ROLE OF TALIN1 PHOSPHORYLATION IN BETA1 INTEGRIN ACTIVATION AND PROSTATE CANCER METASTASIS

Jung-Kang Jin

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ROLE OF TALIN1 PHOSPHORYLATION IN β1 INTEGRIN ACTIVATION AND PROSTATE CANCER METASTASIS

by

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ROLE OF TALIN1 PHOSPHORYLATION IN β1 INTEGRIN ACTIVATION AND PROSTATE CANCER METASTASIS

A DISSERTATION

Presented to the Faculty of
The University of Texas Health Science Center at Houston

and
The University of Texas MD Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment
of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

by

Jung-Kang Jin, M.S.

Houston, Texas

May 2014
Dedication

This dissertation is dedicated to my loving wife Huiling, my parents, my son Christopher and my daughter Jasmina.
Acknowledgements

I express my sincere appreciation to Dr. Gary E. Gallick, for his guidance, mentorship and support.

I wish to thank my committee members, Dr. Sue-Hwa Lin, Dr. David J. McConkey, Dr. Bradley W. McIntyre and Dr. Jeffrey N. Myers for their time, supervision, and contributions.

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I wish to thank Dr. Cai Huang at Department of Molecular and Biomedical Pharmacology, University of Kentucky, Lexington, Kentucky.
ROLE OF TALIN1 PHOSPHORYLATION IN β1 INTEGRIN
ACTIVATION AND PROSTATE CANCER METASTASIS

Jung-Kang Jin, M.S.
Supervisory Professor: Gary E. Gallick, Ph.D.

Talins are adaptor proteins that regulate focal adhesion signaling by conjugating integrins to the cytoskeleton. Talins directly bind and activate integrins but the mechanism by which this occurs is unknown. As integrin activation and overexpression of talins promote prostate cancer metastasis, understanding the mechanism by which talins activate integrins will better elucidate their roles in Prostate cancer metastasis. Phosphorylation of talins on serine 425 has been associated with β1 integrin functions. Work in this dissertation tested the hypothesis that increased talin1 S425 phosphorylation was required for β1 integrin activation and promotion of prostate cancer metastasis.

I first used shRNA to knockdown talins and demonstrated that talin1, but not talin2, is required for β1 integrin activation. Furthermore, talin1 S425 phosphorylation, but not total talin1 expression, correlated with metastatic potential of cultured prostate cancer cells. To directly test whether talin1 phosphorylation activates β1 integrins, I generated talin1 S425 mutants. Expressing a non-phosphorylatable mutant, talin1\textsuperscript{S425A} in talin1-silenced PC3-MM2 and C4-2B4 prostate cancer cells decreased β1 integrin activation, adhesion, motility, and increased the sensitivity of cells to anoikis. In contrast, re-expression of the phosphorylation-mimicking mutant, talin1\textsuperscript{S425D} led to increased β1 integrin activation and generated biologic effects opposite to talin1\textsuperscript{S425A} expression.
Mechanistically, I demonstrated that increased Cdk5 kinase activity correlates with expression of its activator p35, and is responsible for talin1 phosphorylation and β1 integrin activation.

Next, I demonstrated that talin1 phosphorylation is required for Prostate cancer bone metastasis in vivo. In the metastatic PC3-MM2 cells, expression of a non-phosphorylatable mutant, talin1\(^{S425A}\), in talin1-silenced PC3-MM2 cells, abolished their ability to colonize in bone following intracardiac injection, while re-expression of phosphorylation-mimicking mutant, talin1\(^{S425D}\) restored their ability to metastasize to bone. I further demonstrated by immunohistochemical staining that talin phosphorylation is significantly increased in human bone metastases when compared to normal tissues, primary tumors, or lymph node metastases, demonstrating the clinical relevance of talin S425 phosphorylation.

In summary, I have identified a novel signaling pathway whereby overexpression of p35 leads to increased Cdk5 activity, resulting in talin phosphorylation and β1 integrin activation. This signaling axis is responsible for increased metastatic potential of prostate cancer cells. Therefore, talin1, Cdk5 and p35 may be new targets for development of therapies for prostate cancer bone metastasis.
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### Abbreviations

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<tr>
<td>AR</td>
<td>Androgen receptor</td>
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<tr>
<td>CAM</td>
<td>Cell adhesion molecule</td>
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<td>Cdk5</td>
<td>Cyclin-dependent kinase 5</td>
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<tr>
<td>CGH</td>
<td>Comparative genomic hybridization</td>
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<tr>
<td>CRPC</td>
<td>Castration-resistant prostate cancer</td>
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<td>CTC</td>
<td>Circulating tumor cells</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
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<tr>
<td>HBME</td>
<td>Human bone marrow endothelial</td>
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<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
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<tr>
<td>ILK</td>
<td>Integrin-linked-kinase</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
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<tr>
<td>MET</td>
<td>Mesenchymal to epithelial transition</td>
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<tr>
<td>PAK</td>
<td>p21 activated kinase</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<tr>
<td>PIN</td>
<td>Prostate intraepithelial neoplasia</td>
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<tr>
<td>PIP2</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol-3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PIPK1γ</td>
<td>Phosphatidylinositol 4-phosphate 5-kinase γ</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate-specific antigen</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine-binding</td>
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<td>SNPs</td>
<td>Single-nucleotide polymorphisms</td>
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Chapter 1

Introduction
Prostate cancer is the second most common type of cancer in men in the United States. The American Cancer Society estimates that approximately 233,000 men will be diagnosed with prostate cancer with an estimated 29,500 deaths in 2014 [1]. Many risk factors are associated with prostate cancer, including increasing age, family history and race [1-4]. For example, the incidence of prostate cancer rates are especially higher (~60%) in African Americans than in non-Hispanic whites [1, 5]. Other factors such as high fat diet and obesity may also increase the risk of developing prostate cancer [6]. However, the molecular mechanisms by which these risk factors contribute to prostate cancer are still not clear, and thus effective strategies to prevent the occurrence of prostate cancer are also lacking.

Unfortunately, men with early prostate cancer usually have no symptoms. Only when the disease advances, patients may have symptoms such as interrupted urine flow, inability to urinate or pain with urination. Therefore, detection of prostate cancer before these symptoms develop is critical to prevent cancer progression. Currently, the method commonly used to detect early prostate cancer is the prostate-specific antigen (PSA) blood test. PSA is secreted by cells in the prostate glands and can be measured by ELISA from blood samples. An increased level of PSA concentration higher than 4 ng/ml indicates a higher chance of having prostate cancer [7, 8]. However, PSA levels can also be increased by other factors, such as medicines, and increasing age. On the contrary, a PSA level below 4 ng/ml does not absolutely indicate that men do not have prostate cancer [1, 8]. In addition, results of clinical trials of using the PSA test to screen men, thereby reducing prostate cancer are inconsistent. While two European trials of PSA
screening have been reported to reduce mortality about 20~30% [9, 10], one US study has found no reduction in mortality [8, 11]. Based on these results, there are insufficient data to recommend for or against PSA screening for detecting early prostate cancer. Although the use of PSA test for screening early prostate cancer is still controversial [8], the American Cancer Society recommends that men at age 50 should discuss with their health care provider the benefits and limitations of PSA testing [1]. Men at high risk of developing prostate cancer, for example African Americans, should consult their health care provider beginning at age 45 or even earlier [1].

Patients with early stage prostate cancer usually have only local tumors and can be cured by surgery, external beam radiation or radioactive seed implants. The five-year survival rate for localized prostate cancer is close to 100% [1]. However, when metastasis has developed, the five-year survival rate decreases to 28% [1]. Prostate cancer can metastasize to multiple distant organs such as the lungs, liver and brain, but it has a high propensity for metastasizing to the bone. Bubendorf et. al. reported that 80% of the men who had died from prostate cancer had bone metastases [12]. Most current treatments for patients with bone metastases have little effect on improving long-term survival [13, 14]. Therefore, gaining a better understanding of cancer progression to bone metastasis and the mechanisms by which prostate cancer cells metastasize to the bone are critical to develop reagents and therapies for prostate cancer metastasis.
**Genetic alterations in prostate cancer**

Prostate cancer metastasis occurs as results of accumulation of genetic and epigenetic alterations in tumor cells. In regard to genetic alterations in prostate cancer, extensive genomic analyses have identified many copy number alterations and chromosomal rearrangements. Several somatic alterations including, gain of chromosome 8q and losses at chromosomes 3p, 8p, 10q, 13q, and 17p have been identified by comparative genomic hybridization (CGH) [15, 16]. Many key regulatory genes are located within these region, including \textit{NKX3.1} at 8p21, \textit{MYC} at 8q24 and \textit{PTEN} at 10q23, and their roles in prostate cancer will be discussed below.

\textit{NKX3.1 down-regulation}

Recent analyses have demonstrated that expression of \textit{NKX3.1}, a tumor suppressor gene, is downregulated in nearly all prostate cancers and metastases [17]. \textit{NKX3.1} has been shown to be a critical regulator of prostate epithelial differentiation. Deletion of a single \textit{NKX3.1} allele (\textit{NKX3.1}+) or complete knockout (\textit{NKX3.1}++) in prostate epithelial results in hyperplasia and dysplasia in mice at 1 year of age [18]. In older mice with \textit{NKX3.1} knockout (between 1 and 2 years of age), the prostate glands display more severe histopathological alterations resembling human prostate intraepithelial neoplasia (PIN) [19, 20]. Nkx3.1 null mutant mice recapitulate early stages of prostate carcinogenesis [20]. Therefore, these findings are consistent with the tumor suppressor activity of \textit{NKX3.1}, suggesting that \textit{NKX3.1} represents as a gatekeeper gene for prostate cancer initiation [17, 21].
**MYC up-regulation**

The 8q24 chromosomal region containing the *MYC* oncogene is often amplified in advanced prostate tumors suggesting that *MYC* plays a role in late stage prostate cancer [22]. However, studies of prostate cancer have also showed increased *MYC* mRNA in low grade (e.g. Gleason score 6) tissues. Overexpression of MYC has been observed in luminal cells of PIN in the absent of gene amplification [23]. Therefore, these studies suggest a role for *MYC* overexpression in cancer initiation. Furthermore, the effect of MYC overexpression has also been examined using *MYC* transgenic mice. Overexpression of MYC in the mouse prostate induces formation of PIN followed by progression to invasive prostate adenocarcinoma [24]. Together, these results indicate that up-regulation of MYC is a critical oncogenic event associated with prostate cancer progression.

**Loss of PTEN**

*PTEN* was identified as a tumor suppressor gene, and is frequently mutated or deleted in many cancers, including prostate cancer [25-27]. The chromosomal region on 10q23 where *PTEN* is located frequently undergoes allelic loss in prostate cancer, which results in loss of *PTEN* expression in prostate tumors [28]. Recent studies have found that loss of PTEN expression is primarily due to bi-allelic deletion in local prostate cancer [29]. *PTEN* loss has been observed in 20-30% of locally progressive prostate cancers specimens [29, 30]. To investigate the role of *PTEN* loss on prostate cancer development, genetically engineered mouse models have been generated. Prostate-specific deletion of *PTEN* in these mice causes PIN, followed by progression to invasive adenocarcinoma.
[31]. Loss of *PTEN* in heterozygous mutants or conditional *PTEN* deletion in the prostate epithelium also recapitulates many of the events associated with prostate cancer in humans [32]. Knockout of *PTEN* in prostate of mice also promotes progression to castration-resistance [33, 34]. These data indicate that *PTEN* loss in mice is sufficient to initiate prostate cancer and demonstrate the importance of *PTEN* during prostate cancer progression.

**Progression of prostate cancer to castrate-resistance**

During the progression of prostate cancer, tumors usually become resistant to treatments that reduce male hormones, i.e. androgens. The development and progression of prostate cancer are dependent on the androgens which mediate transcription through androgen receptor (AR). In prostate tumor cells, binding of androgen to AR forms a complex that translocates to the nucleus. This complex binds to AR-responsive elements and controls transcription of androgen-regulated genes [35]. Activation of androgen-regulated genes further stimulates cell proliferation and inhibits apoptosis of tumor cells [35, 36]. This dependence of early stage prostate cancer on androgen stimulation serves as the basis for androgen-deprivation therapy [37]. Presently, androgen-deprivation therapy has been widely used by chemical astation to decrease tumor growth [38]. However, patients who have received androgen-deprivation therapy will ultimately progress after an average of 12 to 18 months, as their tumor adapts to the androgen-deprived environment and develops “castrate-resistant” disease [39].
The mechanism of developing castrate-resistant prostate cancer is not fully understood. In castrate-resistant prostate cancer (CRPC) cells, the AR can be activated, and androgen-responsive genes such as PSA [40] continue to be expressed. Several mechanisms have been proposed for the development of CRPC. First, prostate cancer cells can upregulate AR expression and become hypersensitive to androgen stimulation [41], which may occur through selective outgrowth of castration-resistant cells during castration [42]. Second, the AR may be activated by other steroids such as estrogens or be activated in a ligand-independent pathway by receptor tyrosine kinases and growth factors [43-45]. For example, overexpression of HER-2/neu has been shown to activate AR in CRPC cells [46]. Insulin-like growth factor (IGF) also can bind and activate AR during castration [42]. Furthermore, Src kinase is critical for bombesin-induced AR-mediated activity and is required for translocation and transactivation of AR [45]. In another mechanism, the production of androgen is upregulated through intratumoral synthesis by intracrine and paracrine pathways, which also contributes to castrate-resistant in CRPC cells [43]. Clearly, multiple mechanisms have been shown to contribute androgen-independence in CRPA. Currently, new AR antagonists such as MDV3100 [47] and abiraterone [48] have shown promise results in clinical trials. However, most patients treated with these AR antagonists ultimately develop resistance to these agents. More studies for the treatment of CRPC either using AR antagonists alone or in combination with other agents are urgently warranted.
Prostate cancer metastasis and microenvironment

Men with CRPC usually develop metastasis in distant organs and progress to metastatic CRPC. The process of cancer metastasis is very inefficient but highly selective and dependent on the interaction between tumor cells and the target organs. The classic model of metastasis of solid tumors, the “seed and soil hypothesis”, was first proposed by Stephen Paget in 1889 [49]. In Paget’s model, the tumor cells (i.e. seeds) only metastasize to specific organs (i.e. soil) that are fertile for the tumor growth. This concept, while remaining a guiding principle for metastasis does not entirely explain the molecular bases for organ specific metastases. With the accumulation of knowledge from cancer metastasis studies, metastases of solid tumors including prostate cancer is now known to involve multiple steps. Metastatic cancer cells undergo a series of processes including angiogenesis, local migration, invasion, intravasation, circulation, extravasation and colonization in the distant organs. Thus, cancer cells must alter signaling to gain cell properties that are critical to ensure successful metastasis.

Moreover, metastasis is not solely dependent on alteration of cancer cell properties, but also requires bidirectional communication between cells and their surrounding environment. The tumor microenvironment is comprised of stromal cells including fibroblasts, endothelial cells, and immune cells, which are recruited to the tumor. A variety of growth factors, cytokines, chemokines and extracellular matrix (ECM) proteins secreted by stromal cells can promote growth of tumor cells and increase their metastatic potential [50, 51]. In contrast, tumor cells also interact with stromal cells to change their properties in favor of tumor growth. Therefore, interaction between tumor
cells and the stromal compartment is critical to promote cancer progression and metastasis [52]. Most of these intercellular interactions are dependent on cell adhesion molecules (CAMs) and receptors on the cell surface including cadherin and integrins. Various mechanisms of alteration in these molecules have been shown to promote metastasis, including reducing cell-matrix adhesion, increasing migratory ability, surviving in circulation, and colonization in the distant organs [53], which will be discussed below.

**Epithelial plasticity**

In the normal prostate gland, basal cells in the lumen attach to the basement membrane, forming a cell layer underneath normal epithelial cells. Adhesions of epithelial cells to each other and to ECM restrict the migratory capability of epithelial cells [54]. In the initial stage of metastasis, tumor cells have to detach from substratum and migrate from the primary tumor into the surrounding blood or lymphatic vessels. The adhesions of cell-cell and cell-matrix are mediated by forming of intercellular junctions that are composed of protein complexes with CAMs. Therefore, tumor cells must decrease the adhesiveness by altering the expression of different CAMs. For example, expression of most integrins and E-cadherin are decreased in primary prostate tumors [55, 56]. This process is a major feature of epithelial to mesenchymal transition (EMT), which allows cells to acquire a more mesenchymal phenotype, increasing their abilities to migrate and invade the ECM [57]. However, in the distant sites, cancer cells reverse this process for mesenchymal to epithelial transition (MET) to thereby regaining an epithelial phenotype allowing adherence to ECM and growth at the metastatic site. This ability of
cancer cells to switch between different phenotypes through EMT and MET is a feature of “epithelial plasticity” [58, 59].

**Survival in circulation**

The metastatic process is not efficient because most disseminated cancer cells are rapidly destroyed in the circulation. Studies have demonstrated that cancer cells are frequently released from the tumor into the circulation [60]; but only less than 0.1% of these disseminated cancer cells can survive and metastasize [61]. Thus, survival of prostate cancer cells in the circulation is one of the rate-limiting steps in cancer metastasis [62]. Normally, epithelial cells require attachment to the ECM through integrin signaling for survival and growth. Disruption of cell-ECM interaction results in loss of attachment, which impairs integrin-mediated survival signaling and induces apoptosis, a process termed anoikis [63]. Thus, anoikis prevents disseminated normal epithelial cells from colonizing at inappropriate locations. For prostate cancer cells to metastasize in the bone, dislodged cells have to survive in the circulation before colonizing the bone [64]. Therefore, metastatic tumor cells must acquire the ability to survive in the circulation. Constitutive integrin signaling is one of the mechanisms by which prostate cancer cells develop anoikis resistance. In metastatic prostate cancer cells, integrin signaling is often increased leading to activation of downstream FAK/Src and Akt pathways, promoting cell survival in circulation [65, 66].
Development of bone metastasis

Currently, studies of prostate cancer bone metastasis have focused on the bidirectional interactions of bone microenvironment and prostate cancer cells. In the bone microenvironment, surviving prostate cancer cells have to attach to capillary endothelial cells or sub-endothelial basement membranes and then extravasate and grow in the bone to complete the process of metastasis. These later steps in prostate cancer metastasis are the most poorly understood, but are specific to the bone compartment. For example, prostate cancer cells have a higher affinity to bind human bone marrow endothelial (HBME) cells than they bind to other endothelial cells [67]. Thus, bone metastasis likely requires interaction of prostate cancer cells with bone microenvironment for metastasis to develop.

