including implantation of patient-derived tumor tissue (Morton & Houghton, 2007), orthotopic injection of cultured human cell line into mouse prostate (Weerden et al., 2009), and subcutaneous injection of cultured human cell line into immunodeficient mouse (Zhuang et al., 2005). The patient-derived tumor model requires patient subjects, and it would increase the individual variation (Stranger et al., 2007) and the difficulties of replicating the study. Drawbacks of orthotopic model are that the transplantation requires expertise and the tumor growth in mouse prostate would be hard to monitor in time (Weerden et al, 2009). Subcutaneous xenograft model increases the feasibility of the experiments, allows the measurement of tumor sizes, and lowers the difficulties of replicating the study. The most commonly used cell lines for prostate xenograft models are human prostate cancer LNCaP (androgen sensitive), DU145, and PC-3 (androgen non-sensitive) cell lines (Wu et al., 2013; Kumar et al., 2008). Both PC-3 and DU145 are representatives of the early androgen depletion independent prostate cancers (Litvinov et al., 2006), and the LNCaP cells are hormonally responsive from a metastatic lesion of human prostatic adenocarcinoma (Horoszewicz et al., 1983). The LNCaP cell line is usually used to study the regulation of prostate cancer progression in response to hormones (Young et al., 1991).

Compared to cells in culture, the in vivo xenograft tumor can allow studies of more complicated factors, including microenvironment (Polyak et al., 2009), exposure to androgen (Hamilton et al., 1984), blood flow (Lekås et al., 1997), hypoxia (Chan et al.,

2007), interaction between implanted human cells and mouse immune cells (Itescu et al., 1998) as well as body metabolism (Niles et al., 2006). These interactions may lead to the alterations of gene expression and regulation of the development and progression of implanted human prostate cancer cells and prostate tumor. However, such investigation has not been reported to the knowledge of the authors. In addition, the main purpose of animal study is to mimic or estimate the clinical cancer progression, study the mechanisms, and provide preventive or therapeutic strategies. The relevance between the mouse xenograft model and clinical human prostate tumor studies is critical, but it is not well documented in prostate cancer research.

The next-generation sequencing (NGS) technology is a revolutionary tool for the analysis of transcriptomics (Metzker, 2010). The RNA-seq (Mortazavi & Williams, 2008) uses NGS to quantify and identify the global behavior of transcriptomes (Wilhelm & Landry, 2009). Compared to other technologies such as microarrays, this method allows the measurement of a complete set of transcriptomes in cells or tissues with a more precise measurement of transcriptional levels of a gene (Wang et al., 2009). In addition, the transcript structures, such as alternatively spliced transcript isoforms, could also be determined using this method (Wilhelm & Landry, 2009). The general process of this technique includes a conversion of RNA to a library of cDNA fragments and the attachment of adaptors to one or both ends of the fragments. Then, the samples go through high-throughput sequencing from one end (single-end sequencing) or both ends (pair-end sequencing) (Wang et al., 2009). Bioinformatic tools such as CLC genomic workbench were then used to analyze and visualize the next generation sequence (NGS) data (Cánovas et al., 2010). Ingenuity

Pathways Analysis (IPA) (Ingenuity Systems, www.ingenuity.com) can be used to identify enriched bio-function terms and canonical pathways (Huang et al., 2011).

This study compared the global transcriptomes of human prostate cancer LNCaP cells, before and after implanting, in athymic Balb/C Nu/Nu mouse xenograft models by RNA-seq technology. The genetic alterations in human LNCaP xenograft tumor model was then compared to the global expressions of clinical human prostate cancer samples data, and the relevance between animal model and clinical human prostate tumor was compared. Findings are expected to help better understand the characteristics of xenograft model and its relevance to clinical prostate cancer outcomes. Results from this study would also provide valuable suggestions for selecting the best model for different purposes of the experimental prostate cancer studies.

3.3. Materials and Methods

3.3.1. Chemicals and reagents

Cell culture medium RPMI-1640 medium with or without phenol red, fetal bovine serum, and TRIzol reagent were purchased from Life Technologies (Grand Island, NY). Antibiotic agent 100X penicillin-streptomycin mix (pen-strep) was obtained from Sigma-Aldrich (St Louis, MO). Matrigel was obtained from BD Biosciences (Mansfield, MA). TaqMan fast universal PCR master mix, primers CXCL12 Hs00171022_m1, CXCR7 Hs00604567_m1, AR Hs00171172_m1, F3 Hs01076029_m1, CREB3L Hs00962115_m1, FLNA Hs0092465_m1, ITGA1 Hs00235006_m1, RhoB Hs03676562_s1, ACTA2 Hs04406862_m1, and UGT2B15 Hs00870076_s1 were from Life Technologies (Grand Island, NY). All chemicals were analytical reagent grade.

3.3.2. Cell and cell culture

The LNCaP human prostate cancer cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured with RPMI 1640 medium with phenol red containing 10% fetal bovine serum (FBS) and 1% pen-strep at a concentration of 2.5×10^5 cells/mL in 175 cm^2 flasks at 37°C with 5% CO_2.

3.3.3. In vivo xenograft Bioassay

The experimental protocol was approved by the USDA Beltsville Area Animal Care and Use Committee. Male athymic nude mice (BALB/c nu/nu, 20-22 g, 5-6 weeks old; Charles River, Frederick, MD) were individually kept in HEPA filter-top cages, and they consumed food and fresh tap water ad libitum. After 3 weeks on a control AIN-93M diet, LNCaP xenografts were initiated in the mice by subcutaneous injections of 2×10^6 cells in 50 mL of phosphate-buffered saline (PBS) plus 50 ml Matrigel (BD Biosciences, Mansfield, MA) in the flank. Mice remained on their respective diets for 7 weeks after cell injection until tumors reached 2-3 cm^3 in volume and were then sacrificed. Portions of tumor tissues were quickly frozen in liquid nitrogen and stored at -80 ºC for mRNA and protein analysis as described below.

3.3.4. RNA extraction and sequencing using RNA-seq technology

To examine the transcriptome profiles of LNCaP cells and xenograft tumor, total RNA of tumor and cell samples were extracted using Trizol and then purified using DNase digestion and Qiagen RNeasy column following the manufacturer's instructions (Qiagen, Valencia, CA). The integrity of RNA samples was determined using an Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA). An Illumina RNA-seq sample prep kit was used to verify the high-quality RNA (Illumina, San Diego, CA). RNA-seq libraries were obtained using an

Illumina GAIIx sequencer at 40 bp/sequence with a depth of approximately 24.8 million sequences for each sample.

3.3.5. Data analysis and bioinformatics

CLC Genomics Workbench was used to generate expression values (CLC Bio, Aarhus, Denmark). Reads were trimmed by quality control filters and mapped to the human genome to remove murine contaminants. Total gene expression was quantified using the RNA-seq Analysis tool (Chitwood et al., 2013) allowing no more than 2 mismatches per reading. The analysis of canonical pathways, functions, networks for differentially regulated genes were performed using the Ingenuity Pathways Analysis (IPA, Ingenuity Systems, and www.ingenuity.com). The regulations and activation status of pathways were predicted by IPA using z-score and an overlapping *p*-value based on the number of genes, fold-changes, and literature database (Chamorro et al., 2017). Genes or networks with *p*-value less than 0.05 and z-score greater than 2 or less than -2 were considered as significantly altered.

3.3.6. Principal component analysis

The principal component analysis (PCA) was conducted using default programmed covariance-estimation in CLC to identify and visualize differences between treatment groups. The plot was shown in a plot with eigenvector with one eigenvalue. Principal components in plot were grouped by color.

3.3.7. Volcano plot analysis

Volcano plot was used to examine the differences/fold changes of the genes from the mean values. Those with significant changes in transcriptome profiles were analyzed using CLC Genomics Workbench (CLC Bio, Aarhus, Denmark). Differences in genes were shown in

a 2-D scatter plot. Gene expressions were based on log2 ratio in X-axis, and the *p*-value was represented as –log10. The genes colored blue were significantly down-regulated ($p \leq 0.05$; z-score$\leq$-2), and the genes colored with red were significantly up-regulated ($p \leq 0.05$; z-score$\geq$2).

3.3.8. Canonical pathway analysis of data sets

Ingenuity Pathways Analysis (IPA, Ingenuity Systems, and www.ingenuity.com) was used to analyze the changes in canonical signaling events (Dalby et al., 2007). Genes were mapped to the Ingenuity Pathways Knowledge Base and overlaid to a global molecular network. The most altered pathways for targets with significance from IPA library of canonical pathways were determined using right-tailed Fisher's test. P-value was used to indicate the probability of correlation between input genes and the IPA canonical pathway reference database.

3.3.9. Network/pathways graphical representation

The networks of pathways were generated using IPA. Up-stream and down-stream regulators and alterations of genes were graphically represented. Nodes were used to represent genes in networks, and the intensity of nodes indicate the degree of regulation (Li & Capuco, 2008). Genes that were up-regulated were marked by red color, and down-regulated genes were marked by green color.

3.3.10 Effects of androgen on gene expressions

The effects of androgen on LNCaP gene expressions were analyzed as previously described (Wang et al., 2008). Briefly, 2.5×10^5 cells/mL of LNCaP were plated in 6-well plates and cultured in RPMI-1640 media with phenol red and 10% FBS. After 24 h, cell media were replaced by RPMI-1640 media containing 10% CDS without phenol red. After 24 h, cells

were treated with or without 10 nM DHT for 48 h. After 48 h treatment, RNA was isolated and cDNA was synthesized as previously described (Hudson et al., 2012) and expressions of target genes were examined using RT-PCR as described below.

3.3.11 Effects of hypoxia on gene expressions

The effect of hypoxia on gene expressions in LNCaP was investigated as follows. Briefly, LNCaP cells were seeded at 2.5×10^5 cells/mL in 6-well plates in RPMI-1640 media with phenol red and 10% FBS. After 24 h, the media was replaced with fresh media containing 150 μM of $CoCl_2$.

After 48 h treatment, RNA was isolated and cDNA was synthesized as previously described (Hudson et al., 2012) and expressions of target genes were examined using RT-PCR as described below.

3.3.12 Effects of tumor cell-immune cell interaction on gene expressions

The effects of tumor cell-immune cell interaction on gene expressions in LNCaP cells were tested. 5×10^5 cells/mL of u-THP-1 cells were plated with differentiation agent PMA (25 ng/mL). After 48 h, u-THP-1 cells were differentiated into d-THP-1, and the culture media, media collected from d-THP-1 were added to 2.5×10^5 cells/mL of LNCaP cells without/with 10 ng/mL of LPS. After 24 h, RNA was isolated and cDNA was synthesized as previously described (Hudson et al., 2012) and expressions of target genes were examined using RT-PCR as described below.

3.3.13 Effects of subcellular matrix on gene expressions

For 3D cell culturing, prechilled matrigel (4 °C overnight) was mixed with LNCaP cells at a concentration of 0.85 × 106 cells/mL. The mixture was incubated at 37 °C to allow the matrigel to gel. After 30 min, 1.5 mL of cell culture media were added to cells. After 72 h

of cell growth, culture media were removed and cells were washed 3 times by cold PBS. 2 mL of corning cell recovery solution were added to cells and cells were separated from matrigel. RNA was isolated and cDNA was synthesized as previously described (Hudson et al., 2012) and expressions of target genes were examined using RT-PCR as described below.

3.3.14. Real-time PCR analysis of gene expression

Real-time PCR was used to quantify changes in relative mRNA levels as previously described (Hudson et al., 2012). Briefly, 1 μg of total RNA was used for cDNA synthesis using the AffinityScript multi-Temperature cDNA Synthesis kit according to manufacturer's protocol. Real-time PCR was performed using the TaqMan Fast Universal PCR Master Mix following the previous published protocol (Huang et al., 2012). TaqMan gene expression assay was used to detect mRNA levels of CXCL12, CXCR7, AR, F3, CREB3L, FLNA, ITGA1, RhoB, ACTA2, and UGT2B15. TATA-binding protein (Tbp) mRNA was used as housekeeping gene for normalization. Relative expression value was generated using ΔΔCt method as described previously (Huang et al., 2012).

3.3.15. Gene expressions in normal, normal adjacent, primary and metastatic prostate tissues in Silico

AR, CXCR4, S100A07, FLNA, MAPK12, UGT2B15, F3, CXCR7, IQGAP1, and RhoB mRNA expression data were queried through the Gene Expression Omnibus (GEO) using gene name and prostate cancer as key words. Data from accession number GDS#2545, which contain a complete set of normal, normal adjacent, primary and metastatic prostate tissues, were selected for analysis. Gene counts from 4 tissue sets including normal, normal

adjacent, primary and metastatic prostate tissues (171 samples) were analyzed and compared.

3.3.16. Statistical analysis

The false discovery rate (FDR) adjusted p-value (Student's t-test) was used to identify the significance in bio-informatic data analysis, and value less than 0.05 was considered significant (CLC Bio, Aarhus, Denmark). For analysis of pathways using IPA, z-score was used to predict the activation or inhibition of biological functions. Pathways with a Z-score greater than 2 was considered as significantly activated, and that less than -2 was considered as significantly inhibited (IPA, Ingenuity Systems, and www.ingenuity.com).

3.4 Results

3.4.1. Principal component analysis of LNCaP xenograft tumor as compared to LNCaP cells

To obtain a probabilistic interpretation of LNCaP xenograft and cell samples, principal component analysis (PCA) was performed. In this study, two individual sets of LNCaP xenograft tumor samples #1 and #2 were analyzed and compared to the LNCaP cultured cells, to assure the authenticity of changes of genes in animal models compared to *in vitro* cultured cells. Fig.3.1 showed that both sets of tumor samples (red and cyan) were generally well-separated from the cell samples (blue) in the vertical direction. The PCA analysis result indicated a difference between LNCaP cultured cells and xenograft tumor groups.

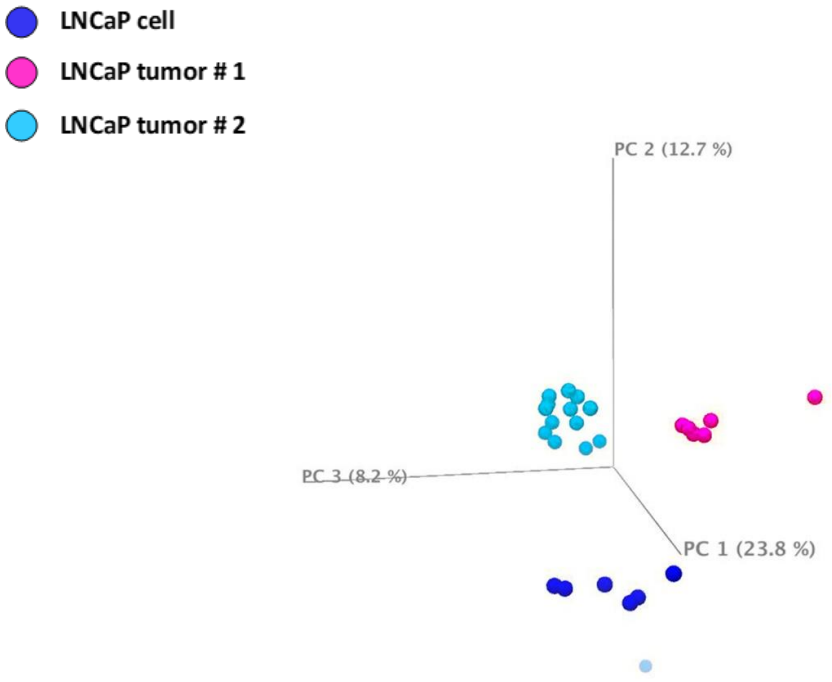

Fig. 3.1 PCA of xenograft tumor as compared to LNCaP cells. Sets of tumor samples were colored in red and cyan and cultured cell samples were colored in blue.

3.4.2. Volcano plot of LNCaP xenograft tumor as compared to cultured LNCaP cells

The changes of global transcriptomic profiles in LNCaP xenograft tumor #1 as compared to LNCaP cultured cells were analyzed using volcano scatter-plot (Fig.3.2). The fold-changes of expressions were represented using a log2 scale on the X-axis, and the *P*-value was represented as -log10 on the Y-axis. Dots with the blue color indicated genes with significant lower expressions, and red color suggested genes with higher expressions. In LNCaP xenograft tumor, more genes with higher expressions were observed compared to LNCaP cells in culture.

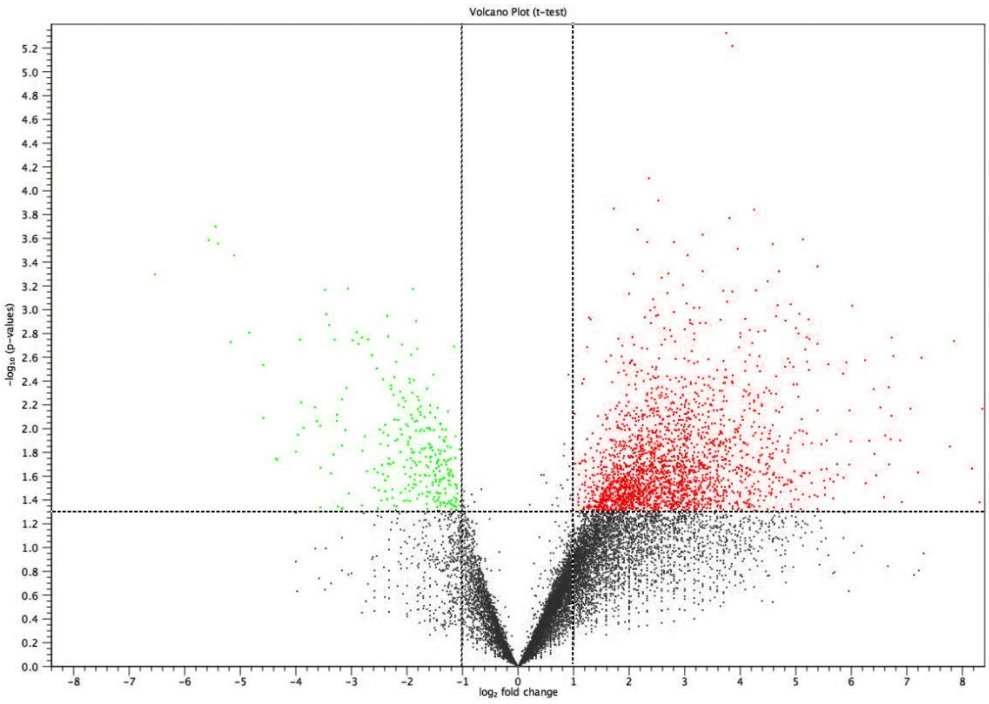

Fig. 3.2. Volcano plot of transcript profiles in xenograft tumor as compared to LNCaP cells. Transcriptome profiles with z-score less than -2 were marked with green color, and with z-score greater than 2 were marked with red color.

3.4.3. Genes expressions in LNCaP tumor compared to cultured cells

The top 10 genes with higher and lower expressions were identified based on their fold changes (Table 3.1a and b). The 4 genes expressed with higher levels in LNCaP xenograft tumors, F3, CREB3L1, ORM1, and RGS2, were increased by 210.04, 182.71, 174.68, and 138.16-fold as compared to LNCaP cultured cells respectively. In the genes with higher expression, F3, CREB3L1, ANGPT2, and THSD7A are involved in cell migration and proliferation (Genecards, NCBI; Carcia-Conesa et al., 2009; Wang et al., 2010; Chaudhari et al., 2014). ORM1 plays a role in infection and inflammation (Dijk et al., 1991). STEAP4 and ADM modulate homeostasis (Edwards et al., 1997; Guo et al., 2011). The expression of RGS2 is associated with the lower expression of androgen-independent AR (Cao et al., 2006).

As for the top 10 genes with lower expressions, FCAMR was the one with lowest expression, by -62.57-fold compared to cultured cells (Table 3.1b). FCAMR has been reported to be involved in the regulation of immune disorders (Takai et al., 2006). In addition, 4 out of 10 of the top genes with lower expressions in LNCaP tumors, including UDP-glucuronosyltransferase 2B15 (UGT2B15), UGT2B17, UGT2B10, and UGT2B28, are from UDP-glucuronosyltransferases family. These genes played pivotal roles in the regulation of androgen metabolism (Nadeau et al., 2011). The UGT2B15, UGT2B17, UGT2B10 and UGT2B28 enzymes exhibited lower expressions, by -62.32, -54.14, -35.44 and -20.85-fold respectively as compared to cultured cells.

Together, the top genes with higher and lower expressions in LNCaP xenograft tumor compared to LNCaP cultured cells indicate alterations of genes in the regulation of androgen, immune response, cell migration and proliferation in the LNCaP tumor.