The bone matrix is composed of 95% of type I collagen and 5% of remaining non-collagen proteins and proteoglycans [68]. Type I collagen, as well as non-collagen matrix proteins such as osteopontin, bone sialoprotein and osteonectin, serve as ECM molecules that abet attachment of prostate cancer cells in the bone [69, 70]. Many of these bone matrix proteins are ligands of integrins, and have been implicated in facilitating colonization of prostate cancer cells in the bone [71]. The cells in the bone marrow include osteoblasts (for bone formation), osteoclasts (for bone resorption), hematopoietic cells, adipocytes and immune cells. Osteoblasts are derived from mesenchymal stem cells in the bone marrow stroma and differentiate to osteocytes when embedded in the bone [72]. The newly formed bone matrix will be hardened by mineralization with deposition of hydroxyapatite crystals to increase resistance to
Differentiated osteoblasts secrete many growth factors, and some of those are embedded in the bone matrix. During bone resorption, osteoclasts secrete acids and proteases to resorb bone and also release these growth factors from bone matrix, which stimulate prostate cancer cells growth in bone [52, 73, 74]. In contrast, secreted factors from prostate cancer cells also stimulate proliferation and maturation of osteoblasts and osteoclasts that, in turn, produce or release even more growth factors and further stimulate metastatic growth of tumor in the bone. This interaction between prostate cancer cells and osteoblasts/osteoclasts and other cells in the bone microenvironment is commonly termed the “vicious cycle” in which tumor growth affects bone remodeling and bone remodeling contributes to tumor growth [52, 74, 75]. Therefore, the bidirectional interactions between bone cells and prostate cancer cells highlight the importance of the bone microenvironment in promoting bone metastasis. Despite the complexity of the bone microenvironment and the experimental difficulty in understanding mechanisms by which prostate cancer cells home to bone, many adhesion molecules have been shown to promote prostate cancer bone metastasis. Among them, cadherin-11 is involved in the metastasis of prostate cancer cells to bone [76, 77]. Treatment of anti-cadherin-11 antibody mAb 2C7 effectively prevented metastasis of PC3-MM2 cells to bone in vivo [78]. Integrin α2β1 has been shown to increase migration and adhesion of cancer cells on collagen, and to promote prostate tumor growth in bone [79, 80].
Therapy resistance

Resistance to therapies is a recurrent problem in prostate cancer bone metastasis. However, the molecular understanding of the mechanisms of resistance is still limited. When disseminated tumor cells colonize distant organs, a small population of tumor cells may enter a quiescent phase and become dormant for years [62, 81, 82]. Unfortunately, most therapies target actively proliferating cells, and thus these quiescent tumor cells are likely to be resistant to the therapies and persist [83]. Eventually, these cells will escape from dormancy and grow leading to metastasis. Although the mechanism of breaking dormancy is unknown, the interaction of microenvironment and tumor cells may be responsible for dormancy and recurrence. Studies have shown that β1 integrin activation is a key regulator to switch dormancy to proliferation and tumor recurrence when supplementation of ECM proteins activates integrin signaling [84]. In breast cancer cells resistant to multiple antitumor drugs, β1 integrin expression was decreased, which correlates drug resistance [85]. These findings suggest that reduced expression of β1 integrins may be important in cancer cell dormancy and resistance to drug treatment. However, in proliferating cells, β1 integrin signaling is increased, leading to activation of the PI3K/Akt survival pathway. This signaling leads to resistance to cytotoxic chemotherapies in breast cancer cells [86]. In addition, β1 integrin expression was also increased upon exposure to ionizing radiation [87]. Therefore, these studies suggest that β1 integrins play an important role in therapy resistance through multiple mechanisms.
Integrins: structure, activation and signaling

While numerous alterations in tumor cells are associated with integrins, aberrant integrin expression or activation has been shown to be involved in nearly every step of cancer metastasis. Thus, I focus on studying the role of integrins in prostate cancer metastasis in this dissertation.

Structure of integrins

Integrins belong to a family of adhesion molecules consisting of 18 \( \alpha \) and 8 \( \beta \) subunits that comprise 24 glycoprotein heterodimers [88, 89]. Integrins bind specific ligands in the ECM and promote diverse processes such as adhesion, migration, survival and proliferation [88, 89]. Although the precise structure for each integrin heterodimer has not been resolved, a study of the X-ray crystal structure of integrin \( \alpha v \beta 3 \) provides a model for integrin structure (Figure 1) [90]. Based on this model, the \( \alpha \) and \( \beta \) subunits of integrins form an extracellular “headpiece” that is connected by a short transmembrane domain to a cytoplasmic tail. The headpiece of the integrin heterodimer is composed of a \( \beta A \)-domain in the \( \beta \) subunit and a \( \beta \)-propeller domain in the \( \alpha \) subunit [91]. The \( \beta A \)-domain is linked to the hybrid domain that acts as a lever, and the angle between the two domains controls ligand binding affinity. Therefore, the affinity of integrins to ligands is tightly regulated by the conformational changes of individual integrin heterodimers [92]. Integrins exist in three principal conformations with different affinities for ligands: an inactivated, “bent” conformation (low affinity), a partially activated, “extended” conformation with a closed headpiece (high affinity), and a “fully” activated conformation with an open headpiece (ligand occupied) [90].
Figure 1. **Integrin domain structures.** Integrins are heterodimers consisting of an α and a β subunit. The inactive integrins are in a bent conformation (see the figure; left), and can be unfolded once activated (see the figure; right). The N-terminal domains of α and β subunit interact and form a headpiece that provides a ligand binding site. The C-terminal domains of each subunit form two legs with transmembrane domains and cytoplasmic tails.

Activation of integrins

Integrins can be activated by binding ligands or interacting with intracellular proteins. Activation of integrins is regulated by bidirectional cellular signaling illustrated in Figure 2 [91]. Traditionally, binding of ECM ligands induces a conformational change leading to activation of integrins. Such activation is generally termed “outside-in” activation, which results in conformational changes leading to opening the hinge between the βA- and hybrid domains in the integrin headpiece as described above [93, 94]. In addition to ligands, other factors can also affect outside-in integrin activation. For example, Mg\(^{2+}\) and Mn\(^{2+}\) can activate and stabilize a high affinity conformation of integrins, while Ca\(^{2+}\) has an inhibitory effect and stabilizes a low affinity conformation [95]. Although inactive integrins can be activated by ligand binding, the affinity of inactive integrins to ligands is much lower than that of activated integrins. Hence, another mechanism to activate integrins and increase binding affinity is through binding intracellular proteins.

Cellular signaling can promote intracellular proteins, such as talins, to bind integrins, which induces conformational changes in integrins leading to their activation. This direction of integrin activation, through intracellular signaling, is usually termed “inside-out” activation. Generally, the two directions of integrin activation stimulate signaling pathways that have different biological consequences in cells. Inside-out signaling is responsible for cell adhesion and controls interactions with ECM proteins, and therefore regulates cell migration and ECM assembly. Outside-in signaling through ECM ligand binding leads to transition of intracellular signaling, which controls cell polarity, survival, proliferation, cytoskeletal structure and gene expression [91].
Figure 2. “Outside-in” and “inside-out” integrin signaling. There are two directions of integrin signaling. In “outside-in” signaling, integrins work as traditional receptors in binding ligands and transmitting signals into the cells (left figure), while in “inside-out” signaling, intracellular proteins, such as talin, bind to the integrin tail and induce a conformational change leading to activation of integrins (right figure). Molecules involved in these procedures are shown in the figure.

However, the two mechanisms of integrin activation are not mutually exclusive, nor independently regulated. For example, inside-out activation of integrin can increase integrin affinity for ligands. This process in turn promotes ligand binding of integrins resulting in outside-in activation. Following activation, integrins bind variety of ligands including collagens, laminins, or RGD-containing proteins (such as fibronectin) [96]. Certain integrin $\alpha$ subunits only pair with specific $\beta$ subunits to form heterodimers and thus create specificity as to which ligands bind which integrins (Figure 3). For the 24 different heterodimers, 12 of them contain $\beta_1$ subunits and only these pairing with $\alpha_1$, $\alpha_2$, $\alpha_{10}$ and $\alpha_{11}$ can bind to collagens, the major component of bone (Figure 3). Thus, $\beta_1$ integrins are likely to play an important role in prostate cancer bone metastasis.

**Integrin signaling**

Integrins have no intrinsic enzymatic activity; thus, their transmission of signaling relies on integrin-associated effectors. Activation of integrins leads to recruitment of focal adhesion kinase (FAK) to their cytoplasmic tails. When integrins cluster, the integrin tails will be brought together, which leads to FAK interaction and auto-phosphorylation of FAK on Y397. Subsequently, Src family kinase and PI3K bind to pFAK Y397 and together induce phosphorylation of other focal adhesion components including paxillin and p130 CAS to initiate signaling [97]. Activation of FAK/Src through phosphorylation is known to activate additional signal pathways including mitogen-activated protein kinases (MAPKs) and PI3K/Akt [98]. Integrin activation induces phosphorylation of p21 activated kinase (PAK) that activates MAPK pathway
Figure 3. Integrin subunits and their ligands. Ligands for leukocyte integrins include E-cadherin, fibrinogen, factor X, intercellular adhesion molecule, inactive complement factor 3b, vascular cell adhesion molecule, and von Willebrand Factor. RGD-containing ligands include bone sialoprotein, fibronectin, latency-associated-peptide of transforming growth factor β1/3, nephronectin, osteopontin, thrombospondin, and vitronectin.

through activating Raf. Another major result of integrin activation is stimulation of the PI3K/Akt signaling pathway. PI3K catalyzes PIP2 to PIP3 that recruits and activates other proteins, including the Akt kinase. While PTEN is frequently lost in prostate cancer leading to activation of the PI3K pathway, integrin-mediated activation of PI3K/Akt has also been shown to promote cancer cell migration and survival [99]. Studies showed that osteopontin, an ECM protein in bone, increases cell migration and adhesion through PI3K/Akt pathway in PC3 cells [100]. β1 integrins also increase the expression of survivin through the Akt pathway, which protects prostate cancer cells from apoptosis induced by the TNF-α [101]. In addition, integrins α5β1 and αvβ3 (but not αvβ1), protect cells from apoptosis by increasing Bcl-2 expression via FAK and PI3K pathways [102, 103].

**β1 integrins in prostate cancer**

In normal prostate glands, the basal cells express integrins that bind beneath the basement membrane. However, during the progression of prostate cancer, these basal cells are usually lost, and integrin expression is altered. Most α and β subunit expression is downregulated, whereas only α2, αIIb, α6, β1, β3 and β6 are upregulated [55]. Among these upregulated integrins, only α2, α6, β3 and β6 subunits have been shown to be overexpressed in metastases [55], but their roles in metastasis are unknown. In prostate cancer cells, β1 integrins contain five different alternatively spliced variants, β1A, β1B, β1C, β1C-2, and β1D [104]. Among the five known β1 integrin cytoplasmic variants, β1A integrins are ubiquitously expressed, whereas β1C levels are reduced in neoplastic prostate epithelium [105]. In addition, prostate cancer cell lines, including PC3, LNCaP
and DU145, mainly express β1A integrins that increase cell proliferation, whereas the β1C variant (not expressed in prostate cancer cells) acted as an inhibitor of proliferation [106].

Expression of β1 integrins has been shown to increase migration and invasion. Several β1 integrins are associated with increased metastasis in prostate cancer, including α5β1 and α2β1 [79, 107, 108]. Trerotola et. al. showed that α5β1 integrins increase migration of prostate cancer cells by associating with talin and relocalizing from focal adhesions to the leading edges of cells [108]. In addition to migration, β1 integrins also form a complex with IGF-IR to promote proliferation of prostate cancer cells in 3D cultures [109]. Studies demonstrated that α5β1 integrins on prostate cancer cells interact with bone stroma, suggesting that they may play a critical role in tumor growth in the bone. Blocking α5β1 integrins by monoclonal antibodies inhibits adhesion of DU-145 prostate cancer cells to bone marrow stromal cells [107, 110]. Another β1 integrin family protein, α2β1, has been shown to increase migration and adhesion of cancer cells on collagen, and promotes prostate tumor growth in the bone [79, 80]. Similar to α5β1, α2β1 was found to mediate adhesion of malignant tumor-derived prostate epithelial cells to human bone marrow stroma, which can also be inhibited by antibodies targeting α2β1 [111].

Little is known about the role of β1 integrin activation in promoting prostate cancer metastasis. Very recently, Dr. Sue-Hwa Lin and collaborators have demonstrated that β1 integrins are constitutively activated in prostate cancer cell lines with high
metastatic potential, but not in cancer cell lines with low metastatic potential [112]. Dr. Lin and collaborators also showed that blocking β1 integrins using a specific anti-β1 integrin antibody (33B6) inhibits adhesion, migration, survival and integrin signaling in prostate cancer cells [112]. Furthermore, treatment of mAb 33B6 by intraprostatic injection suppressed metastasis of PC3-MM2 from the prostate to distant lymph nodes in a spontaneous metastasis mouse model. In an experimental metastasis mouse model following intracardiac injection, blocking β1 integrins by systemic delivery of mAb 33B6 suppressed metastasis of PC3-MM2 cells to bone (Figure 4) [112]. Thus, these studies demonstrated the importance of β1 integrin activation in prostate cancer bone metastasis, and that blocking β1 integrin activation inhibits bone metastasis. Therefore, I focused on studying the role of β1 integrin activation in prostate cancer bone metastasis in my dissertation. Furthermore, I addressed how β1 integrin activation promotes prostate cancer bone metastasis and hypothesized that talin may have a unique role in β1 integrin activation that promotes metastasis of prostate cancer cells.

**Talin in inside-out integrin activation**

Talin is essential to activate integrins by directly binding to the cytoplasmic tail of the β subunit. Although several integrin-associated proteins have been shown to facilitate integrin activation including kindlin and ILK, only talin can directly bind integrins and is sufficient to activate integrins [113]. Overexpression of talin has been observed in several solid tumors, including prostate cancer [114], oral squamous cell carcinoma [115] and ovarian serous carcinoma [116], and generally overexpression correlates with higher
Figure 4. Inhibition of PC3-MM2 cells metastasis in vivo by anti-β1 integrin antibody mAb 33B6. (A) Inhibition of PC3-MM2 metastasis to lymph node in vivo by mAb 33B6. Luciferase-labeled PC3-MM2 cells were injected into the prostate of SCID mice. Mice were treated with IgG or mAb 33B6 (1 mg/kg) before tumor cell inoculation and twice a week subsequently by intraperitoneal injection. Bioluminescence imaging of mice was conducted weekly afterwards to monitor tumor growth and metastasis in vivo. Average numbers of lymph node ± SD metastases detected at the termination of the study. (B) Inhibition of disseminated PC3-MM2 cell metastasis in bone by mAb 33B6. Tumor burden in the femurs of the control and mAb 33B6-treated mice. Luciferase-labeled PC3-MM2 cells were injected into the left ventricle of SCID mice. Mice were treated with IgG or mAb 33B6 (1 mg/kg) before tumor cell inoculation and twice a week subsequently by intraperitoneal injection. Bioluminescence imaging was acquired as above setting. P < 0.05.

tumor grade and metastasis [114-116]. However, little is known about the role of talin in cancer. Studies have shown that knockdown of talin reduces cell migration in ovarian serous carcinoma cells [116]. Overexpression of a dominant-negative form of talin, talin L325R, significantly reduces cell proliferation and increases apoptosis in oral squamous cells [115]. In prostate cancer, talin knockdown decreases cell adhesion, migration and invasion, while overexpression of talin increases anoikis resistance through focal adhesion signaling [114]. However, the role of talin in cancer metastasis and the mechanisms by which talin promotes cancer cell properties were unknown, prior to my work. I hypothesize talin’s role in tumorigenesis occurs through activating β1 integrins, which is a major topic of this dissertation.

Structure of talin

Two distinct talin genes (TLN1 and TLN2, 74% sequence identity) are expressed in vertebrates, with talin1 expressed in nearly every tissue and talin2 normally expressed primarily in the heart, brain, testis and muscles [117-119]. Talins are 270-kDa adaptor proteins that are localized in adhesion complexes between cells and the ECM, where they interact with the β integrin cytoplasmic tail and the actin cytoskeleton [120]. Structurally, talin (including talin1 and talin2) consists of an N-terminal 47-kDa head domain and a 220-kDa C-terminal flexible rod domain (Figure 5) [113, 121]. The talin head consists of an atypical FERM domain including three subdomains (F1, F2, and F3) and a F0 subdomain [122]. The talin head contains binding sites for β integrin tails [123, 124], F-actin [125], phosphatidylinositol 4-phosphate 5-kinase γ (PIPK1γ) [126] and acidic phospholipids such as phosphatidylinositol-4,5-bisphosphate (PIP2) [127]. The talin head
**Figure 5. Domain structure of talin.** Talin is composed of three major domains: FERM (head), linker and rod domain. The FERM domain includes F1, F2 and F3 subdomains. The rod domain includes multiple actin and vinculin binding sites. The Ser425 phosphorylation site in the linker domain is indicated by a red star.

and rod are linked via residues 401-481 amino acid, which contains a calpain cleavage site at 432 amino acid [128]. The talin rod domain is composed of a series of helical bundles that contain multiple actin-binding sites, vinculin binding sites and a second integrin-binding site [129]. Therefore, talin functions as an adaptor protein, as it conjugates integrins to the cytoskeleton [129], and further promotes integrin-mediated signal transduction.