(a) Higher

Symbol	*P*-value	Fold Change	Gene _id
F3	2.63874E-95	210.043	ENSG00000117525
CREB3L1	6.10533E-88	182.705	ENSG00000157613
ORM1	6.7098E-41	174.684	ENSG00000187681
RGS2	2.18059E-89	138.156	ENSG00000116741
COL12A1	1.14882E-76	110.37	ENSG00000111799
STEAP4	6.29119E-54	71.682	ENSG00000127954
ANGPT2	5.17556E-36	67.692	ENSG00000091879
FMOD	4.01081E-64	57.586	ENSG00000122176
THSD7A	1.94077E-23	57.437	ENSG00000005108
ADM	1.27368E-33	52.696	ENSG00000148926

(b) Lower

Symbol	*P*-value	Fold Change	Gene_id
FCAMR	3.20808E-67	-62.565	ENSG00000162897
UGT2B15	9.49572E-71	-62.323	ENSG00000197592
UGT2B17	5.23692E-59	-54.141	ENSG00000197888
GPX8	1.86152E-27	-35.5	ENSG00000164294
UGT2B10	1.4451E-19	-35.444	ENSG00000109181
TP53INP1	4.22144E-31	-31.76	ENSG00000164938
UGT2B28	2.00907E-14	-20.846	ENSG00000135226
UHMK1	1.61483E-55	-19.664	ENSG00000152332
CPS1	4.92146E-49	-19.014	ENSG00000021826
KCNG1	5.24257E-31	-15.132	ENSG00000026559

Table 3.1. Genes with (a) higher and (b) lower expressions in the LNCaP tumor compared to LNCaP cells.

3.4.4. Most altered canonical pathways in LNCaP xenograft tumor compared to cultured LNCaP cells

The major pathways with significant changes were investigated in LNCaP xenograft tumor compared to LNCaP cultured cells using IPA. RhoGDI signaling and signaling by Rho family GTPases were found to be the most activated and inhibited pathways (Table 3.2a and 3.2b).

(a)

Ingenuity Canonical Pathways	*P*-value	Ratio	z-score
RhoGDI Signaling	0.021	0.13	3.13

(b)

Ingenuity Canonical Pathways	*P*-value	Ratio	z-score
Signaling by Rho Family GTPases	0.00069	0.14	-3.89
Actin Cytoskeleton Signaling	2.4E-05	0.16	-3.33
Actin Nucleation by ARP-WASP Complex	0.011	0.18	-3.16
Renin-Angiotensin Signaling	6.3E-05	0.19	-2.99
P2Y Purigenic Receptor Signaling Pathway	0.00069	0.17	-2.99
Cardiac Hypertrophy Signaling	7.4E-05	0.16	-2.87
Melanocyte Development and Pigmentation Signaling	0.00059	0.18	-2.83
Rac Signaling	0.0018	0.16	-2.83
Dopamine-DARPP32 Feedback in cAMP Signaling	0.027	0.13	-2.83
CREB Signaling in Neurons	0.0026	0.14	-2.71

Table 3.2. Most (a) up-regulated and (b) down-regulated canonical pathways in the LNCaP tumor as compared to LNCaP cells.

3.4.5. Regulation of genes in RhoGDI signaling pathway in LNCaP xenograft tumor as compared to LNCaP cultured cells

The regulation of genes associated with RhoGDI signaling pathway in LNCaP xenograft tumor as compared to LNCaP cultured cells was shown in Fig.3.3. In the RhoGDI signaling pathway, inhibition of cadherin, integrin, and Gα caused the lower expressions of guanine nucleotide exchange factors (ARHGEF). Both Rac/Cdc42 guanine nucleotide factor 6 (ARHGEF6) and Rho guanine nucleotide exchange factor 12 (ARHGEF12) exhibited lower expressions. The inhibition of Rho-associated coiled tailing (ROCK) might be related to a higher expression of Myosin-binding subunit MLC phosphatase (MYPT) such

as protein phosphatase12C (PPP1R12C), and myosin light chain 5 (MLC5). On the other hand, the expressions of ezrin/radixin/moesin (ERM) and F-actin were lower.

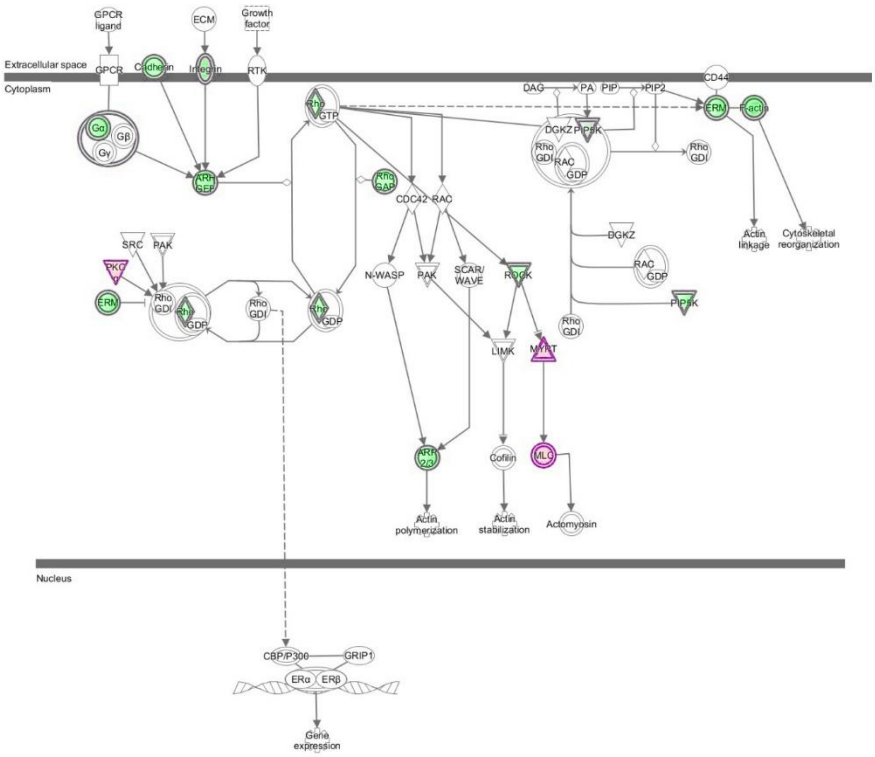

Fig. 3.3. Regulation of genes in RhoGDI signaling pathway in LNCaP xenograft tumor as compared to LNCaP cultured cells. Up-regulated genes were marked with pink color. Down-regulated genes were marked with green color.

3.4.6. Regulation of genes signaling by Rho family GTPases in LNCaP xenograft tumor as compared to cultured LNCaP cells

The regulation of genes involved in signaling by Rho family GTPases in LNCaP xenograft tumor compared to cultured cells was illustrated in Fig. 3.4. Cells interact with extracellular molecules such as extracellular matrix, growth factors, and G-protein coupled receptor ligands and bind to integrin, receptor tyrosine kinase (RTK) and G-protein coupled receptor

(GPCR). These molecules act upon Rac/Cdc42 guanine nucleotide factors (ARHGEFs) and regulate GTPases Rho, Rac, and Cdc42. Both ARHGEF6 and ARHGEF12 were down-regulated. The down-regulation of ARHGEF resulted in an inhibition of the GTPase Rho and further reduced the expression of BORG, ROCK, and PIP5K. The down-regulation of BROG and Septin is critical to the regulation of cytokinesis in endosomal trafficking. The inhibition of Rho also inhibited Rho kinase 1 (ROCK1) and ROCK2. On the other hand, the down-regulation of both ROCK and PIP5K decreased the expression of Rho kinase phosphorylates ezrin-radixin-moesin (ERM) which might result in an inhibition of the actin-membrane linkage. A down-regulation of atypical protein kinase C (PKC) was also observed. As a result, the PAR and PKC complex would regulate the orientation of the microtubule-organizing center (MTOC). Finally, the decrease of ARP2/3 and F-actin might be associated with the regulation of actin polymerization. The down-regulation of IQGAP1 might affect cell-cell adhesion and migration.

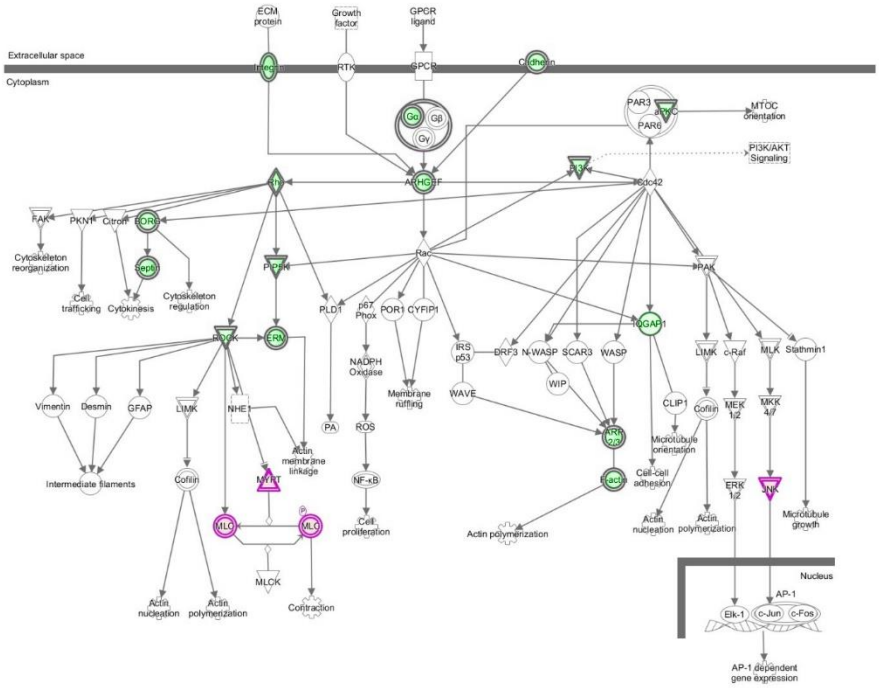

Fig. 3.4. Regulation of genes in signaling by Rho family GTPases in LNCaP xenograft tumor as compared to cultured LNCaP cells.

3.4.7. Genes alteration in (a) androgen-dependent and (b) androgen-independent pathways in LNCaP xenograft tumor compared to LNCaP cell line

A significant inhibition in androgen-dependent pathway associated genes and an increase in androgen-independent pathway related genes were identified (Table 3.3a and 3.3b). For the androgen-dependent pathway, heat shock protein 90 (Hsp 90) is an important therapeutic target for the androgen-dependent prostate cancer (Fang et al., 1996). Both Hsp90AB1 and Hsp90AA2 were significantly decreased in LNCaP xenograft tumor as compared to LNCaP cultured cells. Together with the other two down-regulated notable AR interactors for prostate cancer, FLNA and FKBP4 (Lin et al., 2007), possible inhibition of AR-regulated signaling pathways might exist.

Genes associated with androgen-independent pathways, including MAPK12, Akt3, WNT3A, and IGFBP7 were all up-regulated. MAPK12 is a member of mitogen-activated protein kinase family (Greenblatt et al., 2013). It regulates the transduction of extracellular signals (Taj et al., 2010). MAPK12 is a stress-activated protein kinase, and its activation might be associated with an increase in stress from the microenvironment (Turner et al., 2011). Lin et al. have reported a differential regulation of AR by PI3K/Akt pathway at different stages of prostate cancer development (Lin et al., 2003). The activation of PI3K/Akt pathway is closely associated with the prostate cancer progression. The WNT3A is a gene from WNT family. This gene has been implicated in the regulation of oncogenesis and in developmental processes, such as cell fate and patterning during embryogenesis (Peifer et al., 2000). Finally, IGFBP7 encodes a member of the insulin-like growth factor (IGF)-binding protein (IGFBP) family. IGFBPs bind

IGFs with high affinity and regulate IGF availability in body fluids and tissues and modulate IGF binding to its receptors (Oh et al., 1996). The inhibition of genes in the androgen-dependent pathway and the up-regulation of genes in androgen-independent pathway implicated a shift from androgen responsive to androgen non-responsive status of the cells in xenograft tumor as compared to LNCaP cell line.

(a)

Gene_id	Symbol	Entrez Gene Name	Fold Change	*p-value*
ENSG00000096384	HSP90AB1	heat shock protein 90 alpha family class B member 1	-1.68	1.41E-05
ENSG00000080824	HSP90AA2	heat shock protein 90 alpha family class A member 2	-3.47	0
ENSG00000196924	FLNA	filamin A	-2.36	6.67E-12
ENSG00000004478	FKBP4	FK506 binding protein 4	-2.05	1.27E-07

(b)

Gene_id	Symbol	Entrez Gene Name	Fold Change	*p*-value
ENSG00000188130	MAPK12	mitogen-activated protein kinase 12	1.96	2.65E-08
ENSG00000117020	AKT3	AKT serine/threonine kinase 3	4.56	3.77E-07
ENSG00000154342	WNT3A	Wnt family member 3A	11.17	3.60E-08
ENSG00000163453	IGFBP7	Insulin like growth factor binding protein 7	27.00	9.96E-34

Table 3.3. Genes altered in androgen-dependent and androgen-independent pathways (a) gene alterations in androgen-dependent pathway (b) gene alterations in the androgen-independent pathway. *P* value gets down as "0" represents the value less than 2.234e-30.

3.4.8. Network of AR regulation in LNCaP xenograft tumor compared to cultured cells

Androgen receptor (AR) wxhibited lower expression in LNCaP xenograft tumor compared to cultured cells ($p < 0.05$). As shown in the network of AR regulation (Fig. 3.5), -AR was directly modulated by calpain, COPA, and FKBP4, and indirectly regulated by genes including N4BP2, USP38, HUWE1, and CAND1. Stephen et al have reported that the activation of calpain cleaves the androgen receptor (AR) into an androgen-independent isoform (Stephen et al., 2007). In this study, the calpain was significantly up-regulated, indicating a possible calpain-dependent proteolysis of the AR. The information of COPA (Coatomer Protein Complex Subunit Alpha) is a protein-coding gene which is associated with the regulation of hereditary autoimmune-mediated lung disease and arthritis (Wetkin et al., 2015). The down-regulation of COPA explained the inhibition of AR. In addition, the co-chaperone protein FKBP4 regulates steroid receptor signaling through Hsp90 (Harrison et al., 2007). Down-regulation of Hsp90 accounts for the inhibition of AR (Georget et al., 2002).

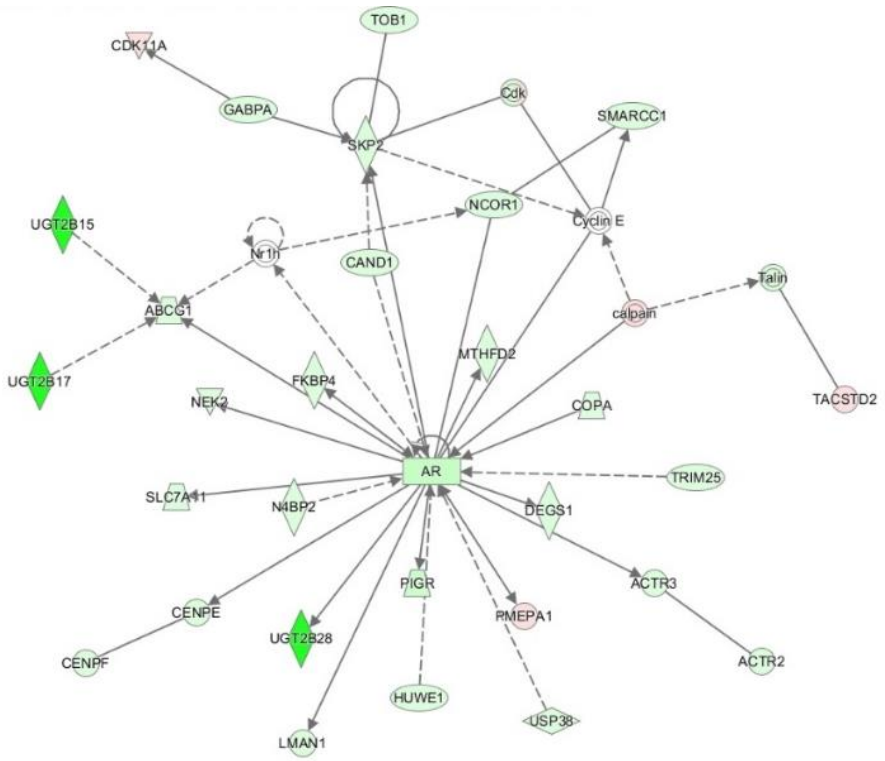

Fig. 3. 5. Regulation of androgen receptor (AR) in LNCaP xenograft tumor as compared to LNCaP cultured cells. Genes with higher expressions were marked with red color. Genes with lower expressions were marked with green color.

3.4.9. Most altered molecular and cellular functions in xenograft LNCaP tumor as compared to LNCaP cultured cells

Across the database, the most significant alteration on biofunctions of LNCaP in xenograft model was in the cellular movement (Table 3.4). IPA analysis identified 302 genes associated with the cellular movement that were differentially regulated in xenograft LNCaP tumor as compared to LNCaP cultured cells (*p*-value: 6.51E-10 to 2.55E-3). In addition, genes regulating cell development, growth, and proliferation, cell-to-cell signaling and interaction, as well as cell death and survival were all changed.

Function Annotation	p-value Range	Number of Genes
Cellular Movement	6.51E-10 - 2.55E-03	302
Cellular Development	7.70E-09 - 1.98E-03	360
Cellular Growth and Proliferation	7.70E-09 - 1.83E-03	339
Cell-To-Cell Signaling and Interaction	3.15E-07 - 1.83E-03	141
Cell Death and Survival	4.96E-07 - 2.63E-03	384

Table 3.4. Most molecular and cellular function alterations in xenograft LNCaP tumor as compared to LNCaP cultured cells.

3.4.10. Alteration of chemokine and chemokine receptors in LNCaP xenograft tumor compared to cultured cells

CXCL12 and its receptor CXCR7 (Table 3.5. $P < 0.05$) were the only chemokine and receptor observed with significant alterations in xenograft model as compared to the cultured LNCaP cells. In prostate cancer, the CXC-motif ligand 12 (CXCL12) and its seven-transmembrane trimeric G-protein–coupled receptor CXC-motif receptor 7 (CXCR7) have been reported to play a role in angiogenesis, invasion and metastasis of prostate cancer (Chinni et al., 2008; Wang et al., 2008). Binding of CXCL12 to CXCR7 modulates a broad range of cellular activities, including cancer cell proliferation, survival, and adhesion (Zheng, Li, et al., 2010).

Gene_id	Symbol	Expr Fold Change	*p*-value
ENSG00000107562	CXCL12	26.70	9.61E-36
ENSG00000144476	CXCR7	-4.12	1.25E-06

Table 3.5. Alterations of chemokines and receptors in xenograft LNCaP tumor as compared to LNCaP cultured cells.

3.4.11. Networks for CXCL12 regulation in LNCaP xenograft tumor as compared to LNCaP cultured cells

CXCL12 has been reported to play a pivotal role in the regulation of invasion and metastasis of prostate cancer to lymph nodes, lung, and bone (Chinni et al., 2008; Wang, Shiozawa et al., 2008). In this study, the CXCL12 was found to be the most up-regulated chemokine (by 26.70-fold) in LNCaP xenograft tumor compared to LNCaP cell line.

The networks of CXCL12 showed that a total of 18 genes related to CXCL12 involved pathways were significantly up-regulated, which includes MMP2, a direct regulator for

CXCL12 (Fig. 3.6). Four genes involved in the CXCL12 down-stream signaling pathways, including S100A7, CCNT1, FOSB, and UHMK, were significantly inhibited. Besides MMP2, C-Jun NH2-terminal kinases (JNK) also regulated CXCL12.

For the downstream pathways, the increase of CXCL12 expression resulted in an indirect up-regulation of TIMP1 and down-regulation of CCNT1. TIMP1 is a cytokine functioning as a tissue inhibitor of matrix metalloproteinases (MMPs). Recent clinical studies have demonstrated that TIMP-1 is elevated in cancer patient plasma and enhances prostate associated fibroblasts proliferation and migration (Gong et al., 2013). *In addition,* CCNT1 functions as regulators for cyclin-dependent kinase (CDK), which is involved in the modulation of the cell cycle (Tahirov et al., 2010).

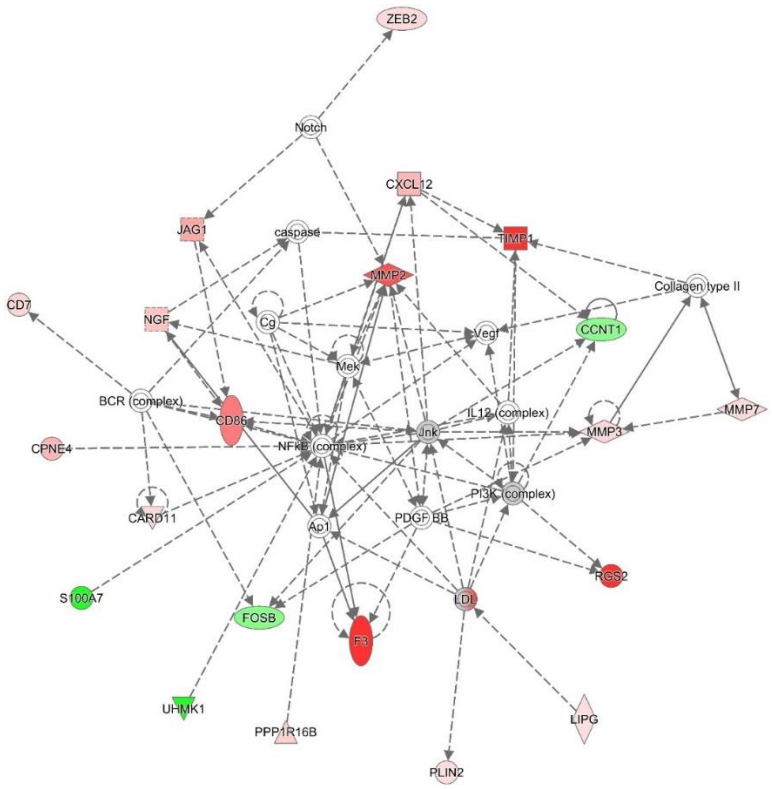

Fig. 3.6. Networks for CXCL12 regulation in LNCaP xenograft tumor compared to LNCaP cultured cells. Up-regulated genes were marked with red color. Down-regulated genes were marked with green color.

3.4.12. Genes alteration associated with cell-matrix adhesion

The overall architecture of a tissue is determined by adhesion mechanisms that involve cell-cell interactions and cell-matrix interactions (Gumbiner et al., 1996). In the progression of prostate cancer, integrins modulate various cell functions including cell migration, survival, and aberrant cellular growth (Fornaro et al., 2001). Integrin and adhesion related genes including ITGA2, ITGA6, ITGAB1, and ICAM5 were changed by -6.12, -2.75, -1.7, and -5.77-fold, respectively (Table 3.6). In contrast, ICAM1 was 5.48-fold higher in tumor samples (Table 3.6).

Gene_id	Symbol	Entrez Gene Name	Fold Change	*p*-value
ENSG00000164171	ITGA2	integrin subunit alpha 2	-6.12	6.30E-20
ENSG00000091409	ITGA6	integrin subunit alpha 6	-2.75	4.22E-14
ENSG00000150093	ITGB1	integrin subunit beta 1	-1.67	1.92E-05
ENSG00000090339	ICAM1	intercellular adhesion molecule 1	5.48	6.03E-07
ENSG00000105376	ICAM5	intercellular adhesion molecule 5	-5.77	7.54E-08

Table 3.6. Genes alteration associated with cell-matrix adhesion.

3.4.13. Most expressed mouse genes in LNCaP xenograft tumor

Gene from mouse source was also examined to identify potential changes of mouse tissues in the tumor site. Filamin A Interacting Protein 1 like (FILIP1L) was the mouse gene with the most expression (Table 3.7). Filamin A is a growth inhibitor in prostate cancer (Desotelle et al., 2012), and it acts as a regulator of the antiangiogenic activity on endothelial cells (Radder et al., 2013; GeneCards, NCBI). LRS2, the leucyl-tRNA synthetase 2, was the second most up-regulated mouse gene. An inactivation of this gene was found in 78% of primary nasopharyngeal carcinoma tissues (Zhou et al., 2009). ARHGAP21, the Rho GTPase activating protein 21, was found to be the third most up-regulated mouse gene.

Gene ID	Symbol	Total gene reads
ENSMUSG00000043336	Filip1l	92308
ENSMUSG00000035202	Lars2	87176
ENSMUSG00000036591	Arhgap21	59437
ENSMUSG00000029635	Cdk8	43540
ENSMUSG00000045999	AY036118	41628
ENSMUSG00000098178	Yam1	36436
ENSMUSG00000038518	Jarid2	36111
ENSMUSG00000023806	Rsph3b	33620
ENSMUSG00000039145	Camk1d	25117
ENSMUSG00000047454	Gphn	24316
ENSMUSG00000021665	Hexb	19576
ENSMUSG00000037965	Zc3h7a	17228
ENSMUSG00000050377	Il31ra	16513
ENSMUSG00000086324	Gm15564	13546
ENSMUSG00000028132	Tmem56	13449
ENSMUSG00000079184	Mphosph8	11986
ENSMUSG00000030255	Sspn	11147
ENSMUSG00000037742	Eef1a1	10128
ENSMUSG00000074305	Peak1	9358
ENSMUSG00000000884	Gnb1l	7412

Table 3.7. Most expressed mouse genes in LNCaP xenograft tumor.

3.4.14. Most expressed mouse (a) chemokine and chemokine receptors and (b) interleukins in xenograft tumor

In the mouse athymic xenograft tumor model, human LNCaP xenograft tumors were surrounded by mouse cells. Interaction of human xenograft tumors and mouse hosts might exist. In this study, selected groups of mouse genes, including chemokine/chemokine receptors as well as interleukins, were investigated. In the most expressed mouse genes, inflammation associated mouse genes such as chemokines (Table 3.8a) and interleukins (Table 3.8b) were expressed (expression values cut-off: 150). The data showed that the most expressed mouse chemokines were from CC and CXC family. CCL6 was the most expressed mouse CC-motif chemokine and CXCL12 was found to be the most expressed mouse CXC-motif chemokine. The two receptors for CXCL12, including CXCR4 and CXCR7, were the most expressed CXC-motif receptors found.

(a)

Gene ID	Symbol	Expression value
ENSMUSG00000018927	CCL6	787
ENSMUSG00000009185	CCL8	782
ENSMUSG00000019122	CCL9	221
ENSMUSG00000061353	CXCL12	219
ENSMUSG00000021508	CXCL14	214
ENSMUSG00000023078	CXCL13	177
ENSMUSG00000018920	CXCL16	175
ENSMUSG00000044337	CXCR7	205
ENSMUSG00000045382	CXCR4	165

(b)

Gene ID	Symbol	Expression value
ENSMUSG00000024810	IL33	190
ENSMUSG00000050377	IL31ra	18098
ENSMUSG00000030748	IL4ra	168
ENSMUSG00000022969	IL10rb	160

Table 3.8. Most expressed mouse (a) chemokine and chemokine receptors and (b) interleukins in xenograft tumor.

3.4.15. Validation of gene expressions in LNCaP xenograft tumors compared to cultured cells

The changes of selected genes in LNCaP xenograft tumor compared to the cultured cells using RNA-seq were further confirmed using RT-PCR. Eight genes including AR, CXCL12, CXCR7, F3, FLNA, RhoB, ITGA1, and UGT2B15 were analyzed. The expression of AR was significantly inhibited, mRNA level of CXCL12 was increased by 27.98-fold, CXCR7 was down-regulated, F3 was up-regulated by 206.05-fold, FLNA was decreased, RhoB was inhibited, ITGA1 was decreased, and UGT2B15 was significantly

down-regulated. All changes of selected genes were consistent with the RNA-seq findings, confirming the observations of transcriptome changes from RNA-seq.

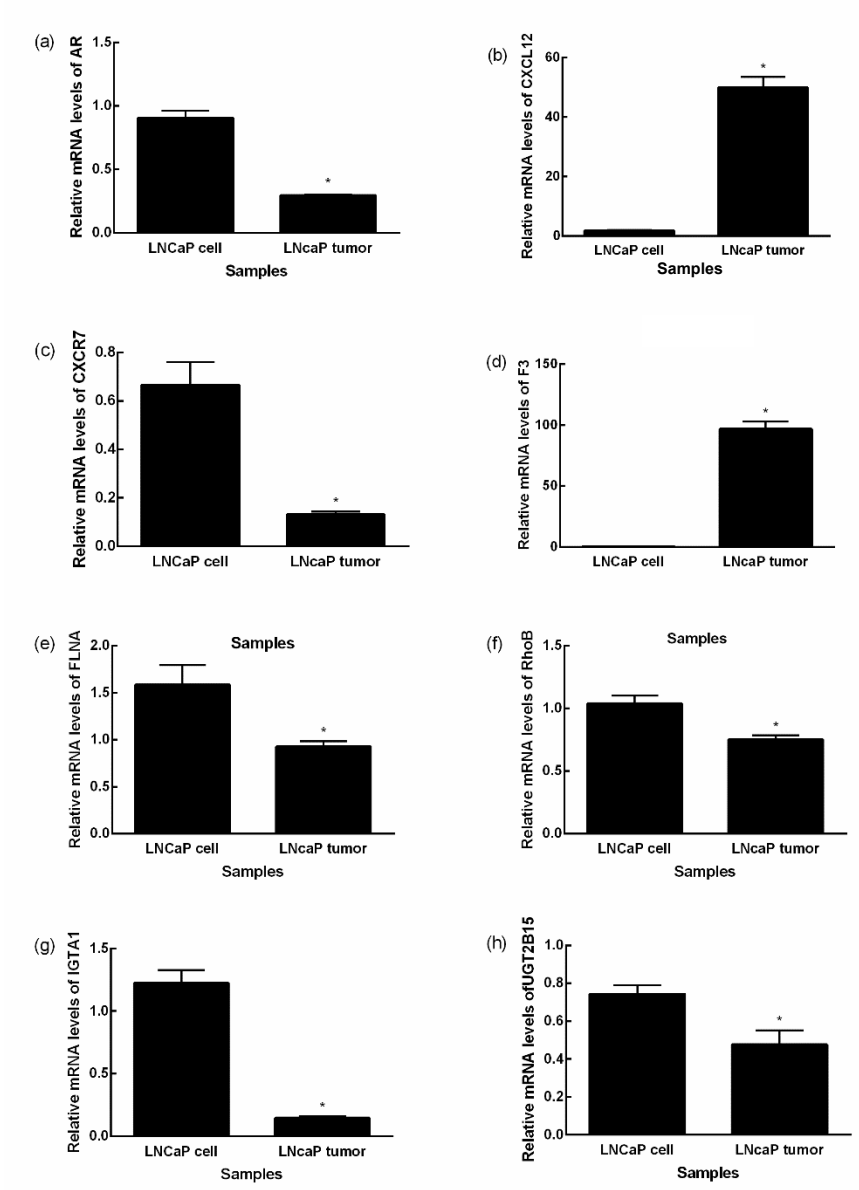

Fig. 3.7 Relative mRNA levels of (a) AR (b) CXCL12 (c) CXCR7 (d) F3 (e) FLNA (f) RhoB (g) IGTA1 (h) UGT2B15 LNCaP cultured cells and LNCaP xenograft tumors. Results were normalized to the normal control and expressed as percent of cells migrated (mean ± SD, n = 3). *P* values ≤ 0.05 were considered as significant and * indicates significantly different from control.

3.4.16. Comparison of LNCaP xenograft model and human clinical outcomes

The alterations of global transcriptomes of LNCaP xenograft tumor as compared to cell culture indicated a difference between the LNCaP cells before and after injection into athymic xenograft mouse host. However, the relevance to clinical findings was not known. Hence, the changes in pivotal genes between LNCaP xenograft tumor and cell culture were further compared to the clinical human prostate samples.

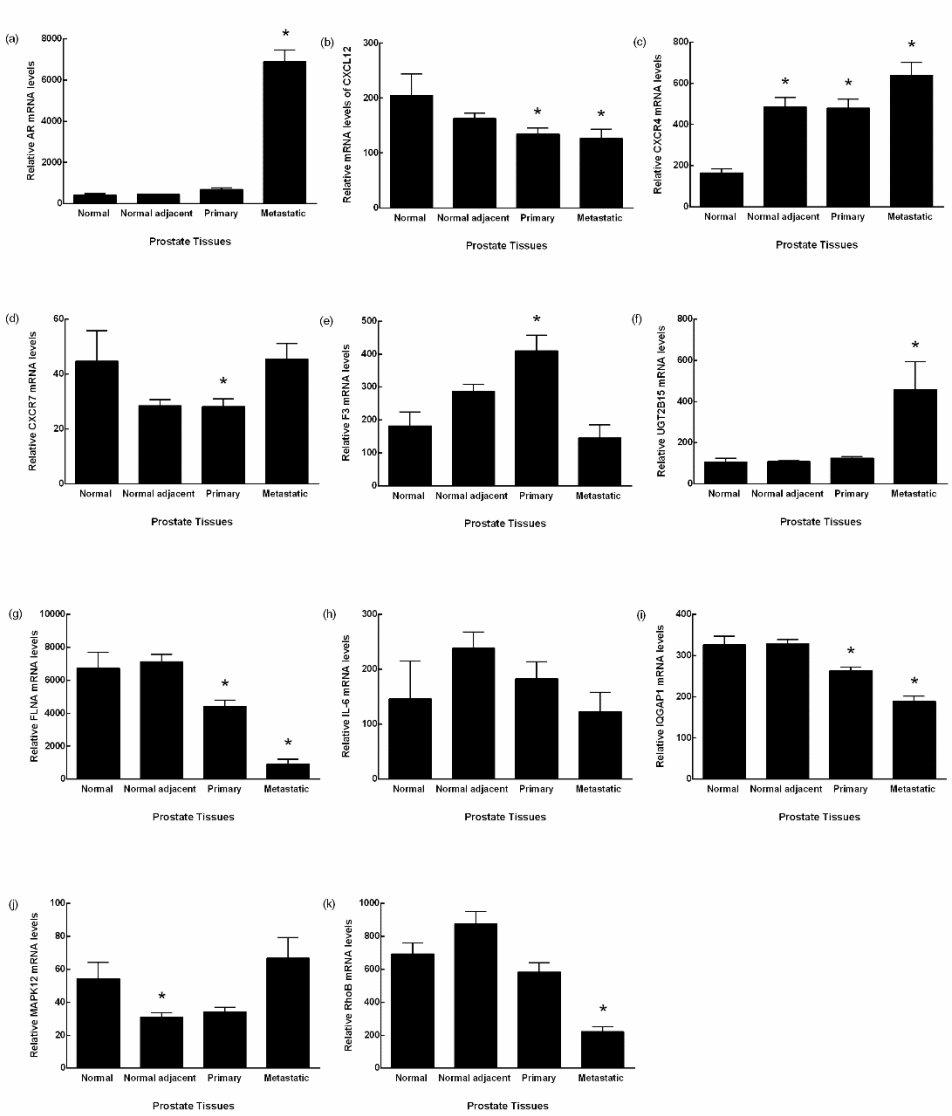

Fig. 3.8. Relative mRNA levels of (a) AR (b) CXCL12 (c) CXCR4 (d) CXCR7 (e) F3 (f) UGT2B15 (g) FLNA (h) IL-6 (i) IQGAP1 (j) MAPK12 (k) RhoB in normal, normal adjacent, primary and metastatic prostate tumors. Results were normalized to the normal control and expressed as percent of cells migrated (mean ± SD, $n = 3$). P values ≤ 0.05 were considered as significant and * indicates significantly different from control.

The comparison of LNCaP xenograft model and human LNCaP cell line showed that mRNA levels of AR and UGT2B15 were the highest in metastatic tumor (Fig. 3.8a, f). CXCL12, FLNA and IQGAP1 expressions were significantly decreased in primary and metastatic tumors (Fig. 3.8b, g, i). In normal adjacent, primary and metastatic prostate tumors, the CXCR4 mRNA level was increased as compared to the control (Fig. 3.8c). In addition, CXCR7 expression was found to be the lowest in primary prostate tissue. Finally, the highest F3 mRNA level was observed in primary prostate tumor (Fig. 3.8e).

3.4.17. The effects of tumor environments on LNCaP gene expressions

3.4.17.1. The effects of androgen

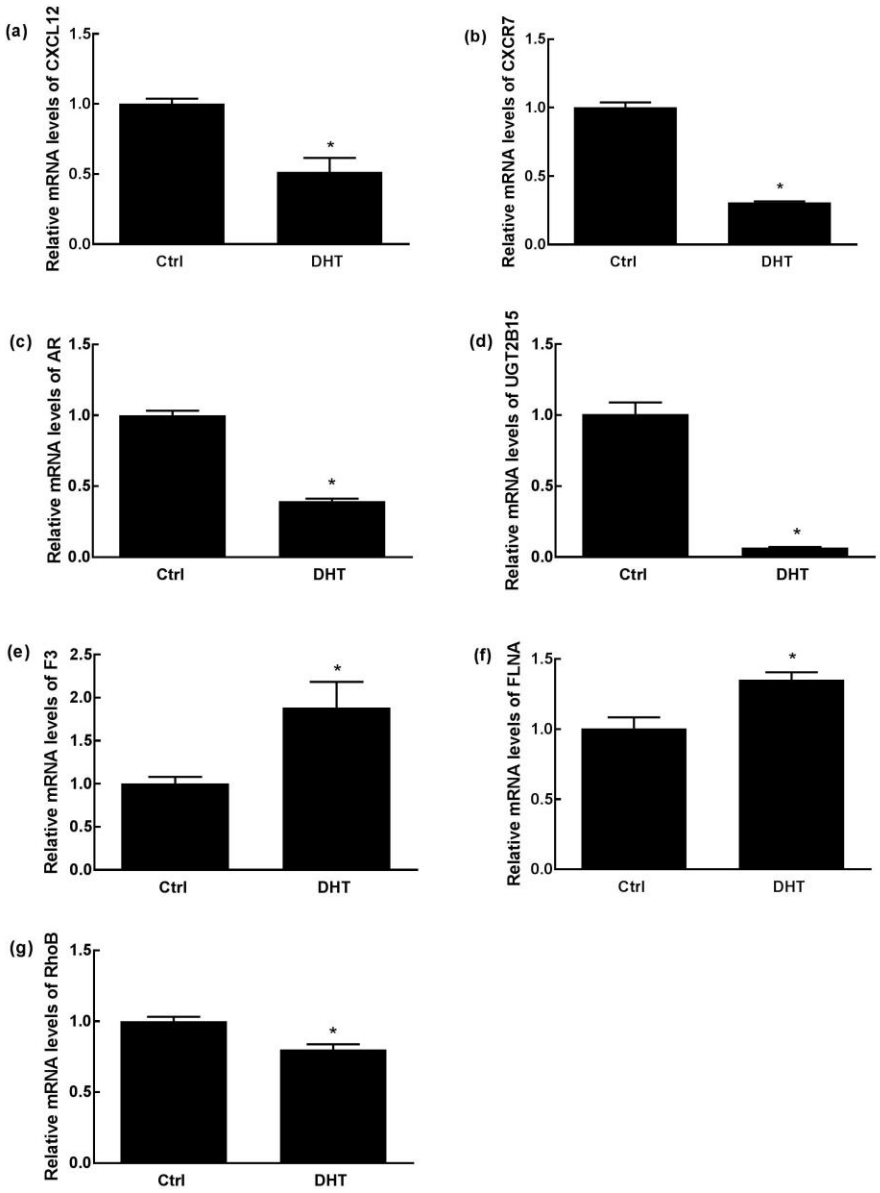

Fig. 3.9 Relative mRNA levels of (a) CXCL12 (b) CXCR7 (c) AR (d) UGT2B15 (e) F3 (f) FLNA (g) RhoB in LNCaP cells treated without/with 10 nM DHT. Results were normalized to the normal control and expressed as percent of cells migrated (mean ± SD, n = 3). *P* values ≤ 0.05 were considered as significant and * indicates significantly different from control.

Relative mRNA level of CXCL12 was significantly down-regulated by 48.45% with the treatment of DHT (Fig. 3.9a). The regulation of CXCL12 by DHT was opposite as compared to the observation in LNCaP xenograft tumor and cultured cells. The expressions of CXCR7 (Fig. 3.9b), AR (Fig. 3.9c), UGT2B15 (Fig. 3.9d) and RhoB (Fig. 3.9g) were significantly decreased by -3.24, -2.52, -14.96, and -1.25 respectively. The mRNA levels of CXCR7 (Fig. 3.7c), AR (Fig. 3.7a), UGT2B15 (Fig. 3.7h) and RhoB (Fig. 3.7f) were consistently down-regulated by DHT as compared to the expression changes in LNCaP xenograft tumor and cultured cells. Besides, F3 was up-regulated by 1.88-fold (Fig. 3.9e). In primary prostate tumor, F3 was also increased significantly (Fig. 3.7d). FLNA was significantly up-regulated by DHT (Fig. 3.9f), with lower expression in LNCaP xenograft tumor as compared to cultured cells (Fig. 3.7e).

3.4.17.2. The effects of hypoxia

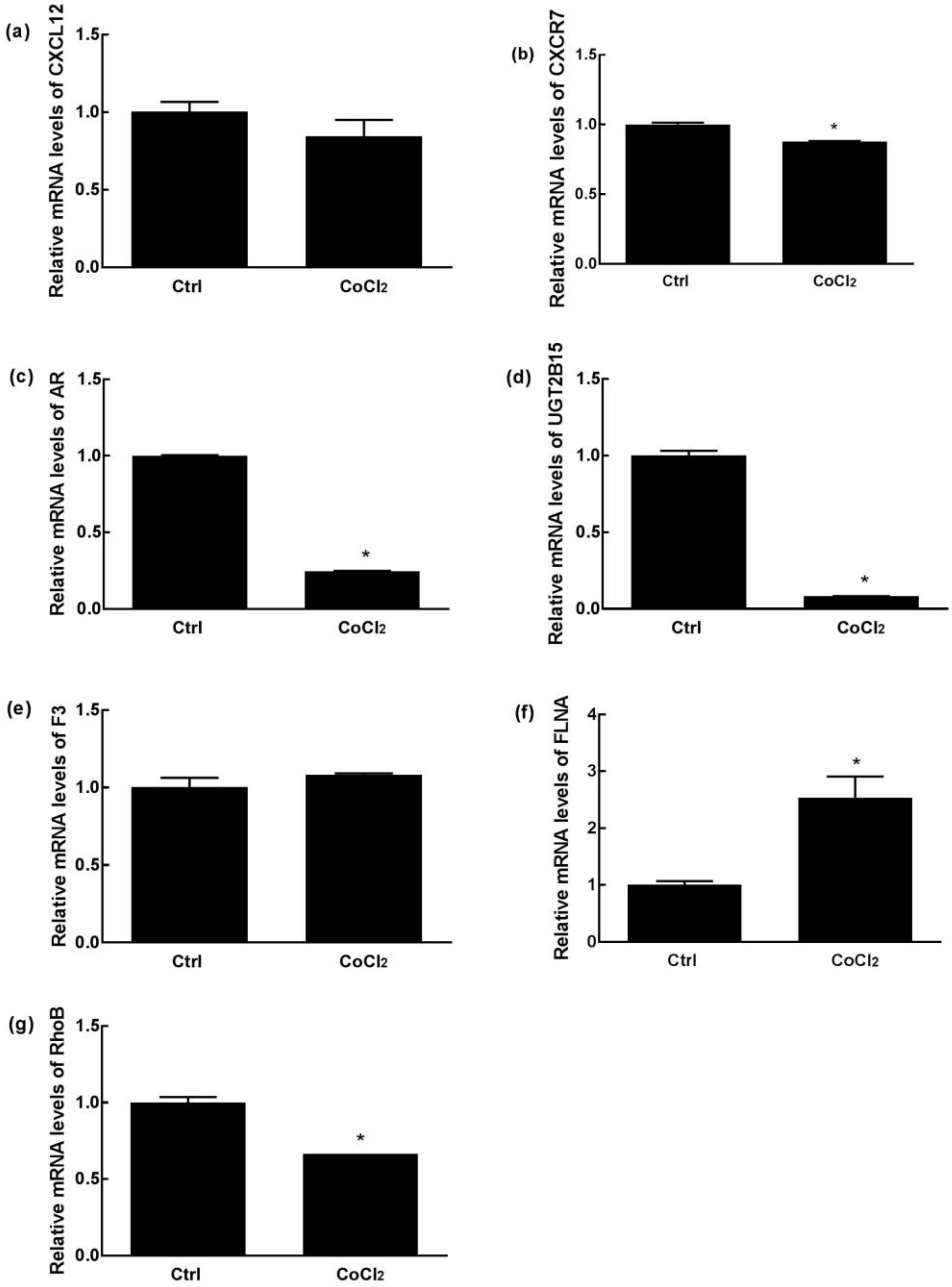

Fig. 3.10 Relative mRNA levels of (a) CXCL12 (b) CXCR7 (c) AR (d) UGT2B15 (e) F3 (f) FLNA (g) RhoB in LNCaP cells treated without/with $CoCl_2$. Results were normalized to the normal control and expressed as percent of cells migrated (mean ± SD, n = 3). *P* values ≤ 0.05 were considered as significant and * indicates significantly different from control.