**Talin-mediated inside-out integrin activation**

Major insights into the principal function of talin were derived from experiments demonstrating that the talin head binds the cytoplasmic tails of β integrin subunits leading to the final step in inside-out integrin activation [124, 130]. The major integrin-binding site at the talin head is located within the F3 subdomain containing a phosphotyrosine-binding (PTB) domain, which interacts with the membrane-proximal NPxY motif in the cytoplasmic tails of the β integrins [131, 132]. Crystallographic data revealed that talin-mediated integrin activation is dependent on binding of the talin F3 subdomain to the β integrin tail in two steps. First, the talin head interacts with the β integrin tail by binding the NPxY motif through its PTB domain (Figure 6). Second, the loop region on the talin F3 subdomain subsequently interacts with the membrane-proximal region within the β integrin tail to displace the integrin tail. The displacement of the integrin tail results in separating the two legs of integrin α/β subunits thereby inducing a conformational change from an inactive bent structure to an extended conformation [122] (Figure 6). Integrins in this ‘primed’, extended form, are activated with their extracellular domains extended and the ligand-binding site exposed, which increases the binding affinity to specific ECM
Figure 6. Talin-mediated integrin activation. (A) Inactive talin can be activated by binding of PIP2, calpain cleavage and possibly through phosphorylation (described in this dissertation) to release the autoinhibitory conformation. (B) During integrin activation, the F3 subdomain on the talin head binds the membrane proximal NPXY motif of β integrin tail. (C) The talin loop structure in the head interacts with the membrane-proximal α helix of the β integrin cytoplasmic tail and separates the connection between integrin α/β tails.

proteins. Ligand binding induces full activation and clustering of integrins. The talin bound with integrin tails then recruits vinculin, FAK, actin and other focal adhesion proteins to form a focal adhesion complex that conjugates integrins with the cytoskeleton (Figure 7).
Figure 7. Talin-mediated integrin activation and subsequent focal adhesion formation. (A) In the inactive state, integrins are in a bent conformation. (B) Binding of talin in the cytoplasmic tail of integrins promotes to a partially activated extended conformation with increased affinity to ECM. (C) Ligand-bound integrins are in a fully activated state. In the cytoplasm, several focal adhesion proteins such as FAK, Src, vinculin and paxillin are recruited to talin for assembly of focal adhesion complexes.

Because integrin activation has to be strictly regulated in normal cells, binding of talin to integrin is tightly controlled. In the cytoplasm, talin forms an antiparallel dimer via a single helix in the C-terminal rod [133]. In this autoinhibitory conformation, the C-terminal talin rod binds the N-terminal PTB domain, which blocks the integrin binding pocket [134]. Therefore, talin itself must be activated prior to binding integrins. How talin is activated is not clear, but the mechanism must involve disruption of autoinhibitory conformation. Several mechanisms have been proposed to relieve this inhibitory conformation, including binding PIP2 [135, 136], calpain cleavage [137] and talin phosphorylation [138]. PIP2 is known to activate talin by inducing conformational changes [135]. Studies of the talin crystal structure revealed that PIP2 on the plasma membrane attracts the positively charged talin head and simultaneously repels the negatively charged talin rod [136]. Such a “pull-push” process relieves the autoinhibitory conformation. In the calpain cleavage model, talin is cleaved by calpain at amino acid 432, yielding an N-terminal 50-kDa globular head domain and a 220-kDa C-terminal rod domain [137]. Thus, the N-terminal head cleaved product can bind to integrin with the FERM domain and is sufficient to activate integrins.

Talin phosphorylation

In addition to the above described mechanisms, phosphorylation of talin has been proposed to induce an active conformation. Hence, in the phosphorylated residues in talin have been mapped using mass spectrometry. Talin is heavily phosphorylated on 30 sites including, serine, threonine and tyrosine residues [139]. Most of the sites are predicted to be phosphorylated by PKA, PKC and cyclin-dependent kinase 5 (Cdk5). Although the
functions of these phosphorylated sites are unknown, talin phosphorylation has been implicated in regulation of membrane-cytoskeleton interactions [140-142]. One of the most heavily phosphorylated sites is on talin1 serine 425, which resides in the talin linker domain [143]. Phosphorylation of talin on S425 was shown to inhibit binding to Smurf1 and prevents talin head ubiquitylation and degradation [143]. Mutation of talin S425 to inhibit phosphorylation results in decreasing cell migration in neural cells [143]. Because talin S425 phosphorylation regulates cell properties associated with integrin functions, talin phosphorylation may plausibly regulate integrin activation. Structurally, phosphorylation of proteins can induce a conformational change [144]; therefore, talin phosphorylation could be one of the possible mechanisms to relieve the inhibitory conformation and lead to talin activation, thus promoting integrin binding and integrin activation. However, the potential role of talin phosphorylation in integrin binding and integrin-mediated signalings has not been assessed previously.

Cdk5 in talin phosphorylation

Studies of Huang et. al. have shown that talin is phosphorylated on S425 by Cdk5 in normal cells [143]. Cdk5 belongs to a member of the cyclin-dependent kinase family of proline-directed protein kinases. Cdk5 was originally isolated from screening of Cdk1 and is 58% and 62% sequence identical with Cdk1 and Cdk2, respectively [145]. Although Cdk5 was cloned as a homologue to other Cdk family proteins, Cdk5 is mostly expressed in terminally differentiated neurons of developing nervous system [146], and regulates the migration of post-mitotic neurons during embryogenesis [147], thus is not involved in cell cycle regulation. However, the mechanisms by which Cdk5 regulates
neuronal migration in the embryonic and adult brains are still unknown. One possible mechanism is through altering microtubule dynamics by phosphorylating proteins that are critical for nucleokinesis, such as Nudel1, FAK and PAK [148-151]. Cdk5 is activated by two non-cyclin regulatory subunits, p35 and p39 [152, 153]. While p39 is specifically expressed in the cerebellum, p35 is expressed in various tissues, including prostate [154-156]. p35 does not show homology with other known cyclins and does not activate other members of Cdk family [152]. Thus, p35 has high specificity for Cdk5 activation [152].

In addition to their roles in neurons, several studies have shown that Cdk5 is activated in non-neuronal cells, including pancreatic cells [157] and corneal epithelial cells [158]. In prostate cancer, Cdk5 and its activator p35 are highly expressed in primary tumors and metastases compared with normal tissues [155, 156]. Cdk5 was shown activated in prostate cancer cells, Strock et. al. demonstrated that overexpression of dominant-negative Cdk5 in prostate cancer cells inhibits lung metastasis in xenograft mice [156]. These results suggest that Cdk5 activity promotes prostate cancer metastasis. Intriguingly, Cdk5 has also been shown to increase migration and cell-matrix adhesion, i.e. functions associated with β1 integrin activation [158, 159]. Recently, a study showed that Cdk5 and p35 are clustered with talin and β1 integrins into lamellipodia during cell spreading [160]. Inhibition of Cdk5 activity using the pan-Cdk inhibitor, roscovitine, prevents binding of talin/integrin, which suggests that Cdk5 activation may be involved in integrin activation. Thus, Cdk5 and p35 may play important roles in talin phosphorylation-mediated integrin activation, which will be studied in this dissertation.
Summary of the problem and hypothesis

Prostate cancer is the most commonly diagnosed cancer in men in the western world with a nearly 29,500 deaths anticipated in 2014 in the United States [1, 161]. Although patients with organ-confined prostate cancer are usually successfully treated, for those with metastases, the 5-year survival decreases to 28% [1]. Prostate cancer has a high propensity for metastasizing to the bone. Thus, understanding the mechanisms by which prostate cancer cells metastasize to the bone will be critical for designing novel therapeutic strategies for metastasis prevention.

Recent studies have implicated β1 integrin signaling in promoting critical steps in prostate cancer metastasis, such as adhesion, migration, invasion and cellular survival. Several β1 integrins on prostate cancer cells have been shown to interact with bone matrix and promote tumor growth in the bone [79, 80, 111]. However, the role of β1 integrins in prostate cancer bone metastasis remains unknown. A better understanding of the β1 integrin signaling pathway by which prostate cancer cells increase metastatic potential is needed. Moreover, the mechanism by which β1 integrins are activated in prostate cancer cells is also unknown. Although, talin has been shown to regulate integrin activation and thus is a candidate for activating β1 integrins in prostate cancer cells, the potential role of its phosphorylation in β1 integrin activation has not been assessed previously. In addition, talin, Cdk5 and p35 have been implicated in prostate cancer metastasis, but whether they are in a signaling axis that promotes β1 integrin activation leading to bone metastasis was not clear prior to my studies.
The goal of this Ph.D. dissertation work was to investigate the roles of talin1 phosphorylation in prostate cancer metastasis and specifically to understand the mechanism of talin1 phosphorylation in activating β1 integrins and the effects in promoting prostate cancer bone metastasis. Therefore, the first question addressed in the dissertation work was whether β1 integrins are activated through inside-out β1 integrin activation in metastatic prostate cancer cells, and what would be the effects to cancer cells. The second question was whether talin1 phosphorylation promotes β1 integrin activation in metastatic prostate cancer cells, and what would be the effects of talin phosphorylation on cancer cells? The third question was what is the mechanism by which talin1 is phosphorylated in prostate cancer cells? The fourth question was whether talin1 phosphorylation promotes prostate cancer bone metastasis in vivo.

The hypothesis tested in this dissertation was that β1 integrins are constitutively activated in metastatic prostate cancer cells through talin1 S425 phosphorylation, which is required to promote prostate cancer metastasis. To test this hypothesis, I first determined the correlation of β1 integrin activation with metastatic potential of cancer cells, and whether β1 integrins were activated through inside-out signaling. Then, I investigated whether talin phosphorylation promotes β1 integrin activation in prostate cancer cells by talin silencing and mutation, and examined the effects of talin phosphorylation on integrin associated functions in cancer cells. For the third question, I determined the potential mechanism that increased p35 expression promotes Cdk5 activity that phosphorylates talin resulting in β1 integrin activation in prostate cancer cells. Finally, I assessed the effects of talin phosphorylation in prostate cancer metastasis.
in vivo and investigated the clinical relevance of talin phosphorylation in prostate cancer tissues. Together, this work addressed the mechanism and biological function of talin phosphorylation and provides a better understanding of the mechanism for prostate cancer metastasis.
Chapter 2

Materials and Methods

**Cell lines and culture condition**

The PC3 and PC3-MM2 cells were gifts from Dr. Isaiah J. Fidler at the M. D. Anderson Cancer Center. The PC3-MM2 is a highly metastatic cell line derived following two passages of PC3-M cells in nude mice of selecting and re-passaging lymph node metastases [162]. LNCaP cells were obtained from the American Type Culture Collection. The C4-2B4 cells were gifts from Dr. Sue-Hwa Lin at the M. D. Anderson Cancer Center. The C4-2B4 cells derived from bone metastasis are a C4-2 subline derived from LNCaP [163]. These cell lines as well as those that were gene-silenced or engineered to express talin1 mutants were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum. To increase cell attachment, β1 integrin knockdown, talin1 knockdown and talin1S425A expressing cells were cultured on poly-D-lysine (Sigma, St. Louis, MO) coated plates. Cell lines were checked every six months and found to be mycoplasma free, and the identity of cell lines was authenticated at the M. D. Anderson Cell Identification Core Facility.
Antibodies

Anti-talin1 (97H6) antibody was purchased from GeneTex (San Antonio, TX). Anti-pan total talin (8d4) and β-actin antibodies were purchased from Sigma. Anti-pteratin S425 antibody and blocking peptide were purchased from ECM Biosciences (Versailles, KY). Anti-active β1 integrin (9EG7) and total β1 integrin (MAR4, 18) antibodies were purchased from BD Biosciences (San Jose, CA). Anti-active β1 integrin (12G10) and talin2 (68E7) antibodies were purchased from Abcam (Cambridge, MA). Anti-Cdk5 (C-8) and p35 (C-19) antibodies were purchased from Santa Cruz (Dallas, TX). Anti-FAK, PARP, cleaved PARP, AKT, pAKT S473, p130 Cas, pp130 Cas Y165, GFP and Myc-tag antibodies were purchased from Cell Signaling (Danvers, MA). Anti-pFAK Y397 and Alexa Fluor 647-conjugated secondary antibodies were purchased from Life Technologies (Grand Island, NY).

Flow cytometric analysis of β1 integrins

Cultured cells (1 × 10^5) were detached by trypsin without EDTA, resuspended in 0.5 ml of ice-cold PBS buffer with 0.5% bovine serum albumin (BSA; Sigma Cat. no. A7030) and 0.04% sodium azide, and incubated on ice for 10 min. Anti-active β1 integrin (9EG7; 1:300), active β1 integrin (12G10; 1:300), total β1 integrin (MAR4; 1:300) antibodies or isotype IgG control were added and incubated on a rocker for 1 hr at 4°C. Cells were centrifuged at 500 x g for 5 min, washed with PBS three times, and then incubated with Alexa Fluor 647-conjugated secondary antibody (1:500) in 1 ml of PBS with 0.5% BSA on a rocker for 30 min at 4°C in the dark. Labeled cells were washed twice with PBS and subjected to FACS analysis using a FACS Canto II flow cytometer.
(BD Biosciences). Labeled cells were fixed and subjected to FACS analysis within one week. To fix cells, labeled cells were washed with PBS and resuspended in 1% paraformaldehyde for 5 min followed by washing with PBS containing 0.5% BSA and stored at 4°C in the dark. Expression of activated and total β1 integrins was quantitated by geometric mean fluorescence intensity using FlowJo software (Tree Star, Ashland, OR).

**Immunoblotting**

Cells were washed with PBS and lysed with RIPA buffer (10 mM Sodium phosphate, pH 7.2, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid, 2 mM EDTA, 1% Triton X-100, 10% glycerol, protease inhibitor cocktail, and PhosSTOP phosphatase inhibitor cocktail; Roche, Indianapolis, IN). Cell lysates were sonicated (Bioruptor XL, Diagenode, Denville, NJ) for 90 sec on ice and centrifuged at 14,000 ×g for 20 min at 4°C. Protein concentration of the supernatants was measured using a DC protein assay kit (Bio-Rad, Hercules, CA). The supernatants (30 µg) were mixed with Laemmli sample buffer, boiled for 5 min, and subjected to SDS-PAGE. Proteins were transferred on PVDF membranes followed by blocking with 5% milk for 1 hr. For phospho-protein immunoblotting, membranes were blocked in 5% BSA for 2 hr. Membranes were incubated with primary antibodies for 1 hr at room temperature, or overnight at 4°C. Then, horseradish peroxidase-conjugated secondary antibodies (1:6500) were incubated with membrane for 1 hr. Membranes were incubated with SuperSignal Femto Substrate (Thermo) and exposed to X-ray films to detect proteins.
Immunofluorescence staining

Cells were cultured on coverglasses (Grace Bio-Labs, Bend, OR) for 18 hr, fixed in 4% paraformaldehyde for 15 min, washed with PBS, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and incubated in blocking buffer (LI-COR, Lincoln, NE) for 1 hr. Antibodies (anti-active β1 integrin 12G10, 1:100; anti-total β1 integrin clone 18, 1:100) were diluted in blocking buffer and incubated for 18 hr at 4°C. Slides were washed with PBS and incubated with Alexa Fluor 647-conjugated secondary antibody for 1 hr. Nuclei were stained using Hoechst 33342 (Life Technologies). Slides were treated with ProLong Gold antifade Reagent (Life Technologies) and images were acquired using a TCS SP5 confocal microscope (Leica, Buffalo Grove, IL) and analyzed using Image-Pro Plus software (Media Cybernetics, Rockville, MD). For fluorescence area quantitation, a threshold of fluorescence intensity was used to define fluorescence area on images. The number of pixels in the area with an intensity above the threshold was counted and normalized to cell count for each image.

Anoikis assays

Cells (1 × 10^6) were detached by trypsin, resuspended in 13 ml of serum-free medium containing 0.05% BSA in a 15 ml conical tube with vent cap, and rotated in an incubator to prevent cell aggregation. For counting live and dead cells, 500 µl of cell suspension was incubated with 2 mM Calcein AM (BD Biosciences) for 30 min at 37°C followed with 4 mM propidium iodide (BD Biosciences) and counted under a fluorescence microscope. For cells expressing talin mutants, live cells were counted by trypan blue exclusion using a Vi-Cell XR cell viability analyzer (Beckman Coulter,
Indianapolis, IN). The cells in the cell suspension were centrifuged at 500 ×g for 5 min and lysed in RIPA buffer at specific times followed by immunoblotting for PARP and cleaved PARP. To detect apoptotic cells using propidium iodide FACS analysis, the cell suspension was centrifuged, fixed by resuspended in ice-cold 70% ethanol and incubated at 4°C for overnight (see appendix for protocol). Cells were centrifuged and washed with PBS and resuspended in 50 µl of PBS with 2 mg/ml ribonuclease A and incubated for 5 min on ice. PBS (450 µl) containing 75 µM propidium iodide was added and incubated for 30 minutes on ice followed by analyzing using FACS analysis. Cells in the sub-G0/G1 were quantitated using FlowJo software.