Hypoxic condition could be induced in-vitro using $CoCl_2$ (Ciafre et al., 2009). The treatment of LNCaP cells with $CoCl_2$ had no influence on the mRNA levels of CXCL12 and F3 mRNA levels in LNCaP cells (Fig. 3.10a, e), while a significantly higher expression of CXCL12 could be observed in LNCaP xenograft tumor as compared to cultured cells (Fig. 3.7b). The relative mRNA levels of CXCR7, AR, UGT2B15, and RhoB were all significantly reduced by $CoCl_2$ (Fig. 3.10b, c, d, g), and the results were consistent with the observation from LNCaP xenograft tumor and cultured cells (Fig. 3.7a, c, f, h). The FLNA was significantly increased by $CoCl_2$ (Fig. 3.10f), while with lower expression in LNCaP xenograft tumor as compared to cultured cells (Fig. 3.7e).

3.4.17.3. The effects of tumor cell-immune cell interaction

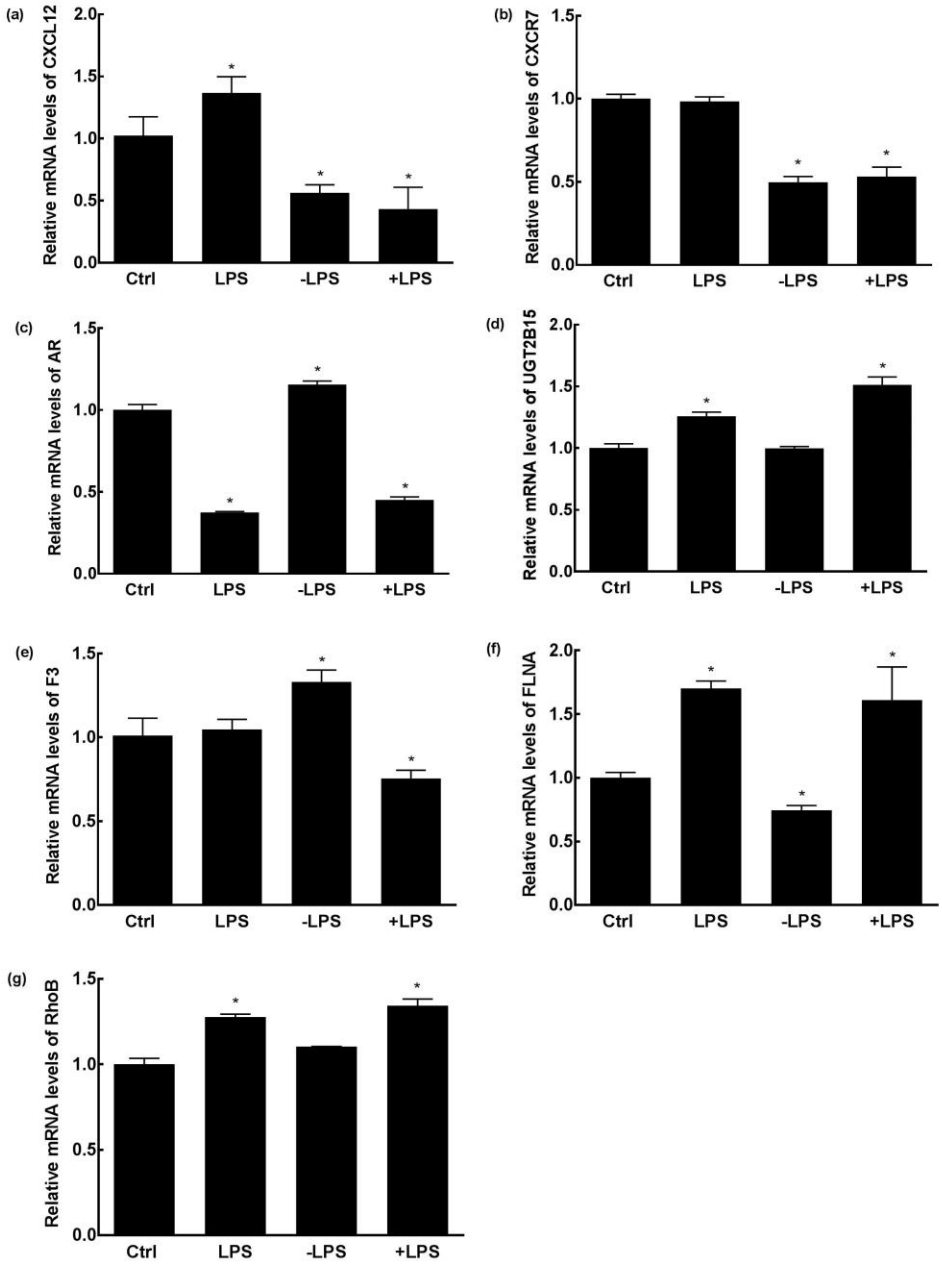

Fig. 3.11 Relative mRNA levels of (a) CXCL12 (b) CXCR7 (c) AR (d) UGT2B15 (e) F3 (f) FLNA (g) RhoB in LNCaP cells treated with RPMI media, RPMI media with LPS, conditioned media without LPS and conditioned media with LPS. Results were normalized to the normal control and expressed as percent of cells migrated (mean ± SD, n = 3). *P* values ≤ 0.05 were considered as significant and * indicates significantly different from control.

CXCR7 mRNA level was significantly down-regulated in LNCaP cells treated with conditioned media (Fig. 3.11b). The effect of tumor cell-immune cell interaction on CXCR7 was consistent with that observed in LNCaP xenograft tumor and cultured cells (Fig. 3.7c). The expression of AR was inhibited by LPS, regardless of the treatment of conditioned media (Fig. 3.11c). CXCL12 mRNA levels were increased by LPS but were significantly down-regulated by conditioned media, regardless of the LPS treatment (Fig. 3.11a). No significant effect of conditioned media could be observed on the regulation of UGT2B15, but LPS stimulation seemed to increase the UGT2B15 mRNA level (Fig. 3.11d). F3 was not affected by LPS stimulation while it was significantly increased by conditioned media treatment. However, the up-regulation of F3 by conditioned media was inhibited by further being treated with LPS (Fig. 3.11e). FLNA levels were significantly induced by LPS, regardless of the conditioned media treatment. The treatment of conditioned media without LPS decreased FLNA expression (Fig. 3.11f). In addition, no significant changes of RhoB mRNA levels could be observed by conditioned media, while LPS significantly up-regulated RhoB expression (Fig. 3.11g).

3.4.17.4. The effects of subcellular matrix

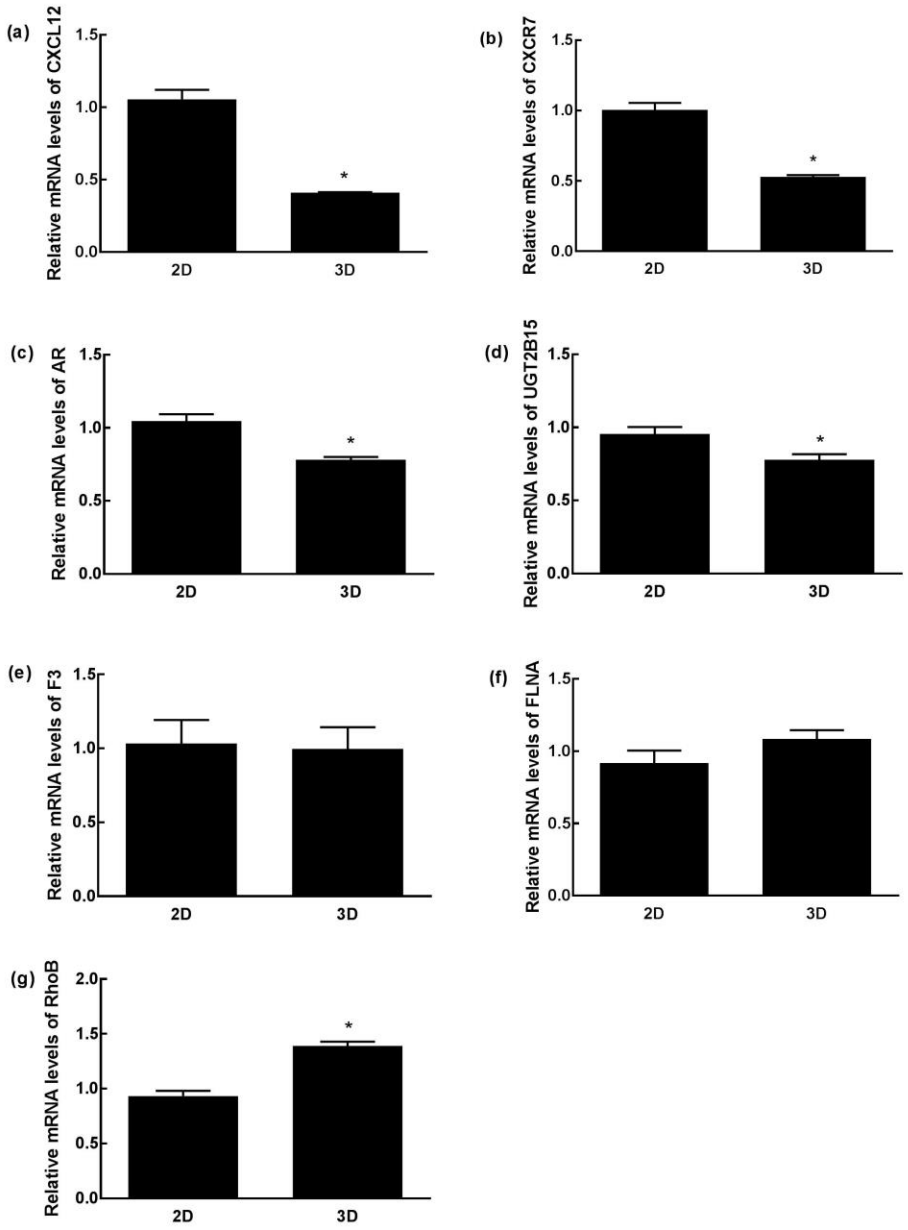

Fig. 3.12 Relative mRNA levels of (a) CXCL12 (b) CXCR7 (c) AR (d) UGT2B15 (e) F3 (f) FLNA (g) RhoB in LNCaP cells cultured in 2D and 3D models. Results were normalized to the normal control and expressed as percent of cells migrated (mean ± SD, n = 3). *P* values ≤ 0.05 were considered as significant and * indicates significantly different from control.

As shown in Fig. 3.12b, c and d, mRNA levels of CXCR7, AR, and UGT2B15 were all significantly down-regulated in LNCaP cultured in subcellular cell matrix, by -1.89, -1.34 and -1.23, respectively (Fig. 3.12b, c,d). The observation was consistent with that of LNCaP xenograft tumor and cultured cells (Fig. 3.7a, c, h). CXCL12 was significantly decreased (Fig. 3.12a) and RhoB was up-regulated by -fold (Fig. 3.12g). The regulation of CXCL12 and RhoB were opposite in cells cultured in subcellular cell matrix as compared to that in LNCaP xenograft tumor model and cultured cells (Fig. 3.7b, f). In addition, no change of F3 or FLNA was observed in LNCaP growing in subcellular cell matrix (Fig. 3.12e, f).

3.5 Discussion

The current study, using the NGS approach, 1) provides molecular information on the comparison of LNCaP cell tumor xenograft vs. its parent celland 2)identifies possible tumor microenvironmental signals that lead to the display of these molecular changes.

3.5.1. Top genes with higher and lower expressions in prostate cancer

Our principal component analysis and volcano plots identified significant differences of gene expression profiles between xenograft tumor sets and cell culture group. The top 10 genes with higher and lower expressions were investigated to find correlations between major genes changed and their functions in LNCaP xenograft tumor as compared to cultured cells.

Coagulation factor III (F3) was the one with highest expression in LNCaP xenograft model as compared to cultured cells. F3 could be activated by immune stimuli or inflammation associated genes such as lipopolysaccharide (LPS), TNF, VEGFA, F2, phorbol-myristate-acetate (PMA), and IL-1β (Genecards, NCBI). The up-regulation of F3 in the tumor sample

would support a pro-inflammatory environment within the xenograft. The increase of F3 would in turn modulate cell migration, adhesion, recruitment, proliferation and extravasation (Clouston et al., 2018; Hisada et al., 2017; Stampfli et al., 2017). The gene with the second highest expression, CREB3L1, has been reported to be regulated by intramembrane proteolysis in response to virus infection (Denard et al., 2011). The up-regulation of this gene again would be suggestive of inflammatory microenvironment in the tumor xenograft. The third highest expressed gene, ORM1, is a gene encodes acute phase plasma protein. The up-regulation of ORM1 is associated with acute inflammation (Shaikhibrahim et al., 2011). The function of this gene has not yet been fully understood, but Boncela et al have reported that this gene might be involved in immunosuppression (Boncela et al., 2001). Similar to F3 and CREB3L1, the up-regulation of ORM1 (by 174. 68-fold) indicated that the progression of prostate cancer in LNCaP xenograft tumor might be associated with the regulation of immune response. Given the proposed immunosuppressive properties of ORM1, we hypothesize that an increase in ORM1 may allow cancer cell to escape immune surveillance which warrants further study.

In addition, it should be noted that the fourth most up-regulated gene, RGS2, is associated with the down-regulation of androgen-independent AR activity (Cao et al., 2006). The increase of RGS2 expression might contribute to the decreased mRNA level of AR. The end results would be inhibition of AR-mediated pathway and is consistent with the development of an androgen non-responsive phenotype of the xenograft.

For the top 10 genes with lower expressions, Fc alpha/mu receptor (FCAMR) was first reported in this study as the gene with the lowest expression in LNCaP xenograft tumor compared to LNCaP cell line (Table. 3.1b). FCAMR is a receptor that has dual specificity

for IgA and IgM (Yang et al., 2013). FCAMR binds to IgA and IgM and may be responsible for mucosal immunity (Ouchida et al., 2012). In the case of IgA, known to be produced by prostate epithelial cell, FCAMR may be responsible for the secretion of IgA. The down-regulation of FCAMR might be associated with the dysregulation of immune system in the prostate (Silva et al., 2017).

UGT2B15 and UGT2B17 were the second lowest expressed genes. Together with UGT2B10 and UGT2B28, these 4 genes are from UDP-glucuronosyltransferase family (Nadeau et al., 2011). They became the most important group that exhibited the lowest expressions in LNCaP xenograft tumor as compared to LNCaP cell culture. Additionally, UGT2B15, UGT2B10, UGT2B17 and UGT2B28, are pivotal regulators for androgen metabolism (Nadeau et al., 2011). Chouinard et al have reported that UDP-glucuronosyltransferase 2B15 (UGT2B15) and UGT2B17 enzymes are major determinants of the androgen response in prostate cancer LNCaP cells. UGT2B15 and UGT2B17 enzymes conjugate dihydrotestosterone (DHT) and metabolites of DHT, which includes androstane-3α, 17β-diol (3α-DIOL) and androsterone (ADT) (Chouinard, 2007). The activation of UGT2B15 and UGT2B17 genes would have a strong impact on the inactivation of androgens in LNCaP cells (Chouinard, 2007).

Together, the top genes with higher and lower expressions in LNCaP xenograft tumor as compared to LNCaP cultured cells highlighted a major effect on the regulation of androgen response, as well as the immune response.

3.5.2. Rho-related pathways identified as major pathways altered in LNCaP xenograft tumor as compared to cultured LNCaP cells

Pathway analysis allows the identification of Rho-related pathways to be top pathways altered in LNCaP tumor xenograft. RhoGDI signaling and Rho family GTPases signaling pathways were the most activated and inhibited pathways identified in LNCaP xenograft tumor as compared to LNCaP cell culture (Table 3.7.2a and 3.7.2b). Rho-family proteins are a branch of Ras family involved in the regulation of cell adhesion, survival, proliferation, and migration in tumor progression (Sahai & Marshall, 2002). Rho GTPases comprise of a large subfamily including Cdc42, Rho and Rac proteins (Malliri & Collard, 2003). Different members from Rho-family seem to play crucial roles in the regulation of tumor development. RhoGDI is known as a suppressor of Rho family GTPases (Dovas & Couchman, 2005). As a binding partner, RhoGDI tightly controls the activity of GTPases and their biological functions. RhoGDI is associated with the development of cancer including prostate (Marcos et al., 2014). Besides, up-regulation of RhoGDI has been reported to be related to colon and prostate cancer metastasis (Yamashita et al., 2012). RohGDI up-regulation has been reported to be associated with increased proliferation and cell migration in prostate cancer (Chow et al., 2012). Our pathway analysis is consistent with these observations and points toward a role for RhoGDI pathway in LNCaP tumor xenograft development. Additionally, there may be a cross talk between Rho-related pathways and androgen receptor mediated pathways. Zhu et al reported RhoGDI down regulation androgen receptor-mediated pathway which is consistent with our observed down regulation of androgen-dependent pathways in the tumor xenograft (Zhu et al., 2013). However, literature on RhoGDI and Rho pathway and cancer development remain

inconsistent. Zhu et. al suggested RhoGDI inhibition might prevent prostate cancer (Zhu et al., 2013). Our results as well as others appeared to indicate the association of RhoGDI with prostate cancer development. Additional studies are necessary to elucidate these discrepancies.

3.5.3. Alteration of androgen-related pathways

In the early-stage of prostate cancer progression, androgens are the main regulators of prostate cancer cell growth and survival (Shahinian et al., 2005). The tumor will eventually develop to become non-responsive to androgen. Our data support that the LNCaP tumor xenograft progressed to a more androgen -independent phenotype than the parent LNCaP cells. As mentioned above, the activation of RhoGDI may regulate androgen-dependent pathways (Zhu et al., 2013). In addition, many genes involved in the modulation of the androgen receptor function were inhibited in the LNCaP xenograft tumor, including Hsp90AB1, Hsp90AA2, FLNA and FKBP4. These proteins are all known to form complex with the androgen receptor and enhance and modulate androgen receptor-mediate biological activities (Bebermeier et al., 2006; Ciocca et al., 2010; Castoria et al., 2011; Stope et al., 2013). Down regulation of these proteins would suggest a deviation of the LNCaP tumor from androgen responsiveness of its parent LNCaP cells. What further supports the deviation from androgen responsiveness and toward androgen non-responsiveness is that many signaling pathways that promote prostate cancer proliferation were up-regulated.

The genes listed as involved in androgen-independent pathway, such as MAPK12, Akt3, Wnt3A, and IGFBP7, all exhibited higher expressions in LNCaP xenograft tumor as compared to cultured cells (Lin et al., 2015). The activation of MAPK12 might be

associated with the stress from the microenvironment (Kamiyama et al., 2015). Also, AR is closely correlated with the expression of PI3K/Akt pathway (Yang et al., 2003). A decrease of AR activity has been found with the activation of the PI3K/Akt pathway in androgen-independent prostate cancer DU145 cells, while other researchers have reported an increase of AR expression by PI3K/Akt pathway in androgen-dependent prostate LNCaP cells (Lin et al., 2003). Wnt3A was also among the top genes with higher expressions in LNCaP xenograft tumor as compared to LNCaP cell line. It has been reported that WNT treatment promotes the self-renewal of prostate cancer cells with stem cell characteristics (Bisson & Prowse, 2009). Up regulation of these genes may thus allow prostate cancer cells to bypass the dependence of proliferative activity on androgen.

Network analysis as illustrated in Fig 3.5 provides additional information regarding an overall down regulation of the androgen-dependent pathway. Calpain, a protein belonging to the family of calcium-dependent, non-lysosomal cysteine proteases (Kishimoto et al., 1989), and COPA (Coatomer Protein Complex Subunit Alpha), a protein coding gene which is associated with the progression of autoimmune interstitial lung, joint, and kidney disease (Nakagawa et al., 1994; Chatterjee et al., 2001; Watkin et al., 2015), were down regulated and might directly result in AR down regulation. The indirect regulators of AR are N4BP2, USP38, HUWE1 and CAND1 (Fig. 3.5), which were down-regulated and identified in the network. The down-regulation of these genes might also contribute to the inhibition of AR.

Besides the genes shown in the network for AR regulation, other possible regulations of androgen-related pathway also exist. RGS2 was one of the top genes with higher expressions (138.16-fold) in LNCaP xenograft tumor as compared to LNCaP cultured cells.

It has been reported that RGS2 down-regulates androgen-independent AR activation as an inhibitor of GPCRs (Cao et al., 2006; Wolff, et al., 2012), which partially explains the decrease of AR in LNCaP xenograft tumor. In addition, the regulation of AR in androgen responsive and androgen unresponsive cells might also be correlated with TGF-β. Zhu et al has reported that in the presence of DHT and TGF-β, AR expression in LNCaP cell line is up-regulated, while the AR in PC3 is down-regulated.

The main challenge with all these regulations described above would be the validation of specific pathway in prostate cancer pathogenesis, which warrant further study.

3.5.4. Alterations and regulations of cytokines, chemokines and their receptors

Cytokines, chemokines and their receptors influence the development of immune-cell infiltration, as well as tumor cell growth, angiogenesis, migration and metastases (Balkwill, 2004). CXCL12 and CXCR7 were significantly altered in LNCaP xenograft tumor as compared to LNCaP cultured cells. CXCL12 was identified as the only chemokine changed in LNCaP xenograft tumor compared to cell culture (Table 3.5). The CXCL12 chemokine could be secreted by many types of cells including immune cells such as primary blood monocytes, (Sánchez-Martín, Estecha et al., 2011) macrophages (Rigo, Gottardi et al., 2010) as well as tumor cells from prostate and other tissues (Sun, Wang et al., 2005; Roy, Zimmerman et al., 2014; Ray, Stacer et al., 2015). The activation of CXCL12 chemokine has been reported to increase the motility of LNCaP and PC3 cells (Singh, Singh et al., 2004) and induction of downstream pathways such as Akt-1 and metalloproteinases, which is involved in the regulation of prostate cancer cell migration (Singh, Singh et al., 2004; Chinni, Sivalogan et al., 2006). CXCR7, the receptor for CXCL12, was significantly down-regulated by -4.12-fold (Table 3.5). The result suggested a pivotal role for the chemokine

CXCL12 and its receptor CXCR7 in the regulation prostate cancer progression in a LNCaP xenograft model.

Network analysis provides additional information regarding proximal and distal molecular changes related to CXCL12. For the regulation of CXCL12, MMP2 was identified as a direct regulator, and the increase of MMP2 might be associated with the up-regulation with CXCL12 mRNA levels (Fig. 3.6). In the down-stream signaling pathways, CXCL12 indirectly up-regulated the expression of genes such as TIMP1and RGS2, and down-regulated S100A7, cyclin T1 (CCNT1), FOSB and UHMK1. The alterations of these genes are associated with the regulation of cell cycle, proliferation of prostate cancer cells and prostate associated fibroblasts (Tahirov et al., 2010; Gong et al., 2013). As mentioned above, RSG2 can regulate AR, therefore it is possible that CXCL12 may act as an endocrine to influence AR and androgen-dependence in LNCaP tumor xenograft.

3.5.5. Alterations of cell adhesion associated molecules

Integrins and cell adhesion molecules are two important families that regulate cell adhesion, junction formation, differentiation, and polarity (Albelda & Buck, 1990). Integrins promote the cell adhesion/attachment and progression of many types of cancers including prostate cancer (Fornaro et al. 2001; Hudson et al., 2012). Alterations of integrin expression in cancer cells might correlate with tumor growth and progression. The integrins ITGA2, ITGA6, ITGB1 and ICAM5 were all expressed lower in LNCaP xenograft tumor as compared to cultured cells (Table 3.6). Integrin ITGA2, ITGA 6 are subunits for collagen receptor and laminin receptor, respectively. Besides, ITGB1 is a subunit for both laminin and collagen receptors (Berno et al., 2005; Gao et al., 2014). The inhibition of these cell adhesion/attachment-related molecules may affect the cell-matrix

adhesion in prostate cancer progression. ICAM was another matrix molecular that was changed. ICAM proteins are ligands for the leukocyte adhesion protein LFA-1 (integrin alpha-L/beta-2). ICAM1 encodes a cell surface glycoprotein which is typically expressed on endothelial cells and cells of the immune system (Rice et al., 1989). ICAM1 but not ICAM5 may be an important risk factor for prostate cancer (Chen et al., 2006).

Together, the up-regulated integrin subunits and ICAM1 might be associated with the progression of LNCaP xenograft tumor from the parent LNCaP cell line.

3.5.6. Expressions of mouse genes in LNCaP xenograft model

The LNCaP xenograft tumor are surrounded by mouse cells in the athymic xenograft mouse model. The genes expressed in the host might have an influence on the human xenograft tumor growth. The top expressed mouse genes identified, such as Filamin A, LRS2 and ARHGAP21, are associated with the regulation of angiogenesis, cell growth, and migration of prostate cancer (Zhou et al., 2009; Desotelle et al., 2012; Lazarini et al., 2013; Radder et al., 2013). Moreover, among the mouse chemokines and cytokines, CXCL12 was the most expressed CXC-motif chemokine. Two receptors for CXCL12, including CXCR4 and CXCR7, were the most expressed mouse CXC-motif receptors found in LNCaP xenograft tumor (Table 3.7.8). CXCL12 and its receptors are involved in the immune-inflammatory response and the regulation of prostate cancer metastasis (Sfanos & Marzo, 2012). These data support that the CXCL12/CXCR4,7 pathway may be an important promoter of LNCaP tumor xenograft. In addition, CCL6 was the most expressed CC-motif chemokine (Table 3.7.8). The CCL6 is secreted by neutrophil and macrophages, and it could be greatly reduced by lacking functional GM-CSF receptors (Croxford et al., 2015). The expression of chemokine CCL6 could improve antimicrobial

immunity, activate peritoneal cells, and recruit natural killer cells and killer dendritic cells (Coelho et al., 2007). These data support that an inflammatory environment exists in the LNCaP tumor xenograft.

3.5.7. Validation of gene expressions in LNCaP xenograft tumors as compared to cultured cells by RT-PCR

The alterations of transcriptome profiles by RNA-seq analysis were validated by RT-PCR. Eight genes including AR, CXCL12, CXCR7, F3, FLNA, RhoB, ITGA1, and UGT2B15 were examined. AR is important in the development and progression of prostate cancer (Heilein & Chang, 2004), and it was discovered to be significantly down-regulated in LNCaP xenograft tumor as compared to cell culture. CXCL12 and its receptor CXCR7 were the only chemokine and chemokine receptor found with significant changes in LNCaP tumor as compared to cell culture. F3 was the most up-regulated gene and was also a regulator for cell recruitment, migration, adhesion, and proliferation (Genecards, NCBI). FLNA and UGT2B15 are from the top genes with the lowest expressions. UGT2B15 is a determinant of the androgen response in prostate cancer (Nadeau et al., 2011). FLNA regulates AR in androgen-dependent pathway (Lin et al., 2007). RhoB and ITGA1 were genes associated with the Rho signaling pathway, which was the most important pathway with changes in this study. The results of mRNA level expressions for these genes using RT-PCR were all consistent with RNA-seq data, and these results validated the results observed from RNA-seq.

3.5.8 Effects of tumor microenvironments

In-vitro models were also used to elucidate potential tumor environmental factor that may influence our observed changes in LNCaP tumor xenograft vs. parent LNCaP cells.

Regulation of CXCL12 was not associated with the androgen, hypoxia, tumor cell-immune cell interaction or subcellular matrix. The CXCR7 changes in LNCaP were related to the levels of androgen, hypoxia, tumor cell-immune cell interaction and subcellular matrix. AR and UGT2B15 were affected by androgen, hypoxia and subcellular matrix. F3 and FLNA were affected only by androgen and tumor cell-immune cell interaction, respectively. The regulation of RhoB was influenced by androgen and hypoxia. Together, tumor microenvironments differentially affected the regulation of CXCR7, AR, UGT2B15, F3, FLNA and RhoB. These data support that tumor microenvironments play a critical role in influencing LNCaP xenograft tumor growth and developments.

3.5.9. Comparison of LNCaP xenograft model and human clinical outcomes

Whether the *in vitro* models using human cells or animal experimental models could predict the clinical outcomes is an important question that needs to be addressed. We queried existing data base for human prostate cancer gene expression data and focused on the following gene set: The AR, chemokine CXCL12 and its receptors CXCR4 and CXCR7, most up-regulated gene F3, one of the most down-regulated genes UGT2B15, AR regulator FLNA and MAPK12, cytokine IL-6, genes involved in Rho signaling pathway including IQGAP1, and RhoB. The results of our analysis were mixed.

The genes associated with hormone regulation, including AR, FLNA, and MAPK12, were all oppositely regulated in clinical prostate tumor samples as compared to the xenograft model. Genes related to Rho signaling pathway, such as RhoB and IQGAP1, were consistently regulated as in human tumor samples. The data suggested the interpretation of biological efficacies by using LNCaP xenograft to extrapolate to clinical outcomes might need caution.

3.6 Conclusion

In this study, the comparison of LNCaP cell culture and LNCaP xenograft tumor showed that genes were differentially regulated in LNCaP xenograft tumor when compared to LNCaP cell culture. The LNCaP cells before and after implantation differed significantly in the expressions of androgen receptor, chemokines, and pathways that might play pivotal roles in the regulation of tumor progression. CXCL12 and CXCR7 were the only significantly altered chemokine and chemokine receptor in LNCaP xenograft tumor as compared to cultured cells. Moreover, a shift from androgen-dependent to androgen-independent status was observed. The gene alterations in LNCaP xenograft model and clinical prostate tissue samples at different cancer progression stages suggested that alterations of genes in LNCaP xenograft tumor and cultured cells are not all the same as compared to clinical outcomes. Genes associated with the regulation of androgen response, such as AR, FLNA, and UGT2B15, were oppositely regulated, while genes involved in Rho signaling pathway, such as RhoB and IQGAP1, were consistently regulated. The regulation of genes in xenograft tumor might be related to the tumor microenvironments, such as androgen, hypoxia, interaction with immune cells and subcellular matrix.

Chapter 4: Elucidating Molecular Effects of Diet-derived Cancer Preventive Compound Phenethyl Isothiocyanate (PEITC) Using RNA-seq

4.1. Abstract

(Phenethyl isothiocyanate) PEITC is a compound naturally derived from cruciferous vegetables. It has been shown that PEITC possesses chemopreventive activity in prostate cancer. However, the mechanisms underlying the effects of PEITC remain clear. We hypothesize that PEITC may act on immune system as inflammation is associated with the development of prostate cancer. However, the effects of PEITC on the regulation of immune responses are not well known. In this study, we seek to extend our understanding of the effects of PEITC on immune system. PEITC was investigated for its effects on bacterial cell membrane component lipopolysaccharide (LPS)-induced inflammatory responses in cultured human leukemia THP-1cells. RNA-seq and bio-informatic analyses of gene expressions were conducted to identify the gene alterations and canonical pathways modulated by PEITC against inflammatory stimuli. Neuroinflammation and PPAR signaling pathways were identified as the most activated and inhibited canonical pathways in THP-1 cells stimulated with LPS stimulation. PKA/cAMP signaling pathway was identified as the primary pathway altered by PEITC in THP-1 cells. Regulation of Histone and PDE families might be the major groups of genes attributing to the inhibitive effect on PKA/cAMP signaling pathway by PEITC. The up-regulation of growth inhibitive and apoptosis inductive gene TIAF1, and down-regulation of biomarkers LRN3, PTH RDS4, might contribute to the suppressive effect of PEITC on THP-1 cell growth. For the first time, three novel genes AC005488.2-6, AC092536.3-1, and AL021393.1-2 were identified as the most down-regulated gene in THP-1 cells stimulated by LPS. Conversely,

AC092536.3-1 and AL021393.1-2 were the most up-regulated genes by PEITC treatment with LPS stimulation. In addition, the pivotal mediators in PKA/cAMP signaling pathway including FPR1, NTN1, P2RY13, PDE6G and PTGDR that were regulated by LPS were reversely mediated by PEITC. The results suggest that PEITC may exert its protective effects through modulation of inflammation.

4.2. Introduction

Prostate tumorigenesis is closely associated with inflammation. In prostate cancer tissue, inflammation lesions are frequently observed in the histological specimens of the adult prostate tissue (Sfanos & Marzo, 2012).

To prevent prostate cancer, bioactive compounds with potential tumor inhibitory effects are especially promising for their low toxicities, inexpensive cost and easy availability (AidaRodriguez-Garcia et al., 2017). The cruciferous-derived compound phenethyl isothiocyanate (PEITC) is one of the compounds considered as an antitumor candidate for its chemopreventive effect on prostate cancer both *in vitro* and *in vivo.* (Xu et al., 2005). Hudson's group has reported that PEITC is able to suppress the growth of LNCaP xenograft tumor and inhibit angiogenesis and cell attachment through cell adhesion molecule (PECAM-1/CD31) *in vivo*. This study also suggested that the suppressive effect on tumor growth by PEITC might not be correlated with the apoptosis or regulation of androgen receptor (Hudson et al., 2012). Indeed, PEITC was reported to significantly down-regulate mRNA expression of IL-6, a typical pro-inflammatory cytokine in LNCaP xenograft tumor (Li et al., 2013). This observation indicated that the suppressive effect of PEITC on prostate tumor might be associated with the regulation of immune response. The mechanisms of anti-inflammatory effects of PEITC were not fully understood, especially on immune cells.

The suppression of lipopolysaccharide (LPS) induced nitric oxide, IL-1β, IL-6, IL-10 TNF-α and COX-2 by PEITC was reported in mouse macrophage RAW264.7 (Tsai et al., 2010; Lee et al., 2011). For human cell, the effect of PEITC was only reported on the human mast cell line HMC-1, and an inhibition of IL-1β and IL-6 expression through caspase-1 pathway was observed (Moon & Kim). *In vivo*, a promotional effect on immune responses in normal and WEHI-3 leukemia BALB/c mice was reported by Tsou's group (Tsou et al., 2013). Kim et al. have shown that hormone-responsive prostate cancer cells secrete C-C chemokine ligand 2 (CCL2), which recruits monocytes through its receptor CCR2 (Kim et al., 2013). Hence, these observations suggest the existence of a possible correlation between the regulation of inflammation and prostate cancer development by PEITC through modulation of immune cells such as monocyte/macrophage.

In humans and mice, the circulation of blood monocytes enables the trafficking of immune cells to the inflamed sites and provides peripheral tissues with major immune defense (Serbina et al., 2008). Monocytes, monocyte-derived macrophages and dendritic cells have pivotal roles against microbial pathogen-induced infection (Serbina et al., 2008). However, the mechanisms of the immune response by PEITC upon inflammation stimuli in human monocytes is not known. The correlation between the regulation of prostate cancer progression and inflammation by PEITC is also poorly studied.

LPS induced immune responses are well-documented in the innate immunity research to mimic the initiation of an inflammation caused by the infection of Gram-negative bacteria (Aderem & Ulevitch, 2000). The human leukemia cell line THP-1 has been extensively used as a valuable model for studying the mechanisms involved in the regulation of immune responses (Heil et al., 2002; Chanput et al., 2014). In this study, we hypothesize

that the suppressive effects of PETIC on prostate tumor growth might be through the inhibition of inflammation. THP-1 exposed to PEITC was tested for its effects on LPS stimulated inflammatory responses. Deep-sequencing RNA-seq technique was used to obtain the global transcriptome information, and canonical pathways with alterations were analyzed and compared using IPA to elucidate potential molecular mechanisms of action.

4.3. Materials and Methods.

4.3.1. Chemicals and reagents

RPMI-1640 medium with phenol red, fetal bovine serum, and TRIzol reagent were obtained from Life Technologies (Grand Island, NY). 100X penicillin-streptomycin mix (pen-strep), lipopolysaccharide (LPS) and PEITC (indicated purity 99%) were obtained from Sigma-Aldrich (St Louis, MO). All other chemicals were of analytical reagent grade and were used without any further purification.

4.3.2. Cells and cell culture

The human monocytic leukemia cell line THP-1 was purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were seeded at 2.5×10^5 cells/mL in 175 cm^2 flasks and cultured with RPMI 1640 medium with phenol red containing 10% fetal bovine serum (FBS) and 1% pen-strep at 37°C with 5% CO_2.

4.3.3. Cell treatment

THP-1 cells were plated in RPMI medium at the density of 2.5×10^5 cells/mL in 6-well plates at 37°C in 5% CO_2. After 24 h, 0, 0.25 and 1 μM of PEITC were added to cells. The treatment was changed freshly every day. After 48 h treatment of PEITC, 10 ng/mL of LPS treatment was added and after 4 h the cells were collected.

4.3.4. RNA extraction and sequencing using RNA-seq technology

Total RNA was extracted using Trizol followed by DNase digestion and Qiagen RNeasy column purification following the manufacturer's protocol (Qiagen, Valencia, CA). The integrity was verified using an Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA). High-quality RNA was processed using an Illumina RNA-seq sample prep kit following the

manufacturer's instructions (Illumina, San Diego, CA). Final RNA-seq libraries were validated and sequenced at 40 bp/sequence using an Illumina GAIIx sequencer at a depth of approximately 24.8 million sequences per sample.

4.3.5. Data analysis and bioinformatics.

Raw sequence reads were scanned for low quality and trimmed by several quality control filters using CLC genomics workbench software (CLC Bio, Aarhus, Denmark). Trimmed reads were aligned to the human genome to remove possible murine contaminants in the sample. The remaining reads were aligned to the human genome. The value for mismatch cost was set to 2, insertion cost was 3, for deletion cost was 3, length fraction was 0.95 and similarity fraction was 1.0.

The molecular processes, molecular functions and genetic networks following butyrate treatment were further evaluated by analyzing differentially expressed genes using Ingenuity Pathways Analysis (IPA, Ingenuity Systems, and www.ingenuity.com). IPA is a software application that enables biologists to identify the biological mechanisms, pathways, and functions most relevant to their experimental datasets or genes of interest.

4.3.6. Principal component analysis

The principal component analysis (PCA) was performed using CLC. The directions of the variability of the data were analyzed using the default programmed covariance-estimation. Each component represents the eigenvector with one eigenvalue. The final data was projected onto space in a plot spanned by the eigenvectors with the simplified version of information of data variability in directions.

4.3.7. Volcano plot analysis

The volcano plot is a special plot showing the significance of changes in genes and transcripts, and the differences of genes are visualized in a 2-D scatter plot using a given comparison. The X-axis represents the fold change between different treatments, and the value is transformed to a log2 scale. The Y-axis exhibits negative log10 of the *P*-values and gives the information of statistical significance. The volcano plot analysis was conducted using CLC using Kal's statistical analysis (Z-score) test. The results of the statistical analysis were represented as a scatter plot with information of the weighted differences of the mean group values and the weighed fold changes of the mean group values. Significantly altered genes (Z-score was less than -2 or greater than 2) were marked with blue color and red color, respectively.

4.3.8. Canonical pathway analysis of data sets

The analysis of canonical pathways identified the pathways from the IPA library of canonical pathways that were most significant to the dataset. Genes from the data set that were associated with a canonical pathway in the Ingenuity Pathways Knowledge Base were considered for the analysis. The significance of the association between the dataset and the canonical pathway was measured by Fischer's exact test and *P*-value determining the probability that the association between the genes in the dataset and the canonical pathway was explained by chance alone.

4.3.9. Functional analysis of data sets

The Functional Analysis identified the biological functions and diseases that were most significant to the dataset. Genes from the datasets that were associated with biological

functions and diseases in the Ingenuity Pathways Knowledge Base were considered for the analysis. Fischer's exact test was used to calculate a *P*-value determining the probability that each biological function and disease assigned to that data set was due to chance alone.

4.3.10. Pathways analysis and network generation

A data set containing gene identifiers and corresponding expression values was uploaded into in the application. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. These genes, called Focus Genes, were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these focus genes were then algorithmically generated based on their connectivity.

4.3.11. Network/pathways graphical representation

A network pathway is a graphical representation of the molecular relationships between gene products. Genes were represented as nodes, and the biological relationship between two nodes was represented as an edge. All edges were supported by canonical information stored in the Ingenuity Pathways Knowledge Base. The intensity of the node color indicated the degree of up- (red) or down- (green) regulation.

4.3.12. Statistical analysis

For RT-PCR and protein experiments, statistical analysis of data was carried out using the GraphPad PRISM program (GraphPad Software Inc., San Diego, CA). Multiple groups of data were analyzed using the analysis of variance (ANOVA) test followed by post-hoc test. All experiments were performed in triplicate, and data were reported as a mean ± standard error of the mean (SEM). P values ≤ 0.05 were considered as significant. For bio-informatic

data analyzed using CLC genomics workbench software, FDR adjusted *P*-value (Student's t-test) was used, and a value less than 0.05 was considered as with significance (CLC Bio, Aarhus, Denmark). For IPA analysis, the z-score algorithm was used to identify the predicted biological functions. Z-score greater than 2 was considered as activated and z-score less than -2 was considered as inhibited (IPA, Ingenuity Systems, and www.ingenuity.com).