**Lentivirus-mediated gene silencing**

To decrease β1 integrin expression, a viral pLKO.1-puro plasmid (Sigma) containing a shRNA sequence 5’-GCCCTCCAGATGACATAGAAA-3’ was used. Two plasmids containing different shRNA sequences were used to decrease talin1 or talin2 expression. sh-Talin1 sequences used were 5’-CCCAGAGTATTAACGCTCCAA-3’ targeting UTR region, and 5’-GCCTCAGATAATCTGGTGAAA-3’ targeting CDS region. sh-Talin2 sequences used were 5’-GACGAATCCAAACACGAAATC-3’ and 5’-ACGATGCGTGTGAGTCATTC-3’. To decrease Cdk5 expression, the sequence used was 5’-CAGAACCTTCTGAAGTGTAAC-3’. A non-targeting shRNA sequence 5’-GCGCGATAGCGCTAATAATTT-3’ in the same vector was used as a sh-control plasmid. For lentivirus production, the pLKO.1-puro plasmid (3 µg) was co-transfected with the packaging plasmid pCMV-dR8.2 dvpr (3 µg) and the envelope plasmid pCMV-VSV-G (0.6 µg) in a ration of 5:5:1 into 293FT cells in one 100-mm plate (Life
Technologies) using Lipofectamine 2000 (Life Technologies). After 24 hr, medium was replaced with 20% FBS. The viral supernatant was collected after 24 h and again at 48 hr, filtered through 0.45 µm filters followed by centrifugation at 20,000 rpm for 2 hr at 4 °C. The viral pellet was resuspended in 200 µl of RPMI medium and stored at -80 °C. Cells cultured in 48-well plates were incubated with 20 µl of virus supernatant in the present of 8 µg/ml of polybrene (Sigma) and centrifuged at 500 xg for 20 min and further incubated for 24 hr. The medium was changed after 24 h and replaced again with 5 µg/ml puromycin after 48 hr and incubated for one week to select stable silenced cells. For Cdk5 knockdown, cells were tranduced and lysed after 48 hrs without selection.

**Immunoprecipitation**

Cells were lysed in lysis buffer containing 50 mM Hepes, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid, 10% glycerol, protease inhibitor cocktail, and phosphatase inhibitor cocktail. Lysates (500 µg protein) were diluted two-fold in immunoprecipitation buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol) and incubated with anti-talin1 (97H6) antibody (5 µg) plus 5 µM cytochalasin D for 3 h at 4 °C. Protein G-coupled agarose beads (50 µl) were added into the mixture and incubated for 1 h at 4 °C followed by washing using lysis buffer once and immunoprecipitation buffer three times, and immunoblotting were subjected.

**Mutagenesis**

To express talin1\(^{WT}\) and mutants in prostate cancer cells, human full-length talin1 cDNA in pEZ-M29-talin1 plasmid was purchased from GeneCopoeia (Rockville, MD).
To generate pWPXL-GFP-talin1\textsuperscript{WT}, full-length talin1 DNA fragments were amplified by PCR using pEZ-M29-talin1 as a template and

5’GCTGTACAAGTCCGGCATGGTTGCACTTTCACTGAAGA3’ (primer #1) and
5’GAGATCTGAGTCCGGACAAGAAAGCTGGGTTGCG3’ (primer #2; see appendix for subcloning protocol). The DNA fragments were subcloned into BspEI partial-digested pWPXL-GFP vector (gift of Dr. Didier Trono, Addgene plasmid #12257) using In-Fusion HD cloning system (Clontech, Mountain View, CA) according to the manufacturer’s instructions (see appendix for plasmid maps). To generate pWPXL-GFP-talin1\textsuperscript{S425A}, the pEZ-M29-talin1 was used as a template for PCR amplification and

5’GACTCAGTGGCCCCCACAAGGATCAACAGTCTCG3’ and
5’GGGCACTGAGTCCTCCAGCATAGACTCCTC3’ (underline denotes mutated site) as complementary mutagenic primers to generate linearized pEZ-M29-talin1\textsuperscript{S425A}, and then recircularized using the In-Fusion system (see appendix for mutagenesis protocol). The pEZ-M29-talin1\textsuperscript{S425A} was then used as the template for PCR amplification with primer #1 and #2 to generate full-length talin1 1-2542\textsuperscript{S425A} DNA fragments, which were inserted into a BspEI partial-digested pWPXL-GFP vector using In-Fusion enzyme. The same procedures above were used to generate pWPXL-GFP-talin1\textsuperscript{S425D} with the following complementary mutagenic primers:

5’GACTCAGTGGACCACAAAAAGTCAACAGTCCTG3’ and
5’GGTCCACTGAGTCCTCCAGCATAGACTCCTC3’ for PCR amplification.
**Transfection**

To generate stable GFP-talin1\(^{\text{WT}}\) and mutant cells, pWPXL-GFP-talin1\(^{\text{WT}}\), mutant plasmids and pWPXL-GFP (empty vector) were transiently transfected into talin1-silenced cells using jetPRIME (Polyplus-transfection, Illkirch, France) according to the manufacturer’s instructions. For talin1 knockdown in PC3-MM2 and C4-2B4 cells, a pLKO plasmid directing expression of shRNA to the talin1 UTR was used, therefore not interfering with expression of GFP-talin1 mutant proteins subsequently expressed. Transient transfected GFP-talin1 mutant cells were sorted for GFP positive cells by a flow cytometer at the M. D. Anderson Flow Cytometry and Cellular Imaging Core Facility to select stable transfected cells with similar levels of GFP expression. For Cdk5 overexpression, plasmids of empty vector (pEF4-myc-His), Cdk5 wild-type (pEF4-myc-His-Cdk5), and dominant-negative Cdk5 (pEF4-myc-His-Cdk5-144N) [143] were transiently transfected using jetPRIME.

**Proliferation assays**

Cells (5 × 10\(^4\)) were cultured on 60-mm CellBind plates (Corning) in triplicate for specific times. Cells were detached by trypsin and live cell number were counted by trypan blue exclusion using a Vi-Cell XR cell viability analyzer.

**Adhesion assays**

Tissue culture treated 96-well plates were coated with fibronectin (5 µg/cm\(^2\); BD Biosciences), or collagen I (5 µg/cm\(^2\); BD Biosciences) for 1 hr at 37°C, then blocked by 1% BSA. Cells were detached by trypsin without EDTA, resuspended in serum-free
medium and incubated in plates at $4 \times 10^4$ cells per well in triplicate for 5 min at 37°C. Plates were washed three times by 100 µl of PBS to remove unattached cells. Attached cells were incubated with 1 µM Calcein AM (BD Biosciences) in PBS for 5 min at 37°C. Fluorescence-labeled cells were lysed in 100 µl of 1% Triton X-100, and the fluorescence intensity was measured using an Envision plate reader (PerkinElmer, Waltham, MA).

**Migration and invasion assays**

Migration assays were performed using collagen I coated (1 µg/cm²) polycarbonate transwell filters in 24-well plates (8-µm pore size; Corning, Tewksbury, MA). For invasion assays, Matrigel coated filters (BD Biosciences) were used. Cultured cells were detached by trypsin, suspended in serum-free medium and incubated in the upper chamber of transwell inserts at a density of $5 \times 10^4$ cells per well. The medium in the bottom chamber was supplemented with 0.1% FBS as a chemoattractant. Cells were allowed to migrate for 8 hr or to invade for 24 hr. Cells adhering to the bottom of the filters were stained with HEMA 3 stain kit (Fisher Scientific, Kalamazoo, MI) and then the cells on the upper side of the filter were removed by cotton tips. Images of filters were acquired at 20× using a microscope with camera (Eclipse Ti-S; Nikon, Melville, NY). Cell numbers on images were counted using Image-Pro Plus software.

**Cdk5 kinase activity assays**

Cdk5 kinase activity was measured using ADP-Glo kinase assay (Promega, Madison, WI) according to the manufacturer’s instructions. Cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5%
deoxycholic acid, 5 mM EDTA, protease inhibitor cocktail, and phosphatase inhibitor cocktail. Cell lysate (500 µg) was incubated with anti-Cdk5 antibody (5 µg) in modified lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% deoxycholic acid, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, protease inhibitor cocktail, and phosphatase inhibitor cocktail) for 1 h at 4 °C followed by incubated with 50 µl of protein A-coupled agarose beads for 3 h at 4 °C. The agarose beads were washed using modified lysis buffer and resuspended in 10 µl kinase reaction buffer (40 mM Tris-HCl, pH 7.5, 20 mM MgCl₂ and 0.1 µg/µl BSA). Cdk5 enzyme reaction was initiated by incubating agarose beads in kinase reaction buffer for 30 min at 30 °C with 170 µM histone H1 peptide PKTPKKAKKL (Enzo Life Sciences, Farmingdale, NY) and 50 µM ATP in a final volume of 25 µl. ADP produced during kinase reaction was measured using ADP-Glo kinase assay according to the manufacturer’s instructions (see appendix for protocol). The reaction mixture (20 µl) was transferred to a 384-well plate, and luminescence read using an Envision plate reader.

**In vivo metastasis assays**

PC3-MM2 sh-control, talin1-silenced with GFP, GFP-talin1WT and GFP-talin1 mutant cells were transduced with lentivirus containing a plasmid directing luciferase expression (pLenti-PGK-Blast-V5-LUC [164], i.e. the Addgene plasmid #19166) as described above, and then incubated with 5 µg/ml blasticidin to select stable transduced cells. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) guidelines of M. D. Anderson Cancer Center. For intracardiac injection (experimental metastasis assays), the cells (1 × 10⁶) were suspended
in 50 µl of PBS and injected into left ventricle of male nude mice. Bioluminescence images were acquired immediately after intracardiac injection to properly ensure distribution of cells in mice, and then were used to monitor tumor growth. For bioluminescence imaging, mice were injected intraperitoneally with 100 µl of 15 mg/ml luciferin in PBS, imaging using a Xenogen IVIS 200 system (PerkinElmer) was performed 10 min later. X-ray and matched bioluminescence images were acquired and superimposed using a Kodak In-Vivo Multispectral Imaging System FX (Carestream, Rochester, NY). For intraprostatic injections (spontaneous metastasis assays), the procedure of Kim et al. [165] was followed. Male athymic nude mice (Ncr-nu/nu; ages 8–12 wk) were anesthetized placed in a supine position. A midline incision was made on the lower abdomen, and the prostate was exteriorized. Cells (5 × 10^5) in 10 µl of PBS were injected into the dorsolateral side of the prostate. The incision was closed with surgical metal clips. The growth of primary tumor was monitored by bioluminescence imaging. Mice with similar sized primary tumors were sacrificed, primary tumors were removed and the lymph node metastases were identified by bioluminescence imaging.

**Immunohistochemistry**

Human prostate cancer tissue samples from primary tumor, lymph node metastases and bone metastases were obtained from the prostate cancer tissue bank at M. D. Anderson Cancer Center, under an approved IRB protocol. To validate the specificity of anti-pteratin S425 antibody, PC3-MM2 cells were fixed in 10% formalin, pelleted and embedded in agarose (see appendix for protocol). Sections of agarose cell blocks were incubated with pteratin S425 antibody alone or simultaneously with corresponding
blocking peptide (ECM Biosciences) followed by using the same procedures in tissue staining. For tissue staining, formalin-fixed, paraffin-embedded sections in 4-µm-thick were de-waxed with xylene, rehydrated in graded concentrations of alcohol followed by heat-induced epitope retrieval at 121 °C for 10 min in citrate buffer (pH 6.0) using a pressure cooker. Tissue sections were treated with 3% hydrogen peroxide for 30 min, washed with PBS, blocked with 5% normal horse serum for 30 min, and incubated at 4 °C overnight with anti-palin S425 antibody. Antibody binding was detected using biotin-streptavidin system (Vector Laboratories, Burlingame, CA) with 3,3′-diaminobenzidine as the chromogen. Hematoxylin was used as the counterstain. The immunostaining results were reviewed and scored by pathologists as described previously [112]. Tissues were defined as positive when over 10% of cells were stained.

**Statistical analyses**

Data are shown as means ± s.d. One-way ANOVA and post Tukey’s test were used to determine significance in metastasis assays. A chi-square test was used to evaluate immunohistochemical analysis. Other assays were determined by two-tailed Student’s t-test. P-values less than 0.05 were considered statistically significant.

Statistical analyses were conducted using GraphPad Prism software (GraphPad Software, La Jolla, CA).
Chapter 3

Constitutive activation of β1 integrins in metastatic prostate cancer cells

**Activation of β1 integrins correlates with metastatic potential of prostate cancer cells**

To examine whether activation of β1 integrins plays a role in prostate cancer metastasis, I determined β1 integrin expression and activation in several available prostate cancer cell lines, including the lymph node-derived LNCaP and its variant C4-2B4, as well as the PC3 and its metastatic variant PC3-MM2 cells. LNCaP and C4-2B4 cells are tumorigenic, however, with low metastatic potential when implanted orthotopically or intracardially. PC3 and PC3-MM2 are tumorigenic and highly metastatic, exhibiting high incidence of metastasis in both spontaneous and experimental bone metastasis models [77, 166]. Using anti-total β1 integrin mAb MAR4, FACS analysis showed that all 4 cell lines expressed high levels of β1 integrins (Figure 8). Next, we examined integrin activation in these cell lines. Western blot showed that the levels of phosphorylation of pFAK Y397 are higher in PC3 and PC3-MM2 than LNCaP and C4-2B4 cells (Figure 9), suggesting that integrin signaling is in a more activated state in PC3
Figure 8. FACS analysis of total β1 integrins in prostate cancer cell lines. (A) FACS analysis using anti-total β1 integrin antibody, mAb MAR4. Cells incubated with secondary antibody only were used as negative controls. (B) Quantitation of geometric mean fluorescence intensity of total β1 integrins.
Figure 9. Immunoblotting of cell lysates for the expression of FAK and phosphorylated FAK Y397. (A) Immunoblotting of pFAK Y397 and total FAK using cell lysates from prostate cancer cells. (B) Quantitation of band intensity in immunoblotting.
and PC3-MM2 cells with higher metastatic potential. We further used the conformation-sensitive antibody 9EG7 to examine whether β1 integrins in these cells were present in the activated conformation. Monoclonal antibody 9EG7 recognizes the EGF-like repeats 1-4 that were exposed in activated β1 integrins [167-169]. The data showed that 9EG7 binds to PC3 and PC3-MM2 strongly (Figure 10). In contrast, 9EG7 showed only modest binding to LNCaP and C4-2B4 (Figure 10). β1 integrin activation was further examined by immunofluorescence staining using another conformation-specific antibody, mAb 12G10, that binds to exposed βA domain during β1 integrin activation [170, 171].

Activation of β1 integrins, determined by fluorescence area, was 20-fold higher in metastatic PC3-MM2 cells as compared to low metastatic C4-2B4 cells (Figure 11). These observations suggest that β1 integrins are present in an activated conformation only in cell lines with high metastatic potential.
Figure 10. FACS analysis of activated β1 integrins in prostate cancer cell lines. (A) FACS analysis using a conformation-sensitive antibody, mAb 9EG7, targeting activated β1 integrins. Cells incubated with secondary antibody only were used as controls. (B) Quantitation of geometric mean fluorescence intensity of activated β1 integrins.
Figure 11. Immunofluorescence staining of activated and total β1 integrins. (A) PC3-MM2 and C4-2B4 cells were stained using a conformation-sensitive antibody for activated β1 integrins (mAb 12G10) and total β1 integrins (mAb 18; bottom panels). Nuclei were counterstained by Hoechst 33342. Scale bar represents 25 µm. (B) Differences in expression by quantitating pixels of immunofluorescence area. *PC3-MM2 compared to C4-2B4, \( P < 0.05 \). **PC3-MM2 compared to C4-2B4, \( P < 0.001 \).
β1 integrin activation correlates with anoikis resistance in prostate cancer cells

Integrin signaling is critical to increase cell survival [172]. Thus, I examined whether increased β1 integrin activation in metastatic prostate cancer cells correlated with cell survival by using an anoikis assay. Single cells from prostate cancer cell lines were placed in suspension with serum-free medium and constantly rotated for 24 and 48 hours to prevent aggregation-induced outside to inside integrin aggregation. The number of viable and dead cells was then determined. As shown in Figure 12, PC3-MM2 has the highest number of viable cells at 48 hours, whereas LNCaP and C4-2B4 cells did not survive in these anoikis conditions. The decreases in viable cell number corresponded to the increases in dead cell number (Figure 12). Analysis of nuclear integrity by FACS of propidium iodide staining showed that the increased dead cell number in anoikis-sensitive cell lines correlated with an increase in the sub-G1 fractions (Figure 13), suggesting that these cells had undergone apoptosis. Consistent with this result, western blot analysis showed a correlation between anoikis sensitivity and increases in PARP cleavage (Figure 14). These observations indicate that the ability of prostate cancer cells to survive in anoikis conditions correlates with the activation status of β1 integrins and also the metastatic potential of these cell lines.
Figure 12. Viable and dead cell number of prostate cancer cells in anoikis conditions. Cells were grown in anoikis condition as described in Materials and Methods. (A) Viable and (B) dead cell numbers were determined by Calcein AM and propidium iodide staining under a microscope, respectively.
Figure 13. FACS analysis of propidium iodide-labeled apoptotic cells. (A) Cells were grown in anoikis conditions for 24 and 48 hr. Cell counts of propidium iodide-labeled cells after 24 hr suspension. Percentage indicated apoptotic cell population in sub-G1. (B) Quantitation of apoptotic cells in sub-G1.
Figure 14. Immunoblotting of PARP and cleaved PARP. PC3 or PC3-MM2 cells grown under anoikis conditions for various lengths of times. Cells were lysed and subjected to immunoblotting.
Silencing of β1 integrins in PC3-MM2 cells by expression of shRNA abrogates anoikis resistance