4.4 Results

4.4.1. Effects of PEITC in LNCaP xenograft tumor and THP-1 cell growth

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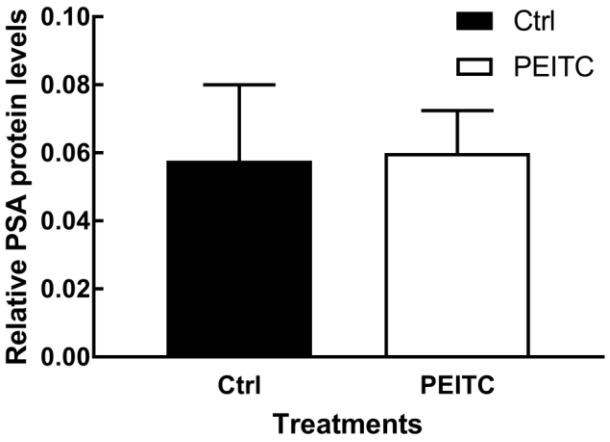


Fig. 4.1 Relative PSA protein levels in LNCaP xenograft tumor treated without or with PEITC. *P* values ≤ 0.05 were considered as significant and * indicates significantly different from control.

We found that the consumption of PEITC in athymic mice, while inhibitingtumor growth, did not lead to a change in PSA protein levels in LNCaP xenograft tumors as compared to that in the control with no PEITC treatment (Fig.4.1).

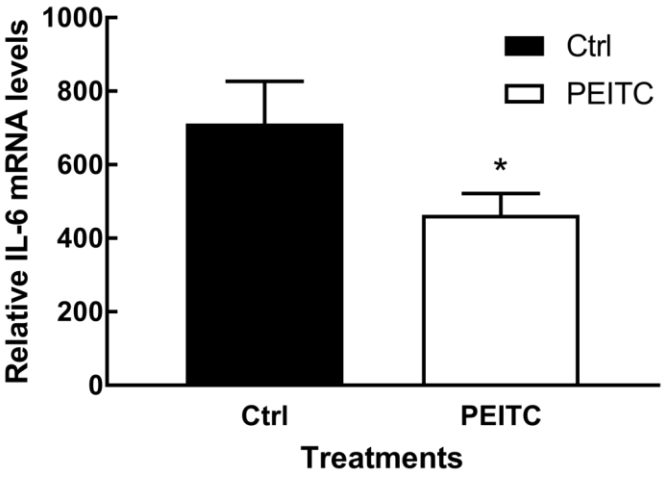


Fig. 4.2 Relative IL-6 mRNA levels in LNCaP xenograft tumor treated without or with PEITC. *P* values ≤ 0.05 were considered as significant and * indicates significantly different from control.

Hudson et al. have reported that the suppressive effect on prostate tumor growth by PEITC might not be caused by the apoptosis of prostate tumor cells or the regulation of androgen receptor (Hudson et al., 2012). However, the mouse inflammatory cytokine IL-6 was significantly down-regulated by PEITC in LNCaP xenograft tumor (Fig. 4.2). These observations suggested that the regulation of prostate cancer progression by PEITC might be inflammation related.

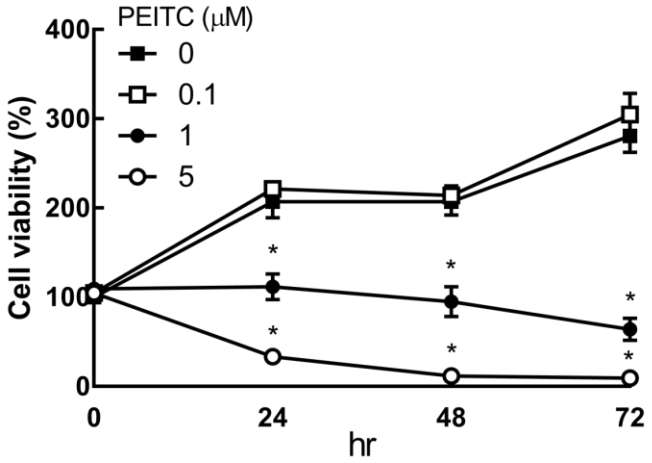


Fig. 4.3 Effect of PEITC on THP-1 growth. Results were compared to the control and expressed as relative mRNA levels (mean ± SD, n = 3). *P* values ≤ 0.05 were considered as significant and * indicates significantly different from control.

We observed in human monocytic leukemia THP-1 cells that PETIC significantly inhibited THP-1 cell growth in a dose-dependent manner (Fig. 4.3). Significant inhibition of THP-1 cell growth was observed after 24 h treatment of PEITC at concentrations of 1 and 5 μM.

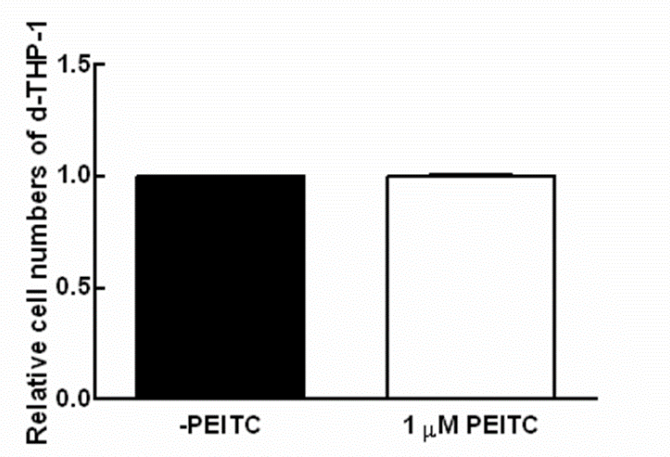


Fig. 4.4 Effect of PEITC on d-THP-1 cell growth. Results were compared to the control and expressed as relative mRNA levels (mean ± SD, n = 3). P values ≤ 0.05 were considered as significant and * indicates significantly different from control.

We also examined the effects of PEITC on macrophage. No significant changes of cell numbers could be observed in differentiated THP-1(macrophage form, d-THP-1) without/with the treatment of 1 μM PEITC (Fig. 4.4) The data suggested that PEITC did not affect cell growth of d-THP-1 (Fig. 4.4).

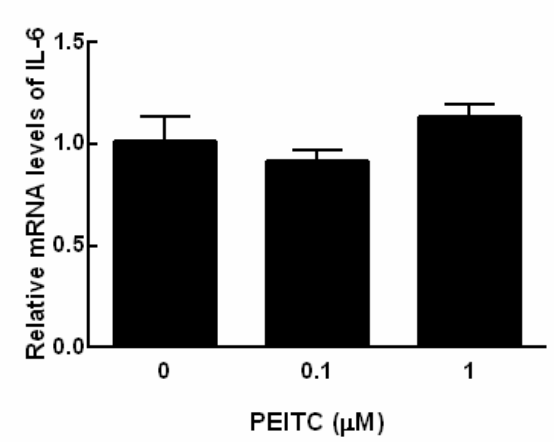


Fig. 4.5 Effect of PEITC on IL-6 in d-THP-1 with LPS. Results were compared to the control and expressed as relative mRNA levels (mean ± SD, n = 3). P values ≤ 0.05 were considered as significant and * indicates significantly different from control.

We also examined the effects of PEITC on IL-6 expression in d-THP-1. Interestingly, we did not observe the effect on LPS-induction of IL-6 expression by PEITC treatment in d-THP-1 (Fig. 4.5).

4.4.2. Concentration effects of PEITC on THP-1 stimulated with/without LPS on global gene expression.

Given the lack of effect on macrophage growth, response of macrophage toward LPS induction of IL-6 but growth inhibitory effect on monocyte by PEITC, we decided to focus on monocyte as the target. We used RNA-seq as a tool to further elucidate the molecular effects of PEITC on THP-1 in the presence and absence of LPS stimulation. The principal component analysis (PCA) identified 6 groups of samples that were well distinguished from each other (Fig. 4.6). Three replicates for each treatment group were clustered together, and different concentrations (0, 0.25, and 1 μM of PEITC) of groups were well separated. The treatments with or without LPS stimulation were clearly differentiated. In summary, samples with/without treatment of different concentrations of PEITC and LPS stimuli were well separated from each other, which suggests gene expression profiles are different form each other.

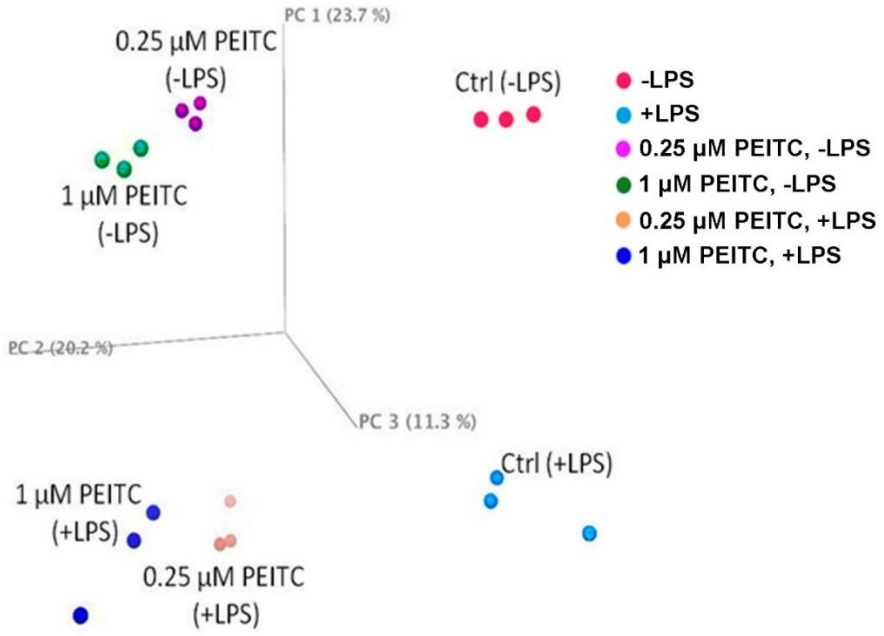


Fig. 4.6 PCA for u-THP-1 treated with/without LPS and PEITC. Sets of u-THP-1 samples without and with LPS stimulation were colored in red and cyan, respectively. U-THP-1 treated with 0.25 μM and 1 μM PEITC without LPS stimulation were colored in magenta and green, respectively. U-THP-1 treated with 0.25 μM and 1 μM PEITC with LPS stimulation were colored in yellow and blue, respectively.

4.4.3. Volcano plots of transcript profiles of THP-1 treated by PEITC with/without LPS

The volcano plots reveal the fold changes and *P*-values of global transcriptome profiling of RNA-seq analysis (Trapnell et al., 2012).

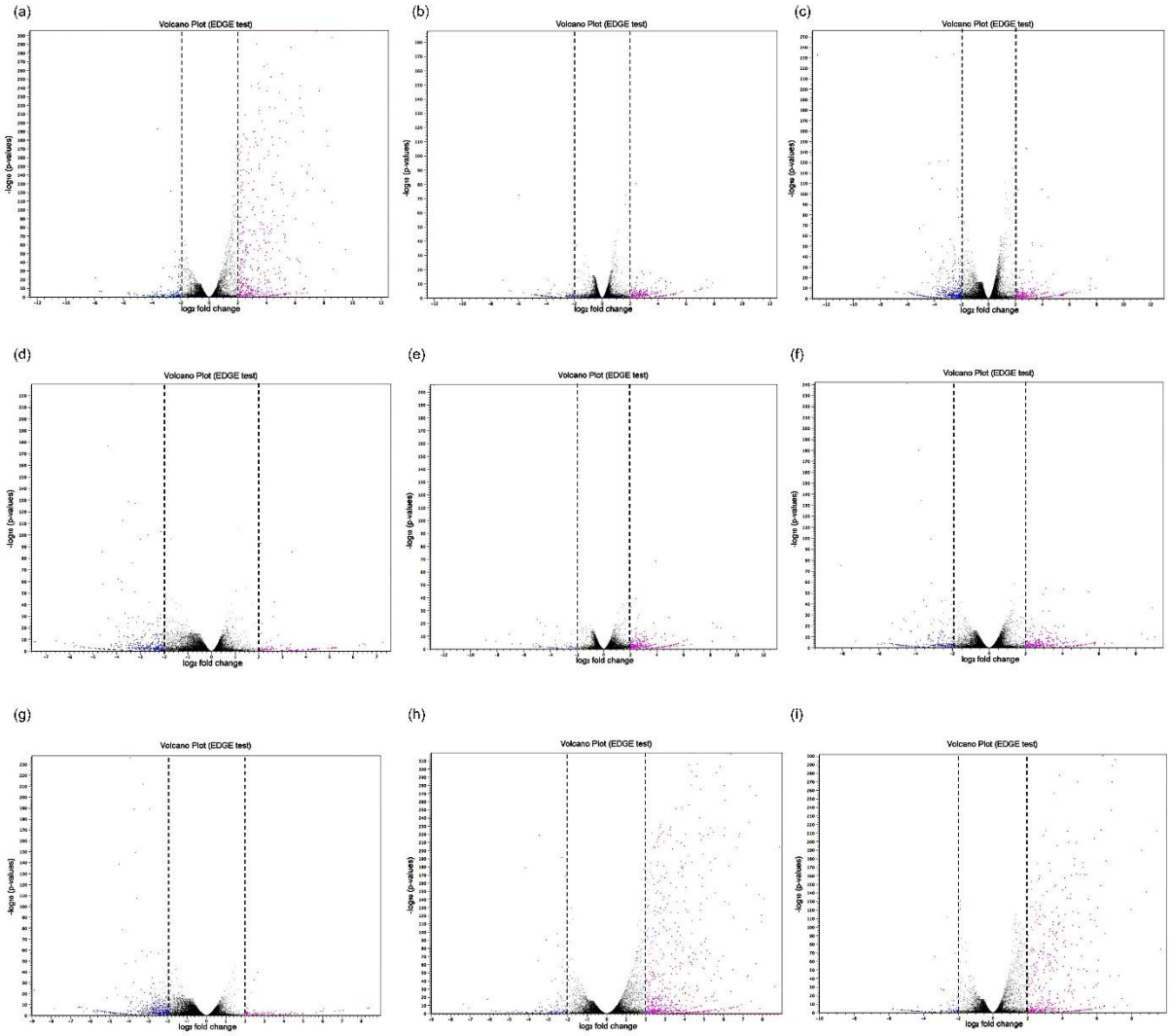


Fig. 4.7 Volcano Plots (a) control vs LPS (b) control vs 0.25 μM PEITC (c) control vs 1 μM PEITC (d) 0.25 μM PEITC vs 1 μM PEITC (e) LPS vs 0.25 μM PEITC with LPS (f) LPS vs 1 μM PEITC with LPS (g) 0.25 μM PEITC with LPS vs 1 μM PEITC with LPS (h) 0.25 μM PEITC vs 0.25 μM PEITC with LPS (i) 1 μM PEITC vs 1 μM PEITC with LPS. Transcriptome profiles with z-score less than -2 were marked with blue color, and with z-score greater than 2 were marked with red color.

In Fig.4.7, each point in the 2D scatters plot represents a statistical result for a single gene. The fold change in horizontal axis was in log2 scale, and the *P*-value in vertical axis was in log10 scale. Genes with up- or down-regulation by more than 2 folds with a *P*-value

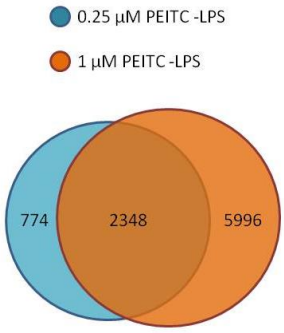
number less than 0.05 were considered significantly changed. The down-regulated genes with the z-score less than -2 were marked with blue color, and genes increased with z-score larger than 2 were highlighted using red color. LPS stimulation significantly altered gene expressions in THP-1, and a greater number of up-regulated genes than down-regulated genes was observed. Fig. 4.7b, c, and d indicated that the changes of transcriptome profiles in THP-1 were correlated with PEITC treatment in a concentration-dependent manner. In Fig. 4.7 (e), (f) and (g), 1 μM of PEITC treatment significantly inhibited the up-regulated genes induced by LPS. The number of inhibited genes was concentration-dependent to PEITC treatment. Moreover, in Fig. 4.7 (h) and (i), the same concentration of PEITC (at 0.25 or 1 μM) significantly altered gene transcriptomes with the treatment of LPS.

Together, the results of volcano analysis indicated that 1) the gene transcriptomes were significantly differed by the treatment of PEITC with/without LPS stimulation and 2) the regulation of genes was concentration-dependent to the PEITC treatment.

4.4.4. Number of overlapped genes in THP-1 by PEITC with/without LPS stimulation

The numbers of overlapped genes in THP-1 treated with different concentrations of PEITC with/without LPS stimulation were summarized.

(a)



(b)

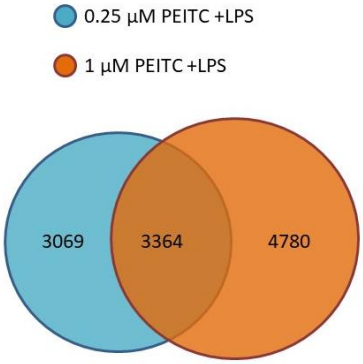


Fig. 4.8 Comparison of the overlapped genes (a) THP-1 treated with 0.25 μM and 1 μM PEITC (no LPS) (b) THP-1 treated with 0.25 μM and 1 μM PEITC (with LPS).

Without LPS induction, 3122 genes were significantly altered in THP-1 treated with 0.25 μM of PEITC. 8344 genes were changed with the treatment of 1 μM of PEITC (Fig. 4.8 a and b). 2348 genes were overlapped in THP-1 with 0.25 and 1 μM of PEITC treatment. After the stimulation of LPS, 6433 and 8144 genes were shown with the difference in THP-1 treated with 0.25 and 1 μM of PEITC, respectively. The number of overlapped genes in THP-1 treated with 0.25 and 1 μM of PEITC with LPS induction reached 3364.

4.4.5. Most up-regulated and down-regulated genes by LPS in THP-1

The respond of human monocytic THP-1 to Gram-negative bacteria cell wall component LPS stimulation was well studied (Chanput et al., 2010), but RNA-seq analysis are less documented. The 20 genes with most up- and down-regulation from our analysis are listed in Table 4.1.

(a)

Symbol	*p*-value	Fold change	Gene ID
CCL4L1	0	5042.69	ENSG00000205020
TNFAIP6	0	895.99	ENSG00000123610
IGKC_1	2.85E-55	724.80	ENSG00000211592
CCL4	0	450.54	ENSG00000129277
SPERT	8.17E-33	401.91	ENSG00000174015
IL1B	0	395.54	ENSG00000125538
INHBA	3.02E-109	379.58	ENSG00000122641
CCL3	1.31E-298	372.80	ENSG00000006075
CCL4L2	0	364.82	ENSG00000197262
CXCL3	5.96E-174	311.83	ENSG00000163734
CXCL10	1.74E-191	288.67	ENSG00000169245
BX323046.3-1	2.32E-08	272.06	ENSG00000213025
AL139130.28	9.69E-123	264.12	ENSG00000215848
C4orf7	2.35E-63	207.25	ENSG00000181617
CCL3L3	1.44E-237	203.15	ENSG00000205021
NR4A3	3.56E-306	181.01	ENSG00000119508
CTGF	4.63E-85	149.43	ENSG00000118523
IL12B	1.07E-136	147.86	ENSG00000113302
IGFBP3	0	146.09	ENSG00000146674
CCL20	2.59E-152	126.50	ENSG00000115009

(b)

Symbol	*p*-value	Fold change	Gene ID
AC005488.2-6	9.16E-23	-244.58	ENSG00000205583
AC092536.3-1	1.94E-07	-202.21	ENSG00000214605
AL021393.1-2	3.94E-07	-182.66	ENSG00000218478
AC008763.9	1.59E-05	-50.78	ENSG00000217570
C11orf89	2.25E-05	-48.10	ENSG00000184682
OFDYP6	0.0016	-47.05	ENSG00000219600
DNAI1	0.00032	-37.31	ENSG00000122735
AP001462.3	0.0031	-32.72	ENSG00000213477
SPDYE8P	0.0035	-32.67	ENSG00000198305
CYYR1	0.026	-25.07	ENSG00000166265
AC074182.6	0.016	-24.04	ENSG00000177855
C10orf99	0.0092	-23.30	ENSG00000188373
AC009758.8-2	0.0087	-23.18	ENSG00000171999
AC120057.9	0.0087	-23.10	ENSG00000213876
FOXS1	0.029	-20.23	ENSG00000179772
GSTT2B	0.024	-19.09	ENSG00000133433
AL035086.12	0.040	-18.22	ENSG00000213089
AL356320.8	0.034	-17.68	ENSG00000215900
FAM3B	0.034	-17.68	ENSG00000183844
C2orf71	0.034	-17.68	ENSG00000179270

Table 4.1 20 most (a) up-regulated and (b) down-regulated genes by LPS in u-THP-1.

* *p*-value gets down as "0" represents the value less than 2.225074e-308.