To examine the causal effect of β1 integrin signaling on prostate cancer cell survival, I used stable expression of shRNA to knockdown β1 integrins in PC3-MM2 cells. As shown in Figure 15, transfection of β1 integrin-specific shRNA reduced the expression of β1 integrins by more than 90%, whereas control non-targeting shRNA did not have significant effects on β1 integrin expression. To examine the effects on integrin-mediated survival pathways, phosphorylation of FAK Y397 and AKT S473 were examined. Knockdown of integrin greatly reduced pFAK Y397 and pAKT S473 phosphorylation (Figure 15). Furthermore, the β1 integrin knockdown cells in suspension were sensitized to anoikis (Figure 16), with a concomitant increase in PARP cleavage (Figure 17). These results suggest that β1 integrin signaling plays a critical role in the survival of highly metastatic PC3-MM2 cells.
Figure 15. Immunoblotting of β1 integrins, pFAK Y397, total FAK, pAkt S473, and total Akt in β1 integrin knockdown cells. A stable PC3-MM2 cell line was derived in which β1 integrins are reduced by expression of shRNA as described in Materials and Methods.
Figure 16. Viable cell number of β1 knockdown PC3-MM2 cells in anoikis conditions. Control and β1 knockdown PC3-MM2 cells were grown in anoikis conditions for 24 and 48 hr as described in Materials and Methods. Viable cell numbers were determined by Calcein AM staining.
Figure 17. Immunoblotting of PARP and cleaved PARP in β1 integrin knockdown cells in anoikis conditions. β1 integrin knockdown PC3-MM2 cells were cultured under anoikis conditions for indicated times. Cell lysates were used for immunoblotting for PARP and cleaved PARP.
Chapter 4
Talin1 phosphorylation regulates β1 integrin activation

**Talin1, but not talin2, activates β1 integrins in prostate cancer cells**

Talin is required for inside-out integrin activation and is overexpressed in prostate cancer [114]. Therefore, I determined whether talin expression contributes to constitutive β1 integrin activation in metastatic prostate cancer cells. Because talins are expressed from two genes, *TLN1* and *TLN2*, I first compared the effect of talin1 and talin2 in β1 integrin activation by gene silencing in PC3-MM2 cells. Silencing of talin1 did not affect the expression of talin2 (Figure 18). While both talin1 and talin2 are expressed in PC3-MM2 cells, talin1 is far more abundant as total talin detected by the pan-talin antibody, 8D4, was significantly reduced in talin1-silenced cells (Figure 18). In addition, talin1-silenced cells possessed a rounded morphology and impaired spreading (Figure 19), a phenotype not observed in talin2-silenced cells (Figure 19, 20). Next, the levels of total and activated β1 integrins in talin1 and talin2-silenced PC3-MM2 cells were determined. Flow cytometric analysis demonstrated that silencing of talin1 (Figure 21), but not talin2 (Figure 22), reduced β1 integrin activation, without affecting expression of total β1 integrins. These results demonstrate that talin1, but not talin2, was primarily responsible for β1 integrin activation in prostate cancer cells.
Figure 18. Immunoblotting of talin1, talin2 and pan-talin in talin1-silenced cells.

Silencing of talin1 in PC3-MM2 cells by shRNA expression using two sequences as described in Materials and Methods. A non-targeting shRNA sequence in the same vector was used as an sh-control.
Figure 19. Effects of talin1 and talin2 silencing on cell spreading. Morphology of talin1- and talin2-silenced PC3-MM2 cells were observed using a light microscope. Scale bar represents 100 µm.
Figure 20. Immunoblotting of talin2 and talin1 in talin2-silenced cells. Silencing of talin2 in PC3-MM2 cells by shRNA expression using two sequences as described in Materials and Methods.
Figure 21. Flow cytometric analysis of activated $\beta_1$ integrins and total $\beta_1$ integrins in talin1-silenced PC3-MM2 cells. FACS analysis using conformation-sensitive antibodies for activated $\beta_1$ integrins and for total $\beta_1$ integrins (top panels). Relative expression of activated and total $\beta_1$ integrins quantitated by fluorescence intensity is shown in the bottom panels. *sh-Talin1 compared to sh-control, $P < 0.005$. 
Figure 22. Flow cytometric analysis of total β1 integrins and activated β1 integrins in talin2-silenced PC3-MM2 cells. FACS analysis using conformation-sensitive antibodies for activated β1 integrins and for total β1 integrins (top panels). Relative expression of activated and total β1 integrins quantitated by fluorescence intensity is shown in the bottom panels.
Talin1 S425 phosphorylation correlates with activated β1 integrins in PC3-MM2 cells

Talin1 has been shown to increase metastatic properties associated with β1 integrin functions in prostate cancer cells [114]. Therefore, I determined whether talin1 expression correlates with β1 integrin activation. I collaborated with the laboratory of Dr. Sue-Hwa Lin to examine talin1 expression in low metastatic LNCaP, C4-2B4, metastatic PC3 and PC3-MM2 cells. The level of talin1 expression was similar in each cell line (Figure 23). These results suggest that increased talin1 expression alone is insufficient to activate β1 integrins. Since talin phosphorylation on S425 is also associated with some integrin functions such as migration [143], we next examined talin1 and talin2 phosphorylation on S425 by immunoblotting. Talin (comprising talin1 and talin2) was highly phosphorylated on S425 in metastatic PC3 and PC3-MM2 cells compared to low metastatic LNCaP and C4-2B4 cells (Figure 23). To examine talin1 phosphorylation specifically, cell lysates were immunoprecipitated with a talin1-specific antibody, followed by immunoblotting with an antibody recognizes talin S425 phosphorylation. Talin1 was highly phosphorylated in PC3 and PC3-MM2 cells (Figure 24), consistent with immunoblotting observed from whole cell lysates (Figure 23). These results demonstrate that levels of talin1 S425 phosphorylation correlate with activated β1 integrins in PC3-MM2 cells.
Figure 23. Talin1 expression and talin S425 phosphorylation in prostate cancer cells.

(A) Immunoblotting of total talin1 and talin S425 phosphorylation using whole cell lysates from prostate cancer cells. (B) Quantitation of band intensity from immunoblotting.
Figure 24. Phosphorylation of talin1 on S425 in prostate cancer cells. (A)
Immunoprecipitation of talin1 followed by immunoblotting of talin S425 phosphorylation in indicated prostate cancer cells. (B) Quantitation of band intensity from immunoblotting.
Talin1 S425 phosphorylation promotes β1 integrin activation

To determine if talin1 S425 phosphorylation were required for β1 integrin activation, I silenced endogenous talin1 and stably expressed either talin1 wild-type (GFP-talin1WT), a non-phosphorylatable mutant (GFP-talin1S425A), or a phosphorylation-mimicking mutant (GFP-talin1S425D) in PC3-MM2 and C4-2B4 cells as described in Materials and Methods. Levels of expression of each form of talin1 were similar (Figure 25). Flow cytometric analysis demonstrated that talin1WT expressed in talin1-silenced PC3-MM2 cells restored β1 integrin activation (Figure 26). In contrast, expression of talin1S425A inhibited β1 integrin activation by 60%, whereas talin1S425D promoted β1 integrin activation by 76% relative to talin1WT. Similar results were observed when talin1WT and mutants were expressed in C4-2B4 cells in which endogenous talin1 was silenced (Figure 27). I further assessed the effects of talin1 phosphorylation on β1 integrin activation by immunofluorescence staining. In agreement with the flow cytometric analysis, fewer activated β1 integrins were expressed in talin1S425A cells relative to talin1WT cells, while talin1S425D cells showed increased clustering of active β1 integrins (Figure 28). Next, I examined whether talin1 phosphorylation affects downstream integrin signaling. The PC3-MM2 talin1S425A cells were decreased in expression of pFAK Y397, pp130 Cas Y165 and pAkt S473 but not total FAK, p130 Cas or Akt (Figure 29), consistent with decreased signaling through activated β1 integrins. Similarly, in talin1-silenced cells, pFAK Y397, pp130 Cas Y165 and pAkt S473 were decreased with no decrease in expression of these proteins; whereas in cells re-transfected with talin1WT, activation of these downstream intermediates was restored. Together, these results demonstrate that talin1 S425 phosphorylation promotes β1 integrin activation and downstream signaling in metastatic prostate cancer cells.
Figure 25. **Expression of talin1 mutants in prostate cancer cells.** (A) Expression of GFP (empty vector), GFP-talin1\(^\text{WT}\) and specified GFP-tagged talin1 mutants in talin1-silenced PC3-MM2 and (B) in talin1-silenced C4-2B4 cells.
Figure 26. Flow cytometric analysis of activated and total β1 integrins of PC3-MM2 talin1 mutants. PC3-MM2 cells expressing talin\textsuperscript{WT} and mutants were subjected to flow cytometric analysis using antibodies for activated β1 integrins (9EG7) and total β1 integrins (MAR4; top panels). Quantitation of fluorescence intensity is shown in the bottom panels. The sh-talin1 knockdown cells were served as a negative control. *\(P < 0.005.\)
Figure 27. Flow cytometric analysis of activated and total β1 integrins of C4-2B4 talin1 mutants. C4-2B4 cells expressing talin1WT and mutants were subjected to flow cytometric analysis using antibodies for activated β1 integrins (9EG7) and total β1 integrins (MAR4; top panels). Quantitation of fluorescence intensity is shown in the bottom panels. The sh-talin1 knockdown cells served as a negative control. *P < 0.005.
Figure 28. Immunofluorescence staining of GFP and activated β1 integrins.

Immunofluorescence staining of GFP and activated β1 integrins (using mAb 12G10) in PC3-MM2 cells expressing talin1WT and mutants. Nuclei were counterstained by Hoechst 33342. Scale bar represents 25 µm.
**Figure 29. Effect of talin1 phosphorylation on activation of downstream β1 integrin signaling.** Immunoblotting of pFAK Y397, total FAK, pp130 Cas Y165, total p130 Cas, pAkt S473 and total Akt in PC3-MM2 cells expressing talin1$^{WT}$ and talin1 mutants. The sh-talin1 knockdown cells served as a negative control.
Chapter 5

Effects of talin1 phosphorylation in prostate cancer cells

**Effects of talin1 S425 phosphorylation on β1 integrin-mediated proliferation and anoikis resistance**

To determine the effect of talin1 S425 phosphorylation on biologic properties of prostate cancer cells, I used the PC3-MM2 and C4-2B4 cells described above expressing talin1^WT and mutants. Expression of talin1^WT, integrin-activating and -inhibiting talin1 mutant proteins had no affect on proliferation rates of PC3-MM2 cells or C4-2B4 cells (Figure 30). However, when cultured in anoikis conditions, 30% of the non-phosphorylatable talin1^S425A-expressing cells died after 24 hr and 40% after 48 hr (Figure 31). In contrast, no cell death was observed in both talin1^WT and talin1^S425D cells after 24 hr. After 48 h, 35% talin1^WT cells died, similar to talin1^S425A, but only 16% talin1^S425D died (Figure 31). To determine if effects on viability were due to changes in apoptosis, propidium iodide staining was performed. A two-fold increase in the sub-G0/G1 population was observed in talin1^S425A cells compared to talin1^WT cells after 24 hr (Figure 32). After 48 hr, the sub-G0/G1 population in talin1^WT cells and talin1^S425A cells was similar, while talin1^S425D cells were still resistant to anoikis (Figure 32). As a second measure of apoptosis, PARP cleavage was examined. PARP cleavage occurred in talin1^S425A cells by 24 hr, but no PARP cleavage was observed in talin1^S425D cells even after 48 hr (Figure 33). Thus, increased anoikis resistance correlated with talin1 S425 phosphorylation in prostate cancer cells.
Figure 30. Effects of talin1 S425 phosphorylation on cell proliferation. (A) PC3-MM2 and (B) C4-2B4 cells expressing talin1 WT and mutants were plated (5 × 10^4 cells) and cultured for indicated times, and then the trypan blue negative cells were enumerated.
Figure 31. Cell viability of PC3-MM2 cells expressing talin1 mutants in anoikis conditions. PC3-MM2 talin1<sup>WT</sup> and mutant-expressing cells were cultured in anoikis conditions as described in Materials and Methods. Surviving cells were counted at indicated times. *Talin1<sup>WT</sup> compared to talin1<sup>S425D</sup>, $P < 0.05$. 
Figure 32. Flow cytometric analysis of propidium iodide-labeled cells in sub-G0/G1. PC3-MM2 talin1\textsuperscript{WT} and mutant-expressing cells maintained in anoikis conditions were harvested at indicated times and stained with propidium iodide, then were subjected to FACS analysis. The percentage of cells in sub-G0/G1 is plotted. \(*P < 0.005.\)
Figure 33. Immunoblotting of total and cleaved PARP of talin1 mutants in anoikis conditions. Immunoblotting of total and cleaved PARP using lysates of PC3-MM2 talin1WT and mutant-expressing cells maintained in anoikis conditions for indicated times.
Effects of talin1 S425 phosphorylation on β1 integrin-mediated adhesion and motility

Next, I determined whether talin1 S425 phosphorylation promoted attachment of metastatic prostate cancer cells to ECM. For these analyses, talin1\(^{WT}\) and mutant-expressing cells were subjected to adhesion to fibronectin and collagen I-coated culture plates. Binding of PC3-MM2 cells expressing talin1\(^{S425A}\) to fibronectin was decreased by 74% and to collagen I by 81% relative to talin1\(^{WT}\) cells (Figure 34). Binding of PC3-MM2 cells expressing talin1\(^{S425D}\) to fibronectin was increased by 66% and to collagen I by 53% relative to talin1\(^{WT}\) cells (Figure 34). Very similar results were observed in adhesion assays when these mutants were expressed in C4-2B4 cells (Figure 35). Next, I determined whether talin1 phosphorylation promotes motility of prostate cancer cells on ECM. Migration assays were performed using collagen I-coated modified Boyden chambers, as described in Materials and methods. The ability of PC3-MM2 cells expressing talin1\(^{S425A}\) to migrate on collagen I was reduced 52% (62% in C4-2B4 cells expressing talin1\(^{S425A}\) as compared to talin1\(^{WT}\) cells, whereas the migratory ability of talin1\(^{S425D}\)-expressing PC3-MM2 cells was increased by 30% (55% in C4-2B4 cells expressing talin1\(^{S425D}\)) relative to respective wild-type transfected cells (Figure 36), again correlating with the level of β1 integrin activation. Similar results were observed in invasion assays, in which cells invaded through Matrigel-coated modified Boyden chambers. The invasive ability of PC3-MM2 cells expressing talin1\(^{S425A}\) was reduced 95% (90% in C4-2B4 cells expressing talin1\(^{S425A}\)) as compared to talin1\(^{WT}\) cells, whereas the invasive ability of talin1\(^{S425D}\)-expressing PC3-MM2 cells was increased by 40% (43% in C4-2B4 cells expressing talin1\(^{S425D}\)) relative to respective wild-type transfected cells.
(Figure 37), demonstrating that talin1 S425 phosphorylation promotes cell migratory and invasive abilities.
Figure 34. Talin1 S425 phosphorylation promotes cell adhesion in PC3-MM2 cells. Adhesion of PC3-MM2 talin1<sup>WT</sup> and mutant-expressing cells on fibronectin and collagen I-coated plates. Adhesion to BSA served as a negative control. *Talin1<sup>WT</sup> compared to talin1<sup>S425A</sup>, P < 0.005. **Talin1<sup>WT</sup> compared to talin1<sup>S425D</sup>, P < 0.0005.
Figure 35. Talin1 S425 phosphorylation promotes cell adhesion in C4-2B4 cells.
Adhesion of C4-2B4 talin1<sup>WT</sup> and mutant-expressing cells on fibronectin and collagen I-coated plates. Adhesion to BSA served as a negative control. *<i>P</i> < 0.0005.
Figure 36. Effect of talin1 S425 phosphorylation on cell migration. (A) PC3-MM2 and (B) C4-2B4 talin1<sup>WT</sup> and mutant-expressing cells were cultured on collagen I-coated membranes in modified Boyden chambers. Cells were allowed to migrate through the membranes for 8 h. *P < 0.0001
Figure 37. Effect of talin1 S425 phosphorylation on cell invasion. (A) PC3-MM2 and (B) C4-2B4 talin1<sup>WT</sup> and mutant-expressing cells were allowed to invade through Matrigel in modified Boyden chambers for 24 h. *<i>P</i> < 0.0001.
Chapter 6

Cdk5 regulates talin1 S425 phosphorylation

**Talin S425 phosphorylation is mediated by Cdk5 which is activated by p35**

Previous works in neuronal cells demonstrated that talin phosphorylation on S425 is catalyzed by Cdk5 [143]. To determine the mechanism by which talin1 S425 phosphorylation is increased in metastatic prostate cancer cells, first I examined whether Cdk5 expression or activity correlated with talin1 phosphorylation in prostate cancer cells. For these studies, both Cdk5 and p35 expression were determined. Expression of Cdk5 by immunoblotting was similar in LNCaP, C4-2B4, PC3 and PC-3MM2 cells (Figure 38). However, levels of p35 expression were increased in PC3 and PC3-MM2 cells compared to LNCaP and C4-2B4 cells (Figure 38). As p35 is known to activate the enzymatic activity of Cdk5, Cdk5 activity was assessed by ADP production through ADP-Glo Kinase Assay kit as described in Materials and Methods. Cdk5 activity was increased in PC3 (2-fold relative to LNCaP) and PC3-MM2 (2.5-fold relative to LNCaP; Figure 39), which correlated with the increased talin1 phosphorylation and β1 integrin activation in these cells. To determine whether Cdk5 was responsible for talin1 S425 phosphorylation in prostate cancer cells, Cdk5 was inhibited in PC3-MM2 cells. Inhibition of Cdk5 by the non-specific Cdk inhibitor, roscovitine, reduced talin1 phosphorylation in PC3-MM2 cells in a time-dependent manner (Figure 40), suggesting that Cdk5 might be responsible for talin1 phosphorylation. To examine directly whether Cdk5-mediated talin1 phosphorylation, Cdk5 was silenced in PC3-MM2 and C4-2B4 cells. The results
**Figure 38. Expressions of Cdk5 and p35 in prostate cancer cells.** Immunoblotting of Cdk5 and p35 using cell lysates from different prostate cancer cell lines.
Figure 39. Cdk5 kinase activity in prostate cancer cells. Cdk5 kinase activity in prostate cancer cells was measured by ADP production as described in Materials and Methods. The sh-Cdk5 knockdown cells were used as a negative control. *PC3 compared to LNCaP cells, $P < 0.0005$. **PC3-MM2 compared to LNCaP cells, $P < 0.0001$. 
**Figure 40. Talin1 phosphorylation was inhibited by roscovitine.** The Cdk inhibitor Roscoitine (20 µM) was added to PC3-MM2 cells. Cells were incubated and lysed at indicated times and immunoblotted for talin1 S425 phosphorylation and total talin1.
demonstrated talin1 phosphorylation was decreased by ~90% (Figure 41). To examine whether Cdk5 kinase activity was required for talin1 phosphorylation and not functioning as an adaptor protein, prostate cancer cells were transiently transfected with a plasmid directing the expression of a dominant-negative Cdk5 [143]. Expression of this dominant-negative mutant inhibited talin1 phosphorylation in both PC3-MM2 and C4-2B4 cells (Figure 42). These results demonstrated that Cdk5 activity, but not expression, promotes talin1 S425 phosphorylation in prostate cancer cells. Next I determined whether Cdk5 regulates β1 integrin activation. Silencing Cdk5 resulted in reduced β1 integrin activation in PC3-MM2 cells (Figure 43), suggesting that Cdk5 is required for β1 integrin activation. Next, to demonstrate that talin1 phosphorylation was mediated in Cdk5-induced β1 integrin activation, I silenced Cdk5 in PC3-MM2 cells in which talin1 was silenced and talin1WT or talin1S425D mutants were re-expressed (Figure 44A). As expected, silencing Cdk5 inhibited β1 integrin activation in talin1WT cells, but did not affect β1 integrin activation in phosho-mimicking talin1S425D cells (Figure 44B). These observations demonstrate that Cdk5 phosphorylates talin1 on S425, and its activity promotes β1 integrin activation.
Figure 41. Immunoblotting of Cdk5, pTalin S425 and total Talin1 in Cdk5 knockdown cells. Silencing of Cdk5 in PC3-MM2 or C4-2B4 cells by shRNA expression was described in Materials and Methods. Cells were infected with lentivirus and lysed after 48 hr.
Figure 42. Immunoblotting of pTalin S425, talin1 and Myc-tag in cells expressing dominant-negative Cdk5. Prostate cancer cells were transfected with Myc-Cdk5 wild-type and dominant-negative fusion construct as described in Materials and Methods, and talin1 phosphorylation was evaluated by immunoblotting.
Figure 43. Flow cytometric analysis of Cdk5 knockdown cells. FACS analysis of activated (9EG7) and total β1 integrins (MAR4) in Cdk5-silenced PC3-MM2 cells (top panels). Quantitation of fluorescence intensity is shown in the bottom panels. *sh-Cdk5 compared to sh-control, $P < 0.0005$. 
Figure 44. Talin1 phosphorylation was mediated in Cdk5-induced β1 integrin activation. (A) Immunoblotting of Cdk5. PC3-MM2 talin1WT and mutant-expressing cells were transducted with a sh-control vector or a sh-Cdk5 vector to silence Cdk5 expression. (B) Activated (9EG7) and total β1 integrins (MAR4) were measured by FACS analysis. *P < 0.005.
Chapter 7

Talin1 phosphorylation promotes prostate cancer bone metastasis \textit{in vivo}

Talin1 S425 phosphorylation is required for prostate cancer bone metastasis

To determine whether talin S425 phosphorylation promoted metastasis in vivo, I used two nude mouse models, one of which examined experimental metastasis, and the other, spontaneous metastasis. For these experiments, the sh-control, talin1-silenced, talin1 wild-type and mutated talin1-expressing PC3-MM2 cells were transduced with a vector directing luciferase expression. To determine bone metastasis of disseminated prostate cancer cells, intracardiac injections were performed (experimental metastasis). Following intracardiac injection of cells, bioluminescence imaging of mice was performed weekly. The presence of tumor in bone was examined by co-localization of X-ray and bioluminescence imaging. A representative image of mice inoculated with cells expressing each construct is shown in Figure 45. No tumor growth observed in any animal in the bone of talin1-silenced and talin1S425A groups. In contrast, 3 of 4 talin1WT mice and 4 of 5 talin1S425D mice demonstrated tumor growth. Tumors were noted in both legs of mice as expected in this model as well as in the heads of mice as previously observed [112, 173] (Figure 46). Thus, blocking talin1 S425 phosphorylation completely inhibited bone metastasis of disseminated PC3-MM2 cells in this model. For tumors that grew, tumor size was estimated by bioluminescence imaging 7, 14 and 21 days post-intracardiac injection. Exponential growth was observed in sh-control, talin1WT and talin1S425D groups, with no difference in talin1WT and talin1S425D observed (Figure 47).
Figure 45. Talin1 S425 phosphorylation promotes bone metastasis in vivo.
Luciferase-labeled PC3-MM2 sh-control, talin1-silenced, talin1\(^{WT}\) and mutant-expressing cells (1 \(\times\) 10\(^6\)) were injected into nude mice intracardially. After 21 days injection, bioluminescence and X-ray imaging of mice were performed. Images are superimposed to localize tumor growth. Arrows indicate tumor growth in femur/tibia junction.
*Talin1\(^{WT}\) compared with sh-talin1 or talin\(^{WT}\), \(P < 0.05\).
Figure 46. Bioluminescence imaging of luciferase-labeled talin1 mutants in mice after intracardiac injection. Luciferase-labeled PC3-MM2 sh-control, talin1-silenced, talin1\(^{WT}\) and mutant-expressing cells (1 × 10\(^6\)) were injected into nude mice intracardially. Shown in the figure is bioluminescence imaging of mice 21 days post-injection.
Figure 47. Tumor burden in the femur/tibia of mice following intracardiac injection.

Tumor growth in the femur/tibia was estimated by luciferase activity of cells (presented as photons/sec).
Next, I examined whether talin1 phosphorylation affects lymph node metastasis (spontaneous metastasis) of prostate cancer cells injected orthotopically into prostate. Mice were sacrificed when primary tumors reached similar sizes (Figure 48), and lymph node metastases were detected and counted by bioluminescence imaging (Figure 49). Lymph node metastases developed in all groups (PC3-MM2 cells expressing talin1\textsuperscript{WT}, talin1\textsuperscript{S425A} and talin1\textsuperscript{S425D} mutants; Figure 49); however, as shown in Table 1, significantly fewer lymph node metastases were observed in talin1\textsuperscript{S425A} mice compared to talin1\textsuperscript{WT} mice. Together, our results are consistent with talin1 S425 phosphorylation promoting metastasis.
Figure 48. Representative primary tumors when mice were sacrificed and examined for lymph node metastases. Luciferase-labeled PC3-MM2 talin1WT and mutant-expressing cells ($5 \times 10^5$) were orthotopically injected into the prostate. Mice were sacrificed when primary tumors reached similar sizes.
Figure 49. Representative bioluminescence imaging of lymph node metastases.
Luciferase-labeled PC3-MM2 talin1 WT and mutant-expressing cells (5 × 10^5) were orthotopically injected into the prostate. Bioluminescence imaging of lymph node metastases of PC3-MM2 talin1 WT and mutant-expressing cells in mice after removing the primary tumor.
Table 1. Effects of talin1 S425 phosphorylation on development of lymph node metastases of talin1-expressing PC3-MM2 cells injected intraprostatically

<table>
<thead>
<tr>
<th>Tumor cells</th>
<th>Tumor incidence</th>
<th>LN metastases incidence</th>
<th>Average # of LN metastases (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Talin1&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>6/6</td>
<td>6/6</td>
<td>3 (2-4)</td>
</tr>
<tr>
<td>Talin1&lt;sup&gt;S425A&lt;/sup&gt;</td>
<td>5/5</td>
<td>5/5</td>
<td>1.8 (1-2)&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Talin1&lt;sup&gt;S425D&lt;/sup&gt;</td>
<td>5/5</td>
<td>5/5</td>
<td>2.8 (2-3)</td>
</tr>
</tbody>
</table>

*ANOVA and Tukey’s test, talin1<sup>S425A</sup> compared to talin1<sup>WT</sup>, P < 0.01

Abbreviation: LN, lymph node.
Chapter 8

Talin1 phosphorylation in human prostate cancer

Talin S425 phosphorylation in human prostate cancer specimens

To determine clinical relevance of talin S425 phosphorylation in prostate cancer, I collaborated with Dr. Sue-Hwa Lin and Dr. Chien-Jui Cheng to perform immunohistochemical analysis on formalin-fixed human prostate tumor specimens. We examined talin S425 phosphorylation in human primary tumors, lymph node metastases and bone metastases. The talin S425 phospho-specific antibody used in the immunohistochemical analysis was validated using a blocking peptide (Figure 50). Representative images of normal glands, primary tumors, lymph node metastases and bone metastases are shown in Figure 51. Talin phosphorylation was not detected in normal or atrophied glands (Figure 51A), while 8 of 20 (40%) of primary tumors and 6 of 13 (46%) lymph node metastases stained positive (Figure 51B). Phospho-talin S425 was observed in 16 of 20 (80%) bone metastases, a significant increased compared to either primary prostate tumors ($P = 0.009$), or to lymph node metastases ($P = 0.043$; Figure 51B). There was no correlation between talin phosphorylation and Gleason score. These data demonstrate increased talin S425 phosphorylation is associated with prostate cancer bone metastasis.
Figure 50. **Validation of specificity of pTalin S425 antibody.** PC3-MM2 cells were pelleted to fix in 10% formalin as described in Materials and Methods. Sections of cell blocks were incubated with pTalin S425 antibody alone or simultaneously with corresponding blocking peptide.
Figure 51. Talin S425 phosphorylation in stages of human prostate cancer. (A) Immunohistochemical staining of talin S425 phosphorylation in primary tumor, lymph node metastases and bone metastases. Arrows indicate negative staining in atrophied glands, and an arrow head indicates negative staining normal glands within the primary tumor. Scale bar represents 100 µm. (B) Percentage of human specimens expressing talin S425 phosphorylation. *Primary tumor compared to bone metastases, $P < 0.005$. **Lymph node metastases compared to bone metastases, $P < 0.05$. 
Chapter 9

Discussion
Cancer metastasis is a complex process involving genetic and epigenetic alterations of cancer cells. While many oncogenes and tumor suppressor genes are associated with cancer metastasis, epigenetic alterations, such as constitutive activation of receptors on the plasma membrane, often deregulate signaling cascades that increase metastatic potential of cancer cells. Integrins are cell surface receptors for various extracellular matrix (ECM) proteins and can be activated through inside-out signaling [174]. When aberrantly activated in cancer cells, integrin signaling promotes multiple steps in the metastatic cascade, including increasing migration, invasion, adhesion and survival. In prostate cancer, activation of several integrins, including α5β1, α2β1 and αvβ3 integrins have functional consequences for prostate cancer metastasis [79, 80, 108, 111, 175-177]. By using a neutralizing anti-β1 integrin antibody (mAb 33B6), Dr. Sue-Hwa Lin’s group demonstrated that inhibiting β1 integrins decreases cell adhesion, migration, cell survival and downstream integrin signaling in vitro [112]. Furthermore, treatment of the anti-β1 integrin antibody, mAb 33B6, suppressed spontaneous metastasis of PC3-MM2 cells from the prostate to lymph nodes in SCID mice. Further, in a mouse model of experimental metastasis, delivery of mAb 33B6 suppressed bone metastasis of PC3-MM2 cells following intracardiac injection [112]. These data suggest that β1 integrin activation is critical for prostate cancer metastasis.

As described in the introduction, integrin activation occurs through an inside-out mechanism or an outside-in mechanism [91, 178]. As inside-out activation rapidly increases integrin affinity for ECM ligands, which in turn facilitates outside-in activation; thus, the two mechanisms of integrin activation are not mutually exclusive [91]. However,
the precise mechanism by which intracellular signaling induces inside-out integrin activation is still unknown. Although several intracellular proteins such as talin, kindlin and integrin-linked-kinase (ILK) are known to facilitate integrin activation, only talin can directly bind β integrins and induce activation [179]. Cellular talin in quiescent cells exists in a closed conformation that requires several molecular steps before assuming an open, integrin binding conformation. However, the regulatory mechanism for talin activation is unknown, and may occur through several different mechanisms including binding PIP2 [135, 136], and calpain cleavage [137]. A potential mechanism not previously studied is talin phosphorylation. Talin is heavily phosphorylated on 30 residues (serine, threonine and tyrosine). One potential important site of phosphorylation to prostate cancer is S425 [139, 143]. Talin S425 is phosphorylated by Cdk5 whose activity is controlled by the activator protein p35. Both Cdk5 and p35 are highly expressed in prostate cancer and associated with metastasis [155, 156]. The talin1 linker domain containing S425 binds the central portion of the rod domain [121], which masks the integrin-binding domain. Thus, phosphorylation of talin1 on S425 is predicted to promote an “open” conformation, thereby exposing the integrin-binding domain [138, 139]. However, the role of talin1 S425 phosphorylation in integrin activation was not determined prior to studies present in this dissertation.

Therefore, I proposed a hypothesis that talin1 S425 phosphorylation promotes β1 integrin activation. Because β1 integrins are receptors for collagens, the major component of bone, I focused on β1 integrin activation and its effect on bone metastasis. Furthermore, I also proposed that talin is phosphorylated by Cdk5 whose activity is increased due to
overexpression of p35 in metastatic prostate cancer cells. Next, I predicted that the increased β1 integrin activation through talin phosphorylation can promote anoikis resistance, adhesion and motility of cancer cells. Using various functional assays, I tested these effects of talin phosphorylation in vitro. Collectively, gaining of these metastatic properties through talin phosphorylation may increase metastatic potential of cancer cells. To test this possibility, I examined metastasis of talin mutants in experimental and spontaneous metastasis models in vivo. Finally, I determined the clinical relevance of ptalin S425 by immunohistochemistry in human prostate cancer samples.

**Activation of integrins in prostate cancer metastasis**

In Chapter 3, I addressed the question whether β1 integrin activation is increased through inside-out activation in metastatic prostate cancer cells. This question was based on the hypothesis that β1 integrins are activated in the metastatic prostate cancer cells, but not in low metastatic cells. To test this hypothesis, the levels of β1 integrin activation were examined in low metastatic (LNCaP, C4-2B4) and high metastatic (PC3, PC3-MM2) prostate cancer cells. The results from works in Dr. Sue-Hwa Lin’s laboratory to which I contributed, demonstrated that the constitutive activation of β1 integrins correlates with metastatic potential of established prostate cancer cell lines [112].

Next, to determine whether β1 integrins were activated through inside-out signaling in metastatic prostate cancer cells, cells were cultured in suspension without ECM ligands to prevent integrin activation by ligand binding or cell aggregation. In this condition, normal cells will undergo anoikis leading to cell death, but metastatic cancer
cells often develop resistance to anoikis by increasing integrin signaling [66]. Indeed, as shown in Chapter 3, β1 integrin activation correlated with anoikis resistance in prostate cancer cells (Figure 12, 13 and 14). Furthermore, knockdown of β1 integrins in PC3-MM2 cells increased sensitivity of cells to anoikis, which suggests that inside-out activation of β1 integrins induces signaling that increases anoikis resistance in metastatic prostate cancer cells. Therefore, I demonstrated in Chapter 3 demonstrated that β1 integrins are constitutively activated through inside-out activation in metastatic prostate cancer cells.

As described in the introduction, β1 integrins have been shown to promote cell properties critical to metastasis. My studies in Chapter 3 provide a molecular mechanism suggesting that β1 integrins are constitutively activated through inside-out signaling. Activation of β1 integrins in prostate cancer was also supported by immunohistochemical staining of downstream effector, FAK. Studies from Dr. Sue-Hwa Lin’s laboratory showed that FAK Y397 phosphorylation is increased in primary tumor and lymph node metastases compared to normal human tissues [112]. Because phosphorylation of FAK Y397 directly results from integrin activation, the results indicate that β1 integrin activation occurs during prostate cancer progression. However immunohistochemical staining of activated β1 integrins cannot be assessed in formalin-fixed clinical specimens as antibodies that recognize activated β1 integrins in these specimens do not exist. In fact, there are only several integrin antibodies that can specifically recognize activated conformation of β1 integrins in cells, such as mAb 9EG7 and 12G10. There are no commercial available antibodies for other activated integrin subunits, such as for β3.
Accordingly, the activation of other integrins associated with prostate cancer progression can only be assessed through activation of downstream signaling. Therefore, whether other β integrins are also activated in prostate cancer cells remains unknown.