Among the 20 most up-regulated genes, 8 were from chemokines families (Table 4.1). The chemokine CCL4L1 was the most up-regulated, by 5042.69-fold (Table 4.1). Other chemokines, including CCL4, CCL3, CCL4L2, CXCL3, CXCL10, CCL3L3, and CCL20, were increased by 450.54, 372.80, 364.82, 311.83, 288.67, 203.15, and 126.50-fold, respectively. Inflammatory cytokines, such as IL-1β and IL-12β, were also among the most up-regulated genes, with an increase of 395.54 and 147.86-fold respectively.

Several novel genes were identified as the most down-regulated genes in THP-1 cells with LPS stimulation (Table 4.1b). AC005488.2-6, AC092536.3-1, and AL021393.1-2 were the 3 most down-regulated novel target genes in LPS induced THP-1. AC005488.2-6, also known as STAG3L1 (Stromal Antigen 3-Like 1), is a Pseudogene. Though pseudogene is not fully functional, it may possess regulatory functions for coding gene expression and reveal a biological role through a coding-independent function for mRNAs (Polisenno et al., 2010). AC092536.3-1 and AL021393.1-2 are novel genes that were the second and third most down-regulated genes in LPS induced THP-1.

4.4.6. 20 most (a) up-regulated (b) down-regulated genes by PEITC with/without LPS stimulation

The 20 most up- and down-regulated genes were identified in THP-1 by PEITC with/without LPS stimulation. One interesting feature is that several highly induced genes are novel and have limited coverage in literature.

(a)

Symbol	Fold change	*p*-value	Gene ID
BX323046.3-1	428.15	3.89E-36	ENSG00000213025
AC068533.7-2	245.85	2.086E-09	ENSG00000214623
AC145132.2-1	191.16	1.74E-18	ENSG00000215006
TIAF1	184.77	4.52E-08	ENSG00000221995
C21orf124	182.61	7.61E-12	ENSG00000215465
AC010531.8-3	109.62	5.77E-06	ENSG00000218256
AL672187.12-2	90.97	2.90E-05	ENSG00000174196
RP11-492M23.2	82.65	5.43E-08	ENSG00000216946
GOLGA6C	74.08	9.88E-06	ENSG00000159289
AL353734.12	63.37	4.69E-06	ENSG00000214178
AL139415.10-1	55.57	2.75E-05	ENSG00000183445
RP11-4M23.4	52.79	5.08E-05	ENSG00000217257
RP11-118F2.2	49.95	8.87E-05	ENSG00000218751
AC025566.22	47.37	0.00017	ENSG00000205412
AL121893.21-2	47.02	0.00019	ENSG00000214612
AL135925.10	46.60	0.00317	ENSG00000165429
AC012533.11	44.87	0.00279	ENSG00000215844
AL135933.11-1	44.61	0.00032	ENSG00000214971
CR848007.7	43.57	0.00451	ENSG00000216469
DPYSL5	42.78	0.00451	ENSG00000157851

(b)

Symbol	Fold change	*p*-value	Gene ID
AC010654.8	-6355.05	1.35E-231	ENSG00000213059
AC004832.3-4	-280.28	5.94E-10	ENSG00000220549
AC136443.3-1	-208.32	3.00E-19	ENSG00000069651
AC008132.12	-118.36	3.06E-06	ENSG00000216340
AL356155.10	-107.84	5.77E-06	ENSG00000203973
AC073655.26-2	-74.63	0.00011	ENSG00000213250
TCEB3C	-61.89	4.88E-06	ENSG00000183791
RP11-628K18.1	-56.44	1.67E-05	ENSG00000215056
AC003111.1-2	-53.11	5.23E-05	ENSG00000209745
AL603965.10-1	-52.98	5.26E-05	ENSG00000204150
KLHDC9	-50.84	9.03E-05	ENSG00000162755
LRRN3	-50.15	0.00019	ENSG00000173114
JSRP1	-47.95	0.00017	ENSG00000167476
OFDYP6	-45.83	0.0032	ENSG00000219600
AC008740.7-2	-45.73	0.0022	ENSG00000205691
AC090772.9-2	-45.73	0.0022	ENSG00000215504
AL109923.29-1	-44.84	0.00034	ENSG00000181741
FAM22F	-39.46	2.72E-06	ENSG00000130950
AC027176.22-1	-38.99	0.0044	ENSG00000214445
CGB7	-37.96	0.0011	ENSG00000196337

Table 4.2 20 most (a) up-regulated (b) down-regulated genes by PEITC (no LPS).

In THP-1 without LPS stimulation, BX323046.3-1, AC068533.7-2, AC145132.2-1 and TIAF1were identified as the 4 most up-regulated genes in THP-1 treated with PEITC, by 428.15, 245.85, 191.16 and 184.77-fold, respectively (Table 4.2a). BX323046.3-1 was for the first time reported as the most up-regulated gene in THP-1 with PEITC treatment in the absence of LPS stimulation. AC068533.7-2 and AC145132.2-1 were novel genes discovered with significant changes and limited documents. For the down-regulated genes, AC010654.8, AC004832.3-4, AC136443.3-1, AC008132.12, AL356155.10 were the 5 most inhibited genes, by -6355.05, -280.28, -208.28, -118.36 and -107.84 folds respectively

(Table 4. 2b). All 5 most down-regulated genes were also novel and limited references were available about these genes.

(a)

Symbol	*p*-value	Fold change	Gene ID
AC092536.3-1	6.37E-11	525.78	ENSG00000214605
AL021393.1-2	4.48E-37	480.13	ENSG00000218478
AC068533.7-2	7.46E-09	395.11	ENSG00000214623
AC005488.2-6	1.39E-13	317.78	ENSG00000205583
AC092375.4-5	3.19E-05	103.43	ENSG00000185710
RP11-91G11.1	0.00016	79.18	ENSG00000218680
RP11-165I9.7	6.75E-06	55.74	ENSG00000220865
RP11-492M23.2	6.75E-06	55.74	ENSG00000216946
AC008763.9	2.25E-05	55.02	ENSG00000217570
RP11-438P9.1	5.84E-05	54.01	ENSG00000218352
AC124068.6	0.0012	53.50	ENSG00000213485
AC125494.2-2	0.0012	53.50	ENSG00000219410
RP11-309H21.1	2.27E-05	51.91	ENSG00000217637
AP000805.4	5.42E-05	48.65	ENSG00000214389
AC106785.3-18	0.00018	44.80	ENSG00000213541
AC145132.2-1	8.089E-52	42.55	ENSG00000215006
RP3-347M6.1	1.56E-12	39.92	ENSG00000217491
XXbac-BPG34I8.2	0.0011	39.16	ENSG00000218347
CTD-2090I13.4	0.00034	38.65	ENSG00000220436
AP000857.4	0.00042	38.52	ENSG00000213305

(b)

Symbol	*p*-value	Fold change	Gene ID
AC000041.8	8.75E-76	-273.01	ENSG00000220855
AC018921.22	8.87E-07	-155.94	ENSG00000205128
AL672207.1	0.00030	-66.64	ENSG00000178146
AL135925.10	0.00058	-58.90	ENSG00000165429
ERN2	1.92E-05	-55.92	ENSG00000134398
7SK_54	6.25E-05	-51.71	ENSG00000201901
FMO3	5.90E-05	-51.43	ENSG00000007933
AC010654.8	7.89E-09	-42.24	ENSG00000213059
HIST1H2BB	9.99E-05	-42.23	ENSG00000196226
HIST1H4K	0.00019	-39.93	ENSG00000197914
HIST1H2AJ	3.05E-23	-38.99	ENSG00000182611
RP11-423O2.1	0.00018	-38.48	ENSG00000218013
RP5-990P15.2	0.0050	-37.41	ENSG00000135820
RP11-345I18.4	0.00061	-33.96	ENSG00000219525
KLHDC9	0.00067	-33.55	ENSG00000162755
RAET1E	0.00068	-33.52	ENSG00000164520
DPYSL5	0.0062	-32.45	ENSG00000157851
L1TD1	0.0012	-31.58	ENSG00000162606
TMEM190	0.0019	-31.10	ENSG00000160472
RP11-522L3.2	0.0013	-29.81	ENSG00000218436

Table 4.3 20 most (a) up-regulated (b) down-regulated genes by PEITC (with LPS).

In LPS-stimulated THP-1 cells, AC092536.3-1, AL021393.1-2, and AC068533.7-2 were the 3 most up-regulated genes by PEITC, with a significant increase of 525.78, 480.13 and 395.11-fold, respectively (Table 4.3a). These genes are novel genes identified to be up-regulated by PEITC in THP-1 with LPS induction. No reference could be found for these three genes. For the down-regulated genes, AC000041.8, AC018921.22 and AL672207.1 were the most inhibited genes by PEITC treatment with LPS induction, by -273.01, -155.94, and -66.64-folds respectively (Table4.3b).

4.4.7. Most activated and inactivated canonical pathways in THP-1 with LPS stimulation

The most activated and inactivated canonical pathways in THP-1 with LPS stimulation were analyzed using IPA.

(a)

Ingenuity Canonical Pathways	*P*-value	Ratio	z-score
Neuroinflammation Signaling Pathway	7.94E-11	0.12	4.12
HMGB1 Signaling	9.55E-09	0.15	4.02
Dendritic Cell Maturation	1.26E-11	0.15	3.4
Cholecystokinin/Gastrin-mediated Signaling	0.0017	0.10	3.16
TREM1 Signaling	6.31E-11	0.24	3.15
Colorectal Cancer Metastasis Signaling	0.00015	0.082	3.13
IL-1 Signaling	0.0032	0.098	3
IL-6 Signaling	3.55E-08	0.15	2.98
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	2.69E-10	0.17	2.84

(b)

Ingenuity Canonical Pathways	*P*-value	Ratio	z-score
PPAR Signaling	6.46E-09	0.19	-3.64
LXR/RXR Activation	9.55E-06	0.12	-3.05

Table 4.4 Most (a) up-regulated and (b) down-regulated canonical pathways in LPS induced u-THP-1.

Neuroinflammation signaling, HMGB1 signaling, and dendritic cell maturation were the 3 most activated canonical pathways in LPS induced THP-1. In addition, LPS also activated interleukin signaling pathways, including the IL-1 and IL-6 signaling. Neuroinflammation signaling pathway is the most activated pathway in LPS induced THP-1 (Z-score: 4.12). The molecules up-regulated in this pathway included IL-1β (395.54-fold), CCL3 (372.80-fold), CXCL10 (288.67-fold), IL-12β (147.86-fold), WNT1 (101.31-fold), IL-6 (93.17-fold), ICAM1 (87.79-fold), CXCL8 (82.80-fold) and tumor necrosis factor alpha TNF

(57.05-fold). HMGB1 signaling was the second most activated pathway identified in LPS induced THP-1 (Z-score: 4.03). Other pathways, such as IL-1 (Z-score: 3.00) and IL-6 (Z-score: 2.98) signaling pathways, were also induced by LPS (Table 4.4). In addition, PPAR signaling and LXR/RXR signaling were the most inhibited canonical pathways by LPS induction in THP-1 (Z-score: -3.64 and -3.05 respectively).

4.4.8. Most altered canonical pathways in THP-1 by PEITC with/without LPS induction

To understand the mechanisms of the effect of PEITC on THP-1 in response to LPS induced inflammation, the canonical pathways modulated in THP-1 by PEITC were analyzed.

Protein kinase A and cAMP-mediated signaling were the two primary inhibited pathways by PEITC in THP-1, regardless of the presence of LPS stimulation (Table 4.5). The z-scores of protein kinase A signaling in THP-1 without and with LPS stimulation were -3.83 and -3.55, respectively (Table 4.5). Moreover, the cAMP-mediated signaling was found to have a z-score of -2.84 and -3.36 in THP-1 without and with LPS induced inflammation, respectively. Cardiac β-adrenergic signaling was inhibited (Z-score: -2.33) in THP-1 by PEITC without LPS induction, but that signaling was not impacted in THP-1 with LPS induction.

The observation indicated that the protein kinase A and cAMP-mediated signaling were the primary pathways changed by PEITC in THP-1 regardless of the LPS stimulation.

(a)

Ingenuity Canonical Pathways	*P*-value	Ratio	z-score
Protein Kinase A Signaling	0.037	0.068	-3.84
cAMP-mediated signaling	0.00081	0.098	-2.84
Cardiac β-adrenergic Signaling	0.030	0.086	-2.33

(b)

Ingenuity Canonical Pathways	*P*-value	Ratio	z-score
Protein Kinase A Signaling	0.00049	0.068	-3.55
cAMP-mediated signaling	0.0079	0.067	-3.36

Table 4.5 Most altered canonical pathways in u-THP-1 treated with PEITC (a) without LPS (b) with LPS.

4.4.9. Genes altered in protein kinase A/cAMP signaling pathway in THP-1 by PEITC with/without LPS induced inflammation

(a)

Symbol	Entrez Gene Name	Fold Change	Expected
AVPR2	arginine vasopressin receptor 2	-3.49	Up
BRAF	B-Raf proto-oncogene, serine/threonine kinase	-2.11	Up
CAMK1D	calcium/calmodulin dependent protein kinase ID	-2.88	Up
DRD4	dopamine receptor D4	-3.87	Up
GPER1	G protein-coupled estrogen receptor 1	-2.23	Up
GRM2	glutamate metabotropic receptor 2	-3.86	Up
GRM8	glutamate metabotropic receptor 8	-2.40	Up
HIST1H1B	histone cluster 1 H1 family member b	-14.65	Up
HIST1H1C	histone cluster 1 H1 family member c	-2.37	Up
HIST1H1D	histone cluster 1 H1 family member d	-7.79	Up
HIST1H1E	histone cluster 1 H1 family member e	-27.94	Up
HIST1H3C	histone cluster 1 H3 family member c	-3.00	Up
HIST2H3C	histone cluster 2 H3 family member c	-5.98	Up
LIPE	lipase E, hormone sensitive type	-2.38	Up
P2RY12	purinergic receptor P2Y12	6.97	Up
P2RY13	purinergic receptor P2Y13	5.68	Up
PDE11A	phosphodiesterase 11A	-2.23	Up
PDE1C	phosphodiesterase 1C	-2.04	Up
PDE3A	phosphodiesterase 3A	-7.81	Up
PDE4B	phosphodiesterase 4B	-2.43	Up
PDE4C	phosphodiesterase 4C	-2.01	Up
PDE4D	phosphodiesterase 4D	-2.91	Up
PDE6A	phosphodiesterase 6A	-2.16	Up
PHKG1	phosphorylase kinase catalytic subunit gamma 1	-2.12	Up
PLCB1	phospholipase C beta 1	-2.03	Up
PLN	phospholamban	-31.77	Down
PPP1R1B	protein phosphatase 1 regulatory inhibitor subunit 1B	-2.56	Up
PRKCE	protein kinase C epsilon	-2.59	Up
PTH1R	parathyroid hormone 1 receptor	-10.93	Up
PTPN14	protein tyrosine phosphatase, non-receptor type 14	-2.004	Down
PYGM	glycogen phosphorylase, muscle associated	-3.77	Up
RGS4	regulator of G protein signaling 4	8.46	Down
RXFP4	relaxin/insulin like family peptide receptor 4	-10.02	Up

(b)

Symbol	Entrez Gene Name	Fold change (LPS)	Fold Change (PEITC -+LPS)	Expected
ANAPC11	anaphase promoting complex subunit 11	2.01	Down	
AVPR2	arginine vasopressin receptor 2	-2.32	Up	
BRAF	B-Raf proto-oncogene, serine/threonine kinase	-2.09	Up	
DRD4	dopamine receptor D4		-2.13	Up
DUSP1	dual specificity phosphatase 1	5.80	2.46	Down
DUSP2	dual specificity phosphatase 2	4.67	2.40	Down
ENPP6	ectonucleotide pyrophosphatase/phosphodiesterase 6	-3.07	Up	
GYS2	glycogen synthase 2		-4.04	Down
HIST1H1B	histone cluster 1 H1 family member b	-9.62	Up	
HIST1H1D	histone cluster 1 H1 family member d	-9.08	Up	
HIST1H1E	histone cluster 1 H1 family member e	-16.88	Up	
HIST1H3C	histone cluster 1 H3 family member c	-4.23	Up	
HIST2H3C	histone cluster 2 H3 family member c	-3.14	Up	
HTR6	5-hydroxytryptamine receptor 6	-2.81	Up	
NTN1	netrin 1	4.52	-2.61	Up
P2RY13	purinergic receptor P2Y13	7.64	-2.32	Up
PDE11A	phosphodiesterase 11A		-2.01	Up
PDE3A	phosphodiesterase 3A		-6.71	Up
PDE4D	phosphodiesterase 4D		-2.25	Up
PDE6G	phosphodiesterase 6G	2.20	-2.49	Up
PLCB1	phospholipase C beta 1		-2.18	Up
PLN	phospholamban		-4.73	Down
PPP1R1B	protein phosphatase 1 regulatory inhibitor subunit 1B	-5.003	Up	
PPP1R3C	protein phosphatase 1 regulatory subunit 3C	2.23	Down	
PRKCE	protein kinase C epsilon		-2.04	Up
PTGDR	prostaglandin D2 receptor	26.65	-2.72	Up
PTH1R	parathyroid hormone 1 receptor	-5.85	Up	
PYGM	glycogen phosphorylase, muscle associated	-2.68	Up	
RXFP4	relaxin/insulin like family peptide receptor 4	-2.64	Up	

Table 4.6 Protein kinase A/cAMP signaling pathways in THP-1treated with PEITC (a) without (b) with LPS stimulation.

All the genes changed in protein kinase A and cAMP signaling pathways in THP-1 by PEITC were listed in Table 4.6.

The *P*-values for protein kinase A and cAMP signaling pathway were 0.0372 and 0.0008, and the z-scores were -3.84 and -2.84 (Table 4.5), indicating that these two pathways were significantly inhibited in THP-1 by PEITC treatment without LPS stimulation. Histone cluster H1 was a large group with alterations by PEITC in THP-1 without LPS induction. Histone H1 family is a group of potential biomarkers for various cancer types (Scaffidi, 2016). All the genes in histone H1 family, including HIST1H1B, HIST1H1C, HIST1H1D, HIST1H1E, and HIST1H3C, were down-regulated. The other group of genes with alterations by PEITC treatment were phosphodiesterases (PDE). In this study, PDE11A, PDE1C, PDE3A, PDE4B, PDE4C, PDE4D and PDE6A were all significantly down-regulated.

With LPS induction, genes in histone and phosphodiesterase families remained in the top groups of genes regulated by PEITC in THP-1 (Table 4.6.4b), Other groups of genes, such as dual-specificity phosphatases (DUSP), were also identified to be altered by PEITC in THP-1 in response to LPS induced inflammation. The DUSP1 and DUSP2 were up-regulated by 2.46 and 2.40-fold, respectively. PEITC also changed the expression of protein phosphatases in THP-1 with LPS induced inflammation. PPP1R1B was down-regulated by -5.00-fold, and PPP1R3C was up-regulated by 2.23-fold.

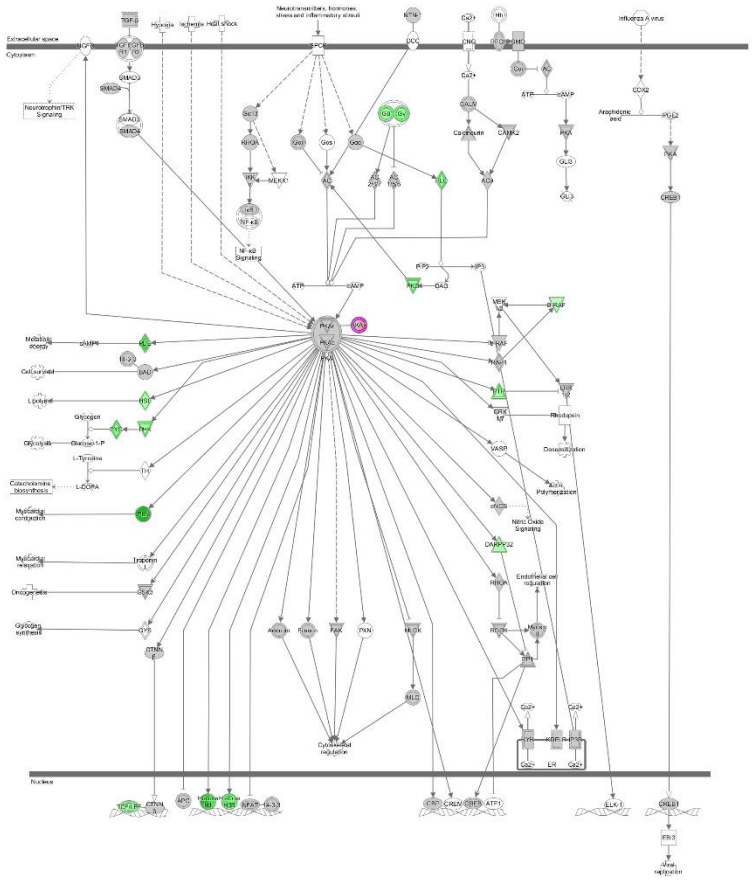
Together, the protein kinase A/cAMP signaling pathway was inhibited by PEITC in THP-1 with/without LPS induced inflammation. The histone H1 and phosphodiesterases

families were the primary groups of genes altered by PEITC in THP-1 regardless of LPS induced inflammation. In addition, expressions of DUSP and protein phosphatases were changed by PEITC in THP-1 in response to LPS stimulated inflammation.

4.4.10. Protein Kinase A/cAMP signaling pathway network analysis in THP-1 treated by 1 μM of PEITC with/without LPS induced inflammation

The signaling pathways of protein kinase A/cAMP regulation were analyzed using IPA.

(a)



(b)

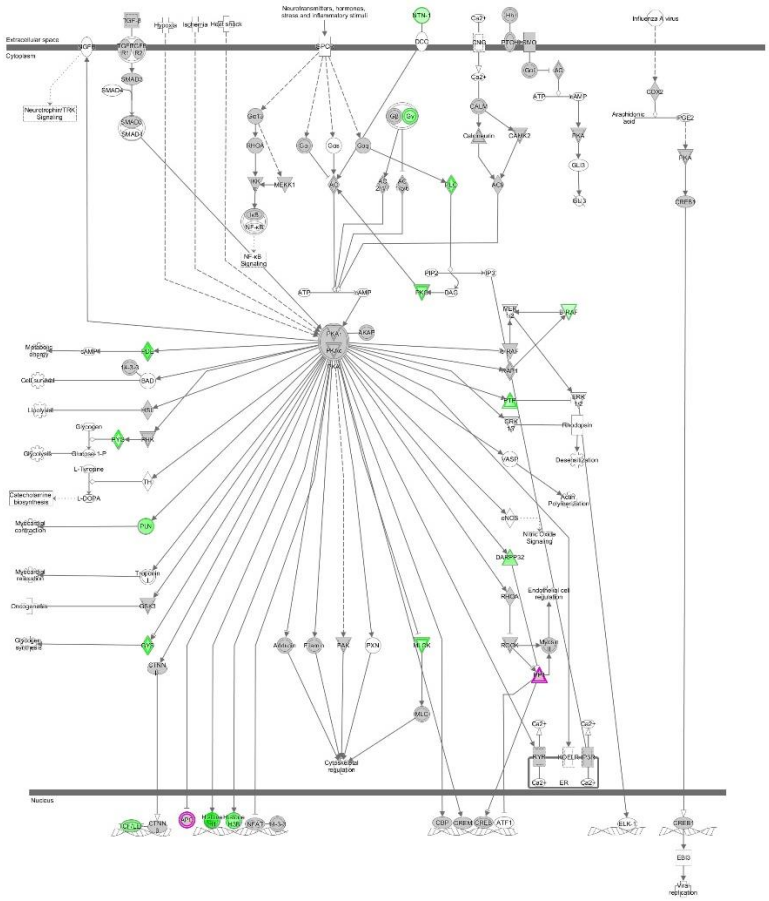


Fig. 4.9 Protein Kinase A/cAMP signaling pathway in THP-1 treated by 1 μM of PEITC (a) without (b) with LPS stimulation. Up-regulated genes were marked with red color. Down-regulated genes were marked with green color.

In THP-1 with/without LPS induced inflammation, PEITC treatment inhibited Gγ, PLC, PKC, PDE, PYC, PLN, B-RAF, PTP, DARPP32 (PPP1R1B), TCF/LEF, histone H1 and histone H3B, indicating that these genes involved in protein kinase A/cAMP signaling were regulated by PEITC in THP-1 regardless of the presence of LPS induced inflammation (Fig. 4.9 a and b).

Furthermore, in response to the LPS induced inflammation, NTN-1, PP-1, and APC were differentially regulated by PEITC in THP-1 as compared to the situation with no inflammation challenge. The NTN-a was significantly down-regulated, and PP-1 and APC were up-regulated by PEITC in THP-1 with LPS stimulation. The changes of genes in kinase A/cAMP signaling pathway indicated that Gγ, PLC, PKC, PDE, PYC, PLN, B-RAF, PTP, DARPP32 (PPP1R1B), TCF/LEF, histone H1 and histone H3B were primary genes from kinase A/cAMP signaling pathway regulated by PEITC in THP-1 regardless of the LPS induced inflammation. In addition, NTN-1, PP-1, and APC were differentially regulated by PEITC upon the challenge of LPS stimuli.

4.5 Discussion

In this study we seek to elucidate the effects of PEITC in immune cells that may contribute to its protective effects against prostate cancer. Our RNA-seq approach allowed us to identify several novel genes and the aspect of PEITC on monocyte/macrophage.

4.5.1. Effects of PEITC in LNCaP xenograft tumor and THP-1 cell growth

PEITC is a dietary compound present in cruciferous vegetables (Fahey et al., 2001). *In vivo* study of PEITC showed that the dietary compound PEITC was an anti-cancer candidate for prostate cancer (Li et al., 2013). Interestingly, mRNA expression of PSA, a androgen responsive gene, was not changed by PEITC treatment (Fig. 4.1). PSA is a well-known chymotrypsin-like serine protease in the human prostate gland (Horil et al., 2007). The effect of PEITC on PSA protein level indicated that the regulation of LNCaP xenograft tumor growth by PEITC was not associated with hormone related signaling pathways (Fig. 4.1). Instead, the inflammatory cytokine IL-6 was significantly down-regulated in LNCaP xenograft tumor by PEITC (Fig. 4.2). These findings suggested a relationship between the

suppressive effect of PEITC on LNCaP xenograft tumor growth and immune response. We also did not observe effects of PEITC on d-THP-1 macrophage cell growth and IL-6 mRNA expression (Fig. 4.4, Fig. 4.5), indicating that the regulation of tumor growth by PEITC might not be related to an effect on macrophages.

However, we did observe growth inhibitory effects on monocytes, suggesting that the primary target of PEITC is monocytes and a lower IL-6 mRNA level may be caused by this.

4.5.2. Most up-regulated and down-regulated genes by LPS in THP-1

The results of PCA and volcano plot analyses suggested that 1) gene transcriptomes of different sample groups differed from each other and 2) the global gene transcriptomes of THP-1 were significantly altered by PEITC with/without LPS stimulation in a dose-dependent manner.

As expected, LPS induction of THP-1 monocytes resulted in the up-regulation of different chemokines and cytokines. One feature from our analysis identified gene that appeared to be more sensitive to LPS, based on fold induction, than some of the more common LPS-responsive genes such as IL-1, IL-6 etc. Among the 20 up-regulated genes, C-C motif chemokine ligand 4 like 1 (CCL4L1) was most up-regulated. CCL4L1 is clustered on the q-arm of chromosome 17, and the secretion of CCL4L1 functions in inflammatory and immunoregulatory processes (NCBI gene cards). It is an inflammation associated gene (Gekonge et al., 2012) expressed in both monocytes and B lymphocytes (Lu et al., 2004). Also, it has been shown that CCL4L1 had a chemotactic effect on leukocyte recruitment (Menicucci et al., 2017) and could attract THP-1 cell migration (Fischer et al., 2007). The

overexpression of CCL4L1 in THP-1 with LPS stimulation might be involved in the regulation of THP-1 migration. The second most up-regulated gene Tumor Necrosis Factor Alpha-Induced Protein 6 (TNFAIP6) was increased by 895.99-fold. TNFAIP6 is known as a regulator of extracellular matrix stability and cell migration (NCBI gene cards). Schmelzer and Doring's reported that TNFAIP6 was the most up-regulated gene (by a 180-fold increase) in THP-1 monocytes by LPS stimulation (1 μg/mL) (Schmelzer & Doring, 2010). As compared to Schmelzer and Doring's observation, LPS concentration used in this study was 100 times lower (10 ng/mL) and was physiologically available (Huang et al., 2012). Based on these results, we reason that these genes may serve as more sensitive markers to assess for the exposure to LPS/gram negative bacteria.

Another interesting feature is the identification of several novel and less documented genes that were induced by LPS exposure. For the most down-regulated genes, AC005488.2-6, AC092536.3-1, and AL021393.1-2 were first reported in this study as the 3 most decreased genes by LPS induction in THP-1. Prior articles about AC092536.3-1 and AL021393.1-2 were limited. In addition, AC005488.2-6 (also known as STAG3L1) was reported by Pezzi et al as a gene expressed in both mouse and human organisms, especially in testis (Pezzi et al, 2000). The data from a comparison of LNCaP cell line and xenograft tumor from chapter 3 showed that AC005488.2-6 was significantly up-regulated by 2.16-fold in LNCaP tumor as compared to LNCaP cells (Data from Chapter 3). AC005488.2-6 was also associated with the synaptonemal complex in immunolocalization and was involved in chromosome pairing and maintenance of synaptonemal complex structure during the pachytene phase of meiosis (Pezzi et al., 2010). These findings support a novel biological role of AC005488.2-6 in mediating LPS induced inflammation.

4.5.3. 20 most up-regulated and down-regulated genes by PEITC in THP-1 with/without LPS stimulation

In THP-1 without LPS stimulation, BX323046.3-1, AC068533.7-2, AC145132.2-1, and TIAF1 were the 4 most up-regulated genes by PEITC treatment. BX323046.3-1, which is also called COX20P1 (cytochrome C oxidase assembly factor pseudogene1), was a novel gene and was only reported by King et al as one of the significant molecules expressed in squamous cell carcinoma (King et al., 2013). This gene was discovered as the most up-regulated (by 428.15-fold) gene by PEITC (1μM) in THP-1 without LPS stimulation. The AC068533.7-2 and AC145132.2-1 were novel genes with no reference. TIAF1 (TGFB1-induced anti-apoptotic factor 1) is another important gene discovered among the most up-regulated genes by PEITC in THP-1 without LPS stimulation. TIAF1 possesses growth inhibition and apoptosis induction effect on U937 monocytes (Khera & Chang, 2003). In this study, TIAF1 has been increased by 184.77-fold, providing possible mechanisms for the growth inhibition of monocytic THP-1 by PEITC treatment. For the down-regulated genes, AC010654.8, AC004832.3-4, AC136443.3-1, AC008132.12, AL356155.10 were novel genes discovered as the most inhibited ones by PEITC in the absence of LPS induced inflammation. No relevant literature could be found about the bio-functions or regulations of these five genes. Further research needs be conducted to elucidate the effects of these genes on THP-1 by PEITC. LRRN3 is among the most down-regulated genes by PEITC in THP-1 without LPS induced inflammation. LRRN3 is one of the genes commonly implicated in multiple cancers including leukemia, prostate, breast, lung cancers (Pihur et al., 2008). The down-regulation of LRRN3 (by -50.15-fold) by PEITC might be associated with the suppressive effect of PEITC on THP-1 leukemia cell growth.

In THP-1 with LPS induced inflammation, some genes were oppositely regulated by PEITC as compared to those in THP-1 with LPS stimulation only. AC092536.3-1, AL021393.1-2, AC068533.7-2 and AC005488.2-6 were the 4 most up-regulated genes by PEITC. Interestingly, as compared the most altered genes changed in THP-1 with LPS stimulation, the most three down-regulated genes including AC005488.2-6 (STAG3L1), AC092536.3-1 and AL021393.1-2 by LPS only, were among the 4 most up-regulated genes by PEITC treatment in THP-1 with LPS induction. The results indicated that PEITC functioned against LPS induced inflammation in THP-1 cells.

In contrast, there were also genes regulated by PEITC consistently in THP-1 with/without LPS induction. The expression of AC068533.7-2 and RP11-492M23.2 were increased in THP-1 with PEITC without LPS induction. They were also elevated in THP-1 with PEITC treatment with LPS stimulation. In addition, AC010654.8 was the most down-regulated gene in THP-1 treated with PEITC without LPS, and it also belonged to the 20 most down-regulated genes in THP-1 treated with PEITC and LPS induction. The data suggested that AC068533.7-2, RP11-492M23.2, and AC010654.8 were specific targets of PEITC in THP-1. Overall, the data from our analysis of up- down-regulated genes by PEITC in THP-1 with/without LPS indicated that PEITC was able to modulate LPS induced immune response.

4.5.4. Most activated and inactivated canonical pathways in THP-1 with LPS stimulation

Qin et al. have reported the correlation between pro-inflammatory factors (such as TNF and IL-1β) and the progression of neurodegenerative diseases using adult wild-type mice administrated with systemic LPS or TNF-α (Qin et al., 2007). In the progression of neuroinflammation, monocyte chemoattractant proteins and their receptors were

particularly important in modulating central nervous system (CNS) (Conductier et al., 2010; Ransohoff et al., 2007). The activation of neuroinflammation signaling pathway by LPS indicated an essential role that monocytes play in LPS induced chronic neuroinflammation and progressive neurodegeneration.

HMGB1 signaling was the second most activated pathway identified in LPS induced THP-1. High mobility group 1 protein (HMGB1) is a chromatin component produced by activated monocytes (for example, by LPS induction), macrophages or necrotic cells. The HMGB1 pathway triggers inflammation and functions as a late mediator of inflammation (Bonaldi et al., 2003). The expression of HMGB1 would promote cell recruitment and secretion of proinflammatory cytokines (Venereau et al., 2012). The activation of a HMGB1 signaling pathway by LPS in monocytic THP-1 cells might enhance transcription and mediate the response to infection, inflammation, and injury (Lotze & Tracey, 2005). In addition, hyperacetylation of HMGB1 by monocytes might redirect it towards secondary secretion (Bonaldi et al., 2003). The activation of this pathway might affect the response of THP-1 to inflammation and monocyte migration.

The LPS stimuli activated toll-like receptors, induced NF-κB and up-regulated inflammatory cytokines IL-1 and IL-6 (Guha & Mackman, 2001). The activation of IL-1 and IL-6 signaling pathway confirmed the up-regulation of IL-1 and IL-6 by LPS in THP-1 found using RNA-seq, and the result was also consistent with that of mRNA expressions of IL-1 and Il-6 in LPS induced THP-1 using RT-PCR.

PPAR and LXR/RXR signaling were the most inactivated canonical pathways in THP-1 with LPS stimulation. PPAR and LXR were nuclear receptors coordinating in lipid metabolism and inflammation (Hong & Tontonoz, 2008). PPAR-γ agonists were able to

inhibit the production of inflammatory cytokines by monocytes (Jiang et al., 1998). Moreover, LXR ligands could inhibit the expression of inflammatory cytokines such as IL-6 with the inhibition of LPS (Joseph et al., 2004). In brown adipocytes, treatment with IL-1β and LPS inhibited the mRNA expression of PPAR-γ (Mracek et al., 2004). The inhibitive effect on PPAR signaling pathway might be attributed to the LPS stimuli and up-regulation of IL-1β in THP-1 monocytes.

Together, the most activated and inactivated pathways in THP-1 with LPS stimulation confirmed the inflammatory response of THP-1 by LPS induction, and the alterations of these pathways might affect the regulation of neuroinflammation response, cell trafficking, and cytokine release by THP-1 cells.

4.5.5. Most altered canonical pathways in THP-1 by PEITC with/without LPS induction

Protein kinase A signaling, cAMP-mediated signaling and cardiac β-adrenergic signaling were the three pathways significantly inhibited in THP-1 by PEITC treatment without LPS induction (Table 4.6.5). In addition, with LPS stimulation, protein kinase A signaling and cAMP-mediated signaling were the most inhibited pathways. The protein kinase A is an enzyme that regulates growth, development, memory, and metabolism. The cellular effects of protein kinase A could be actuated by cAMP to transfer phosphates from ATP to protein substrates (Calipel et al., 2006; Trewhella, 2006). cAMP was one of the well-studied secondary messengers regulating gene expression, cell growth and differentiation, and apoptosis (Chin et al., 2002). In addition, cAMP-dependent kinase A signaling was demonstrated to have gross effects on carcinogenesis, such as the regulation of cell growth and proliferation (Merkle & Hoffmann, 2011). Our results indicated that protein kinase A and cAMP-mediated signaling are the primary pathways regulated by PEITC in THP-1

monocytes regardless of the stimulation of LPS. The down-regulation of protein kinase A/cAMP-mediated signaling pathways might affect the regulation of apoptosis, cell differentiation and proliferation of u-THP-1. The inhibitory effects of PEITC on THP-1 cell growth might be caused by the inhibition of protein kinase A and cAMP-mediated signaling pathways.

4.5.6. Genes altered in protein kinase A/cAMP signaling pathway in u-THP-1by PEITC with/without LPS induced inflammation

The genes from histone H1 family were the primary groups that were inhibited by PEITC in u-THP-1 regardless of LPS stimulation. In this study, all members of H1 family were down-regulated by PEITC. Scaffidi has reported that the main histone H1 family members, including HIST1H1A, HIST1H1B, HIST1H1C, and HIST1H1E, are generally up-regulated in cancers as compared to normal tissue. The up-regulation of histone H1 genes also resulted in increased cell proliferation (Scaffidi, 2016). In ovarian cancer cell line OVCAR-3, HIST1H1C and HIST1H1E were highly expressed (Medrzycki et al., 2014). The depletion of HIST1H1E resulted in inhibited cell survival of breast cancer T47D cells, although apoptosis was not detected. In addition, a loss of HIST1H1C slowed down breast cancer cell proliferation, and G1 arrest was observed (Millan-Arino et al., 2014). The down-regulation of histone H1 family members suggested a possible regulation of cell proliferation and survival by PEITC through the alterations of histone proteins.

Phosphodiesterases (PDE) was another group of genes with significant alterations by PEITC in THP-1. Overexpression of PDEs has been described in various cancer pathologies (Savai et al., 2009). PDEs inhibitors are popular targets for anti-cancer therapy, and the inhibition of PDE isoforms might induce apoptosis and cell cycle arrest and provide

anti-cancer effects (Savai et al., 2009). The data in this study suggested that PEITC might be a potential PDEs inhibitor, and the inhibition of activities of PDEs might also be correlated with the suppressive effects of PEITC on cell growth.

In addition, DUSP1, DUSP2, NTN1, P2RY13, PDE6G, and PTGDR were significantly up-regulated, and FPR1 was decreased in THP-1 by PEITC with LPS stimulation (Table 4.6.7b). As compared to THP-1 with only LPS stimulation, PEITC reversely regulated FPR1, NTN1, P2RY13, PDE6G, and PTGDR. Overexpression of FPR1 showed suppressive function and inhibition of angiogenesis in gastric cancer progression (Prevete et al., 2014). The netrin-1 (NTN1) is a neuronal guidance molecule correlated with the coordination of inflammatory responses and is hypoxia-inducible factor 1α (HIF-1α) dependent (Rosenberger et al., 2008). In this study, LPS significantly increased the expression of NTN1, while PEITC inhibited both HIF-1α (*P*-value: 0.002) and NTN1 in LPS induced THP-1, indicating possible regulation of inflammation and hypoxia status by PEITC. PDE6G is a protein-coding gene involved in the oncogenic signaling pathway (Muller, 2016). The decrease of PDE6G mRNA expression implicated a suppressive effect of PEITC on tumor or cancer cell growth through the alteration of oncogenic drivers. Prostaglandin D2 receptor (PTGDR) is a typical G protein-coupled receptor as well as a pro-inflammatory gene (Northoff et al., 2008). It was strongly up-regulated by LPS in THP-1 and inhibited by PEITC treatment.

In LPS induced u-THP-1, NTN-1, PP-1, and APC were three genes that were differentially altered by PEITC. Besides, the hypoxia-inducible factor 1α (HIF-1α) was significantly elevated (*P*-value: 0.002). The PEITC treatment was demonstrated to inhibit the HIF-1α as well as the HIF-1α dependent molecule NTN1 in LPS induced THP-1 cells. The PP1

protein complex group seemed not to be regulated by LPS. But DARPP32 (up-stream regulator of PP1) was down-regulated by -2.559-fold in THP-1 by PEITC without LPS and inhibited by -5.003-fold with the LPS stimulation. The greater inhibitive effect from PEITC on DARPP32 in THP-1 with LPS treatment as compared to that without LPS stimulation might cause an obvious alteration in the PP1 expression.

Together, the primary genes changed in protein kinase A/cAMP signaling pathway in THP-1 by PEITC suggested that genes from histone 1 family and PDEs were the primary regulators of protein kinase A/cAMP signaling pathway. The inhibition of this pathway might account for the regulation of cell growth by PEITC, and the changes of FPR1, NTN1, P2RY13, PDE6G, and PTGDR by PEITC upon LPS stimulation suggested a regulation of immune response by PEITC.

4.6. Conclusion

The regulation of LNCaP xenograft tumor growth by PEITC might not be related to androgen associated signaling pathways. More likely, correlation between suppressive effect on LNCaP xenograft tumor growth by PEITC and immune response might exist. For immune cells, monocytes but not macrophages were most affected by PEITC treatment. AC005488.2-6, AC092536.3-1, and AL021393.1-2 were discovered as most down-regulated novel genes in THP-1 monocytes with LPS stimulation. PEITC affected THP-1 cells mainly through PKA/cAMP signaling pathway. Genes involved in PKA/cAMP signaling pathway including FPR1, NTN1, P2RY13, PDE6G, and PTGDR were conversely regulated by LPS and PEITC. PEITC up-regulated growth inhibitory and apoptosis inductive gene TIAF1, and it down-regulated cancer biomarkers LRN3, PTH, RDS4, and genes from histone and PDE families. The regulation of these genes might be

associated with the observed suppressive effect of PEITC on THP-1 cell growth. LPS also triggered the activation of neuroinflammation signaling pathway and inhibited PPAR signaling pathway. Finally, the most inhibited genes AC092536.3-1 and AL021393.1-2 by LPS were reversely regulated by PEITC. Together, *in vivo* and next generation sequencing results suggested that tumor inhibitory effects of PEITC may be caused by the inhibition of monocyte growth and inflammation. Moreover, the inhibitory effect of PEITC on monocyte may be related to the regulation of PKA/cAMP-mediated pathway.

Appendix:

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