Currently, whether β integrins have overlapping functions or distinct functions in promoting prostate cancer metastasis is unclear. As I demonstrated the effects of β1 integrin activation in this dissertation, β3 integrins have also been shown to increase adhesion, migration and invasion in prostate cancer cells [180]. In fact, for β integrins other than β1, many effects of β3 integrins, such as the ability to increase migration, appear to be overlap with β1 integrins. Thus, the functions of β3 integrin in promoting metastatic potential of cells may be similar to β1 integrins during prostate cancer metastasis. Despite integrins’ functions may be similar in metastasis, integrins may be activated in different compartments of tumor microenvironments.

In this dissertation, I only focused on the β1 subunit of integrins in prostate cancer metastasis. Nevertheless, the α subunits of integrins are also involved in prostate cancer metastasis. Prostate cancer cells with high expression of α6 integrins are more invasive in a mouse model system [181]. For αv subunit, overexpression of αv integrins increases cell adhesion and invasion in prostate cancer cells [177, 182]. In this dissertation, β1 integrins were activated to increase migration of cancer cells on collagen and promote bone metastasis, but which α subunits pairing with activated β1 integrins is unknown. Integrin α subunit functions to determine the binding specificity to ECM proteins. β1 integrins dimerizing with several α subunits, including α1, α2, α10 and α11, are able to
bind collagen (Figure 3). Among those α integrins, only the α2 subunit is overexpressed in prostate cancer and was shown to increase tumor growth in bone [79]. Therefore, α2β1 integrins could be one of the candidates to bind phosphorylated talin and become activated in this study.

**Talin1 phosphorylation regulates β1 integrin activation**

The inside-out activation of β1 integrins has received less attention in cancer research, as most studies focus on ECM-induced integrin signaling. The studies in Chapter 3 demonstrate that inside-out activation of β1 integrins promotes anoikis resistance, but the mechanism was unknown. Therefore, I determined the mechanism of inside-out activation of β1 integrins in metastatic prostate cancer cells. Previous studies demonstrate that β integrins are activated by talin binding [183]. In vertebrates, talin has two genes, *Tln1* and *Tln2*, that share 74% amino acid sequence similarity [184]. However, which talin is critical to activate β1 integrins in metastatic prostate cancer was not known. In Chapter 4, I demonstrated that talin1 is abundantly expressed in PC3-MM2 cells compared to talin2 (Figure 18). Knockdown of talin1 reduced β1 integrin activation and inhibited cell spreading, but this phenomenon was not observed in talin2 knockdown cells (Figure 19, 21 and 22). In adult mice, talin1 deficiency causes abnormal embryonic development and is embryonically lethal [185]. In contrast, talin2 knockout mice are viable and fertile [185]. Studies using specific antibodies targeting to talin1 and talin2 reveal different subcellular localization. Only talin1 is localized in the podosomes of adhesion structure, suggesting that talin1 has a direct function in cell-ECM interactions [184]. A study also showed that overexpression of talin1 increases integrin signaling in
Therefore, the results in Chapter 4 are consistent with other studies, suggesting that talin1 is critical for \( \beta_1 \) integrin activation and cannot be compensated by talin2 in metastatic prostate cancer cells.

However, when I examined the expression of talin1 in prostate cancer cells, the immunoblotting of talin1 confirmed the result from Dr. Sue-Hwa Lin’s laboratory and indicated that the levels of talin1 in prostate cancer cell lines did not change or correlate with metastatic potential of these cells (Figure 23). This result was unexpected because talin1 was shown to be overexpressed in metastases compared to primary tumor tissues in prostate and other cancers [114-116]. While we cannot explain why talin1 expression did not increase in metastatic prostate cancer cells, studies from other laboratories also showed inconsistent results with respect to talin1 expression. In those studies, most metastatic cancer cell lines did not show higher expression of talin1 compared with low metastatic cancer cell lines, even though talin1 is overexpressed in human metastases specimens [114, 115].

The similar levels of talin1 expression in prostate cancer cell lines indicated that a mechanism other than talin protein level may be responsible for activating \( \beta_1 \) integrins. Because talin1 phosphorylation on S425 controls cell migration associated with \( \beta_1 \) integrin signaling [143], I tested whether talin1 phosphorylation on S425 activates \( \beta_1 \) integrins. For this reason, I examined the phosphorylated talin1 by immunoblotting and immunoprecipitation of talin1 in prostate cancer cell lines. I again confirmed the data from Dr. Sue-Hwa Lin’s laboratory and demonstrated that talin1 phosphorylation is
correlated with metastatic potential of these cells (Figure 23 and 24). In addition, talin1 phosphorylation also correlated with β1 integrin activation in prostate cancer cells, which implies a possible role of talin phosphorylation in activating β1 integrin activation.

To directly test whether talin1 phosphorylation activate β1 integrins, I generated three plasmids encoding full-length human talin1 wild type, a non-phosphorylatable mutant, talin1\(^{S425A}\), and a phosphorylation-mimicking mutant, talin1\(^{S425D}\). To avoid effects from endogenous talin1, I transfected these plasmids into talin1-silenced PC3-MM2 and C4-2B4 cells and selected stable transfected cells (Figure 25). By using these cells in flow cytometric analysis and immunofluorescence staining, I demonstrated that talin1 phosphorylation activates β1 integrins in two prostate cancer cell lines (Figure 26, 27 and 28). Furthermore, talin1 phosphorylation initiated β1 integrin signaling required to increase cell adhesion, migration, invasion and survival in anoikis in prostate cancer cells, but did not affect cell proliferation (Chapter 5). To my knowledge, this is the first evidence that demonstrates talin phosphorylation is important in activation of β1 integrins.

In a previous study, Ratnikov et. al. revealed 30 phosphorylation sites on talin using mass spectrometry [139]. Although the function of talin phosphorylation was unknown, Ratnikov et. al. first proposed that talin phosphorylation may regulate integrin activation, because many residues in talin head where the integrin binding site located are heavily phosphorylated [139]. Later, the same group found that talin S425 phosphorylation functions to prevent ubiquitylation and degradation of the talin head [143]. Up to now, no other study has reported any function of talin phosphorylation on S425 or any other sites. The reason may due to lack of phospho-specific antibody to other talin phosphorylation
sites. Therefore, when new antibodies are generated, researchers may able to study the functions of other phosphorylation sites on talin.

β1 integrins contain several alternatively spliced variants as described in the introduction. While the β1A variant was shown to act as a stimulator of cell proliferation, the β1C variant acted as an inhibitor for integrin signaling. Previous studies have demonstrated that talin binds to only the β1A and β1D isoforms, but not to β1C integrins [186]. Therefore, talin phosphorylation is likely to activate β1A or β1D integrins in prostate cancer cells, but this possibility remains to be determined in future studies.

In this dissertation, I used full-length human talin1, because studies have shown that only full-length talin is capable of forming focal adhesions and initiating proper adhesion-dependent signaling [187]. Talin can be cleaved by calpain to yield a 50-kDa N-terminal head domain and a 220-kDa C-terminal rod domain. In fact, most studies only used the talin head because it can directly bind to the β subunit and is sufficient to activate integrins. Previously, Huang et. al. showed that the talin head phosphorylation on S425 prevents ubiquitylation and protein degradation of the talin head [143]. However, in my dissertation, non-phosphorylatable talin1^{S425A} mutant was not degraded as the full-length protein level was similar compared to that of talin1 wild type or talin1^{S425D} mutants (Figure 25). Perhaps, ubiquitylation of non-phosphorylated talin only occurs after cleavage of the talin head, which remains to be determined.
As described in the Introduction, talin has to attain an open conformation to bind the cytoplasmic tails of β integrins [130, 188]. Based on previous structural analyses, full-length talin1 exists in a compact, auto-inhibited conformation with its integrin-binding domains masked [121]. Phosphorylation of talin has been proposed to relieve the inhibitory conformation [138, 139]. In this dissertation, talin1 phosphorylation was required to activate β integrins as shown in Chapter 4. Furthermore, in Chapter 5, talin1 phosphorylation induced β1 integrin signaling leading to changes of cell properties related to the functions of β1 integrins. Therefore, phosphorylated talin1 may present as an active and open conformation in order to activate integrins. However, whether phosphorylation induces a conformational change of talin was not studied in this discussion and is still unknown. Besides phosphorylation, several integrin-associated proteins, including kindlin and RIAM [189, 190] also have been implicated in promoting an open conformation of talin. Since talin also functions as an adaptor protein, phosphorylation of talin1 may recruit those integrin-associated proteins to facilitate integrin activation. Therefore, future studies of protein association with phosphorylated talin1 are warranted to provide an understanding of integrin activation, and whether these proteins or lipids binding to talin are required for its “open” conformation.

**Talin1 phosphorylation by Cdk5/p35**

Previous studies from Huang et. al. showed that talin1 is phosphorylated by Cdk5 in neural cells [143]. In fact, Cdk5 has been found to regulate cell adhesion and migration in non-neuronal cells [158, 159]. A study using Cdk inhibitor, roscovitine, was able to inhibit Cdk5 activity and β1 integrin activation in keratinocytes, which suggests that
Cdk5 activity is associated with β1 integrin activation [159]. However, whether talin1 is phosphorylated by Cdk5 in metastatic prostate cancer cells was unknown. Therefore, in Chapter 6, I hypothesized that Cdk5 activity is increased in metastatic prostate cancer cells leading to talin1 phosphorylation. First, I demonstrated that expression of p35 is increased in PC3 and PC3-MM2 cells and correlated with increased Cdk5 kinase activity in prostate cancer cells (Figure 38 and 39). This result is in agreement with immunohistochemical analyses of Cdk5 and p35 from other groups. Studies showed that when compared with human normal tissues, Cdk5 and p35 are overexpressed in primary prostate tumors compared with normal tissues [155]. Furthermore, Strock et. al. demonstrated that overexpression of dominant-negative Cdk5 in rat prostate cancer cells inhibits lung metastasis in subcutaneous xenograft mice [156], which suggests that Cdk5 activity is required to promote metastatic potential of prostate cancer cells. A recent study demonstrated that Cdk5 and p35 are clustered with talin and β1 integrins into lamellipodia during cell spreading [160]. In agreement with this result, I demonstrated that Cdk5 activity is required for talin1 phosphorylation, and for β1 integrin activation (Figure 40-43). Moreover, knockdown of Cdk5 in phosphorylation-mimicking talin1S425D cells does not significantly decrease β1 integrin activation, compared to talin1WT, which suggests that talin1 phosphorylation is mediated in Cdk5-induced β1 integrin activation (Figure 44). Therefore, increased p35 expression promotes Cdk5 activity in metastatic prostate cancer cells, which further phosphorylates talin1 leading to β1 integrin activation.

Although the mechanism by which p35 is upregulated in metastatic prostate cancer cells in this studies is unknown, defining this mechanism will provide further
insights into how the p35/Cdk5/talin/integrin activation axis contributes to metastasis. In rat neuroblastoma cells, TGF-β signaling has been shown to increase Cdk5 kinase activity by upregulating p35 protein expression through induction of the transcription factor Egr-1 [191]. In prostate cancer, TGF-β serves as a tumor promoter [192], and that Egr-1 is also overexpressed in aggressive prostate cancer cells [193]. TGF-β also enhances EMT, which increases the migratory capability of prostate cancer cells. One logical possibility for p35 overexpression in metastatic prostate cancer cells is through increased TGF-β and/or Egr-1 signaling. However, future studies are required to determine this potential mechanism of increased p35 expression.

**Talin1-phosphorylation in bone metastasis**

Studies in Chapter 7 assessed the effects of talin1 phosphorylation in prostate cancer metastasis *in vivo*. Using these PC3-MM2 talin1 mutants with the luciferase expressed, I tested whether talin1 phosphorylation is required for bone metastasis (experimental metastasis model) and lymph node metastasis (spontaneous metastasis model). In the bone metastasis model, imaging analysis revealed no tumor growth in legs of mice injected with talin1S425A cells (Figure 45-47). Although tumors also appeared in the mouse heads, this phenomenon is common in this model and seems not relate to bone metastasis [173]. These results suggest that talin1 phosphorylation is required for bone metastasis, possibly by increasing the interaction of β1 integrins on cells with bone stroma. During prostate cancer bone metastasis, integrin heterodimerization usage is altered to increase interaction with ECM in the bone matrix [194]. Several α1 integrin family proteins on prostate cancer cells have been shown to interact with both the bone
matrix and stromal cells. For example, expression of $\alpha_5\beta_1$ integrins facilitates adhesion and spreading of PC3 cells on fibronectin [107]. Blocking $\alpha_5\beta_1$ integrins by monoclonal antibodies inhibits adhesion of DU-145 prostate cancer cell to bone marrow stromal cells [107, 110]. Another $\beta_1$ integrin family protein, $\alpha_2\beta_1$, has been shown to increase migration and adhesion of LNCaP cells on collagen I, and promotes bone metastasis in vivo [79, 80]. Similar to $\alpha_5\beta_1$, $\alpha_2\beta_1$ was found to mediate adhesion of malignant tumor-derived prostate epithelial cells to human bone marrow stroma, which can also be inhibited by antibodies targeting $\alpha_2\beta_1$ [111]. These results suggest that interactions between tumor cells and bone matrix/stroma through $\beta_1$ integrins are critical for tumor cell colonization in the bone. Since talin1 phosphorylation leads to constitutive activation of $\beta_1$ integrins in metastatic prostate cancer cells, my work suggests that talin1 phosphorylation promotes bone metastasis through increasing $\beta_1$ integrin activation on cancer cells thus leading to increased interaction with bone stroma.

To understand whether talin1 phosphorylation is specific to bone metastasis, I also examined roles of talin1 S425 phosphorylation in spontaneous lymph node metastasis following orthotopic implantation of talin1 mutant cells. In contrast to the effects of talin1 mutants on bone colonization, lymph node metastases were observed in mice injected with cells expressing wild-type and talin1$^{S425A}$ that is unable to activate $\beta_1$ integrins (Figure 48 and 49). Although the number of lymph node metastases in talin1$^{S425A}$ was decreased, incidence of lymph node metastasis was not changed (Table 1). Our results were consistent with a recent study of Barthel et al. who found that $\beta_1$ integrins were highly activated in prostate cancer cells derived from bone metastasis, but
not in LNCaP variants selected form lymph node metastasis [175]. Therefore, these results imply that β1 integrins are less important to lymph node metastasis than to bone metastasis [175]. However, my results do support that β1 integrin activation does, in general, promote prostate cancer metastasis.

The results in Chapter 7 demonstrated that inside-out β1 integrin activation is required for prostate cancer bone metastasis. As described in the introduction, integrin signaling through inside-out and outside-in direction are not separate events in cells, because the two mechanisms of integrin activation interact with one another. Therefore, outside-in β1 integrin signaling through binding ECM proteins may also contribute to bone metastasis in vivo. Previous studies from Dr. Sue-Hwa Lin’s laboratory demonstrated that blocking of β1 integrin binding with ligands using an anti-β1 integrin antibody (mAb 33B6) decreased cell adhesion on ECM matrix. Treatment of mAb 33B6 reduced metastatic tumor growth in bone in vivo [112]. In addition, from my studies in Chapter 5, β1 integrin activation through talin phosphorylation increased ECM binding as cells strongly adhered on collagen-coated matrix. These data suggest that integrin outside-in signaling through binding ECM proteins is equally important for bone metastasis. Prostate cancer cells with activated β1 integrins through talin phosphorylation are likely binding bone ECM proteins, which facilities colonization of cancer cell in the bone.

Finally, to support the finding of talin1 phosphorylation in prostate cancer bone metastasis, I collaborated with Dr. Sue-Hwa Lin and Dr. Chien-Jui Cheng to examine
talin phosphorylation in human tissues. Using an antibody specific to phospho-talin S425, we demonstrated that talin phosphorylation is more highly expressed in bone metastases than in lymph node metastases or primary tumors, with no talin phosphorylation observed in normal or atrophied (non-tumorigenic) glands (Figure 51). Therefore, these data are consistent with our in vivo studies and provide a clinical evidence that talin S425 phosphorylation is critical for prostate cancer bone metastasis.

**Integrins, talin1 and Cdk5 as therapeutic targets**

*Integrins*

Targeting integrins using inhibitory antibodies is a logical approach to inhibit aberrant integrin signaling in cancer cells. In studies from Dr. Sue-Hwa Lin’s laboratory, using neutralizing antibody mAb 33B6 successfully inhibited metastasis of prostate cancer cells in mice [112]. In fact, other functional blocking β1 integrin antibodies such as mAb AIIB2 and JB1a have been shown to inhibit tumor growth and metastasis in breast cancer [195, 196]. Therefore, targeting β1 integrins by antibodies has great potential to affect tumor growth and/or metastasis in prostate cancer. Currently, many integrin antibodies are being developed for several diseases including solid tumors [197]. For example, the M200 Volociximab, an anti-α5β1 integrin antibody, was used in a clinical trial (phase II) to assess the effect to patients with various solid tumors [198]. A panel of integrin antibodies targeting αvβ3, including MEDI-522 (phase II), CNTO95 (phase II) and Cilengitide (phase II) was evaluated for the effects of integrin-mediated angiogenesis in patients with metastatic CRPC [199]. Results from these studies will hopefully lead to better treatment strategies for prostate cancer. A concern of integrin-
targeting therapy is the potential overlapping in integrin functions; therefore biomarkers that identify which integrins are activated to target would be important to optimal anti-integrin therapy.

**Talin**

Although talin1 may not be a druggable target for prostate cancer, talin1 phosphorylation may be used as a biomarker for integrin activation in prostate cancer bone metastases. From the results in chapter 8, talin is highly phosphorylated in prostate cancer bone metastases compared with lymph node metastases or prostate tumors. Thus, talin phosphorylation may be a useful biomarker for bone metastasis. Recently, a study found that talin1 is presented in the serum samples from patients with hepatocellular carcinoma, and the serum level of talin1 is significantly higher compared to the healthy group [200]. Phosphorylated talin1 may also present in the serum from prostate cancer patients with bone metastasis. Another possible approach to detect phosphorylated talin1 is to isolate circulating tumor cells (CTCs) from prostate cancer patients. Although whether talin is phosphorylated in CTCs is unknown, the talin1S425D mutant cells were resistant to anoikis as shown in chapter 5, which suggests that talin1 phosphorylation may be required for CTCs to survive in the circulation. Therefore, talin1 phosphorylation in prostate CTCs may be a potential biomarker for prognosis in patients.

**Cdk5**

As increased Cdk5 activity was showed responsible for talin1 phosphorylation in this study, targeting talin phosphorylation through inhibiting Cdk5 activity will be a
reasonable strategy to develop prostate cancer therapy. In chapter 6, talin1 phosphorylation was inhibited by Cdk inhibitor, roscovitine (Figure 40). Therefore, treatment of a selective Cdk5 inhibitor should inhibit β1 integrin activation in prostate cancer cells as well as cancer metastasis. However, no selective Cdk5 inhibitor has been developed to date. The (R)-ros covitine or Selicitelib/CYC202 is current being evaluated in clinical trials for antitumor activity in advanced solid tumors [201]. Although roscovitine has an inhibitory effect on Cdk5 activity, it can also inhibit other Cdns, including Cdk1 and Cdk2. Therefore, developing a more selective Cdk5 inhibitors would be necessary to explore the effect of Cdk5 in prostate cancer metastasis.

**Future perspectives**

This dissertation clarified the role of many proteins implicated in prostate cancer metastasis. While p35, Cdk5, talin and integrins have all been associated separately in increasing metastatic potential, my work is the first to put them together in a single pathway. This dissertation further demonstrated the role of talin phosphorylation as a regulator for β1 integrin activation (Figure 52). However, many questions remain to be determined in the future. Bone microenvironment is known to facilitate cancer cell colonization and tumor growth. This dissertation demonstrated that β1 integrin activation on cancer cells facilitates metastasis of prostate cancer cells in the bone. Therefore, future studies should focus on determining the interaction between cancer cells and the bone microenvironment. Generally, studies are needed to determine what is the role of β1 integrin activation in the bone microenvironment during prostate cancer metastasis. Do β1 integrins on cancer cells affect bone cells or bone remodeling? On the other hand, do
bone cells affect β1 integrin activation on cancer cells? While α and β subunits of integrins control ligand specificity, it may be necessary to identical which α subunit is pairing with β1 integrins. In addition, whether β1 integrins heterodimerize with particular α subunits in different stages of prostate cancer progression? The answers to these questions will provide important information for understanding prostate cancer bone metastasis.

In this dissertation, talin phosphorylation promoted integrin activation leading to metastasis. However, cancer metastasis involves multiple mechanisms that are required to change cell properties, and integrin activation could be just one of them. Another mechanism that is possibly involved in integrin activation is epithelial plasticity. As mentioned in the introduction, EMT is required for increasing cancer cell motility. In our results, talin1 phosphorylation increased migration and invasion in C4-2B4 cells, which implies that EMT may also occur during integrin activation. In fact, studies have shown that expression of β1 integrins enhances EMT in non-small cell lung cancer cells [202]. In breast cancer cells, αvβ1 and αvβ6 integrins also induce EMT after treated with an ECM protein, tenascin. Therefore, whether talin1 phosphorylation can induce ECM should be determined in the future studies.

A difficult question regarding epithelial plasticity is the role of integrin activation in MET. If β1 integrin activation can induce EMT in the primary tumors, cancer cells in bone metastases should have MET and inactive β1 integrins. However, according to our clinical date, talin was mostly phosphorylated in the bone metastases, suggesting that β1
integrins are still activated in these tissues. Therefore, further studies should determine whether β1 integrins on cancer cells are not required for MET to occur. Furthermore, MET can explain why cancer cells in the metastatic sites show similar epithelial phenotypes as epithelial tumor at the primary site. Therefore, other integrins which are highly expressed in the primary site may also show higher expression in the bone metastases.

Because talin1 has been shown to activate β3 integrins [124], whether talin1 phosphorylation can activate β3 integrins in prostate cancer cells also remains to be determined. In addition to the roles of β1 integrins, β3 integrins may also have roles in tumor growth in the bone. β3 integrins specifically bind to bone ECM proteins such as osteopontin and bone sialoprotein. In my studies, talin1 phosphorylation did not affect cell proliferation, suggesting that β1 integrins may be not responsible for prostate cancer cell proliferation (Figure 30). Thus, inside-out activation of β3 integrins may increase the binding of these bone ECM proteins and promote tumor growth in the bone.

For talin phosphorylation, a difficult question to answer is how phosphorylation of talin structurally induces β1 integrin activation? Because talin1 has to be activated prior to activate β1 integrins, it is plausible that phosphorylation may induce a conformational change in talin1 leading to talin1 activation. To test this hypothesis, protein structure modeling analyses may be useful to predict the conformational change of phosphorylated talin1. However, the interaction between phosphorylated talin1 and β1
integrins may be more complex than we thought, and these analyses are likely to be difficult due to the other 30 phosphorylation sites also present in talin.

Although this dissertation focused on motility and survival of cancer cells, integrins affect other properties of cancer cells. One of the major problems in cancer therapy is developing drug resistance in cancer cells. Treatments of kinase inhibitors for prostate cancer usually develop drug-resistance, which has been shown due to increased β1 integrin signaling [84]. Indeed, studies have shown that β1 integrin signaling is increased to activate PI3K/Akt survival pathway for developing resistance to cytotoxic chemotherapies [86]. In addition, β1 integrin expression is increased upon exposure to ionizing radiation, which has been suggested to be a mechanism for therapy resistance [87]. From this dissertation, β1 integrin signaling was shown constitutively activated in metastatic prostate cancer cells. Therefore, β1 integrins may play an important role in therapy resistance and may control recurrence of metastasis. Thus, targeting β1 integrins together with kinase inhibitor may be a better strategy to treat prostate cancer.

Furthermore, cancer cells metastasized into the distant organs usually enter into a period of dormancy or senescence and form micrometastases. Studies have shown that β1 integrin signaling is required to break the dormancy in cancer cells [84]. Therefore, talin1 phosphorylation and β1 integrin activation may play roles in escaping senescence and resuming proliferation of cancer cells leading to tumor growth in the bone.
Conclusions

In this dissertation, I demonstrated the importance of talin1 S425 phosphorylation for inside-out activation of β1 integrins and promotion of prostate cancer bone metastasis. In metastatic prostate cancer cells, β1 integrins are constructively activated through inside-out integrin activation, which correlated with metastatic potential of cells. The levels of talin1 S425 phosphorylation, but not total talin1, also correlate with metastatic potential of prostate cancer cell lines. Besides, talin phosphorylation is significantly increased in human bone metastases compared to primary tumors or lymph node metastases. Using phosphorylation-mimicking and non-phosphorylatable talin1 mutants, I demonstrated that talin1 phosphorylation is required for β1 integrin activation in metastatic prostate cancer cells. Talin1 phosphorylation on S425 plays an important role in cell adhesion, migration, invasion, and anoikis in vitro and metastasis of prostate cancer cells to bone in vivo. Mechanistically, I demonstrated that Cdk5 activity is increased in highly metastatic prostate cancer cells as compared to low metastatic prostate cancer cells. In addition, increased Cdk5 activity in metastatic prostate cancer cells is due to overexpression of p35. Furthermore, talin1 phosphorylation is required for Cdk5-induced β1 integrin activation. In summary, I identified a novel mechanism for β1 integrin activation through Cdk5-mediated talin1 S425 phosphorylation in metastatic prostate cancer cells (summarized in Figure 52). Therefore, I conclude that integrins, talin1, Cdk5 and p35 could be potential targets for development of novel therapies for prostate cancer bone metastasis.
Figure 52. Model of β1 integrin activation by talin1 S425 phosphorylation in metastatic prostate cancer cells. (1) Expression of p35 is increased in tumor cells of high metastatic potential, which increases Cdk5 activity leading to talin1 phosphorylation. Phosphorylation of talin1 on S425 may induce a conformational change leading to talin1 activation. Binding of additional proteins may facilitate this conformational change. (2) Phosphorylated talin1 binds to the cytoplasmic tail of β1 integrin and (3) increases β1 integrin activation and (4) promotes integrin signaling, leading to increased survival, adhesion, motility and metastatic potential in prostate cancer cells.
Appendix
Propidium iodide staining for FACS Analysis

1. Trypsinize cells and centrifuge cell suspension.
2. Add 5 ml of PBS and centrifuge cells at 500 ×g for 5 min.
3. Add 5 ml of 70% ethanol (cold). Make sure that cells are dispersed in the ethanol and not clumpy. If clumpy, gently disperse cells using a pipetman.
4. Leave cells in ethanol at 4°C overnight for fixing.
5. Spin cells at 500 ×g for 5 min and resuspend cells in 5 ml PBS.
6. Resuspend cells in 50 µl PBS, plus 3.3 µL RNAse (30 mg/mL) and incubate at 4°C for 5 min.
7. Add 450 µl PBS and 25 µl PI (1 mg/ml). Incubate at 4°C for 30 min.
8. Analyze immediately after incubation at 4°C, in order to prevent clumping of the cells.
Subcloning talin1 from pEZ-M29 to pWPXL-GFP plasmid

1. PCR amplify *talin1* DNA fragment using pEZ-M29-talin1 plasmid as a temple, primer set 5’GCTGTACAAAGTCCGGCATGGTTGCACTTTTCACTGAAGA3’ (primer #1) and 5’GAGATCTGAGTCCGGACAAGAAAGCTGGGTTGC3’ (primer #2) and Phusion hot start II DNA polymerase (Thermo Scientific).

2. PCR reaction setup:
   - Water: 31µl
   - GC buffer: 10µl
   - 10 mM dNTP: 1µl
   - 10 µM primer #1: 2.5µl
   - 10 µM primer #2: 2.5µl
   - 50 ng DNA: 2 µl
   - DNA polymerase: 1µl
   - Total volume: 50 µl

3. Cycling: after initial denaturation, 98°C 10 sec, 68°C 10sec, 72°C 1 min: 56 sec for 29 cycles then 72°C 5 min.

4. Digested pWPXL-GFP using BspE1 enzyme (Thermo Scientific) at 55°C for 30 min, and then purify DNA fragments of full length pWPXL-GFP.

5. Incubate 4 µl partial-digested pWPXL-GFP and 4 µl talin1 PCR product with 2 µl of 5× In-Fusion HD enzyme premix at 50°C for 15 min.

6. Continue to the transformation procedure according to manufacturer’s instructions.
1. PCR amplify whole pEZ-M29-talin1 plasmid using complementary mutagenic primer set 5′GACTCAGTGGCCCCCAAAAAGTCAACAGTCCTG3′ and 5′GGGCCCCACTGAGTCCTCCAGCATAGACTCCTC3′ (underline denotes mutated site) for talin1\textsuperscript{S425A} mutation. For talin1\textsuperscript{S425D} mutation, using mutagenic primer set: 5′GACTCAGTGGACCCCAAAAAGTCAACAGTCCTG3′ and 5′GGTCCACTGAGTCCTCCAGCATAGACTCCTC3′.

2. PCR reaction setup:
   - Water 31µl
   - GC buffer 10µl
   - 10 mM dNTP 1µl
   - 10 µM primer #1 2.5µl
   - 10 µM primer #2 2.5µl
   - 50 ng DNA 2 µl
   - DNA polymerase 1µl
   - Total volume 50 µl

3. Cycling: after initial denaturation, 98℃ 10 sec, 59℃ 10 sec, 72℃ 3 min: 30 sec for 29 cycles then 72℃ 10 min.

4. To recircularize plasmid, incubate 1 µl talin1\textsuperscript{S425A/D} PCR product with 2 µl of 5× In-Fusion HD enzyme premix and 7 µl water at 50℃ for 15 min.

5. Continue to the transformation procedure according to manufacturer’s instructions.

6. The pEZ-M29-talin1\textsuperscript{S425A/D} plasmids were used as temples to PCR amplify full length talin1\textsuperscript{S425A/D} fragments and subclone into BspEI partial-digested pWPXL-GFP vector according to the subcloning protocol.
Plasmid: pEZ-M29-talin1
OmicsLink Expression Clone of EX-R0040-M29
Catalog No.: EX-R0040-M29
Accession No.: AF078828 ORF Length: 7626 bp
Whole Plasmid Size: 13791 bp
Description: Homo sapiens talin mRNA, complete cds.
Vector: pEZ-M29 Antibiotic: Ampicillin
Stable Selection Marker: Neomycin
**Plasmid: pWPXL-GFP**

Addgene plasmid: 12257

Whole Plasmid Size: 10510 bp

Antibiotic: Ampicillin

Stable Selection Marker: GFP
Plasmid: pWPXL-GFP-talin1\textsuperscript{WT}
Whole Plasmid Size: 18172 bp
Antibiotic: Ampicillin
Stable Selection Marker: GFP

Plasmid: pWPXL-GFP-talin1\textsuperscript{S425A}
Mutation on 5539-5541 (TCC to GCC) in pWPXL-GFP-talin1\textsuperscript{WT} plasmid to replace serine with alanine on 425 amino acid.

Plasmid: pWPXL-GFP-talin1\textsuperscript{S425D}
Mutation on 5539-5541 (TCC to GAC) in pWPXL-GFP-talin1\textsuperscript{WT} plasmid to replace serine with aspartic acid on 425 amino acid.
ADP-Glo™ Activity Assay Protocol

Reaction Components

CDK5/p25 Kinase Enzyme System
(Promega, Catalog #: V3231)
CDK5/p25, Active, 10μg (0.1μg/μL)
Histone H1, 1ml (1mg/ml)
Reaction Buffer A (5X), 1.5ml
DTT solution (0.1M), 25μl

ADP-Glo™ Kinase Assay Kit
(Promega, Catalog #: V9101)
Ultra Pure ATP, 10 mM (0.5ml)
ADP, 10 mM (0.5ml)
ADP-Glo™ Reagent (5ml)
Kinase Detection Buffer (10ml)
Kinase Detection Substrate [Lysophilized]

Reaction Buffer A (5X)
200mM Tris-HCl pH 7.5, 100mM MgCl2 and 0.5 μg/μL BSA.

Assay Protocol

The CDK5/p25 assay is performed using the CDK5/p25 Kinase Enzyme System (Promega; Catalog #: V3231) and ADP-Glo™ Kinase Assay Kit (Promega; Catalog #: V9101). The CDK5/p25 reaction utilizes ATP and generates ADP. Then the ADP-Glo™ Reagent is added to simultaneously terminate the kinase reaction and deplete the remaining ATP. Finally, the Kinase Detection Reagent is added to convert ADP to ATP and the newly synthesized ATP is converted to light using the luciferase/luciferin reaction. For more detailed protocol regarding the ADP-Glo™ Kinase Assay, see the technical manual #TM313, available at www.promega.com/tbs/tm313/tm313.html.

Step 1. Thaw the ADP-Glo™ Reagents at ambient temperature. Prepare Kinase Detection Reagent by mixing 1ml of 2X Buffer by combining 400μl Reaction Buffer A, 1μl DTT and 399μl of dH2O.

Step 2. Prepare 1ml of 2X Buffer by combining 400μl Reaction Buffer A, 1μl DTT and 399μl of dH2O.

Step 3. Prepare 1ml of 250μM ATP Assay Solution by adding 25μl ATP solution (10mM) to 500μl of 2X Buffer and 475μl of dH2O.

Step 4. Prepare 1ml of 0.25mM solution of substrate Hpepride.

Step 5. Prepare diluted CDK5/p25 in 1X Buffer (diluted from 2X buffer) as outlined in sample activity plot. (Note: these are suggested working dilutions and it is recommended that the researcher perform a serial dilution of Active CDK5/p25 for optimal results).

Step 6. In a white 96-well plate (Coming Cat #: 3912), add the following reaction components bringing the initial reaction volume up to 20μl:

Component 1. 10μl of diluted Active CDK5/p25
Component 2. 5μl of 0.25mM solution of substrate Hpepride
Component 3. 5μl of 2X Buffer

Step 7. Set up the blank control as outlined in step 6, excluding the addition of the substrate. Replace the substrate with an equal volume of distilled H2O.

Step 8. At the same time as the CDK5/p25 kinase reaction, set up an ATP to ADP conversion curve at 50μM ATP/ADP range as described in the ADP-Glo™ Kinase Assay technical Manual #TM313.

Step 9. Initiate the CDK5/p25 reactions by the addition of 5μl of 250 μM ATP Assay Solution thereby bringing the final volume up to 25μl. Shake the plate and incubate the reaction mixture at 30°C for 15 minutes.

Step 10. Terminate the reaction and deplete the remaining ATP by adding 25μl of ADP-Glo™ Reagent. Shake the 96-well plate and then incubate the reaction mixture for another 40 minute at ambient temperature.

Step 11. Add 50μl of the Kinase Detection Reagent, shake the plate and then incubate the reaction mixture for another 30 minute at ambient temperature.

Step 12. Read the 96-well reaction plate using the Kinase-Glo™ Luminescence Protocol on a GloMax® Microplate Luminometer (Promega; Cat #: E6501).

Step 13. Using the conversion curve, determine the amount of ADP produced (nmol) in the presence (step 6) and absence of substrate (step 7) and calculate the kinase specific activity as outlined below. For a detailed protocol of how to determine nmols from RU's see ADP-Glo™ Applications Database at http://www.promega.com/applications/cellularanalysis/celsignaling.htm

Kinase Specific Activity (SA) (nmol/min/mg)

|ADP (step 6) – ADP (step 7)| in nmol | (Reaction time in min) | Enzyme amount in mg|

FOR IN VITRO RESEARCH PURPOSES ONLY. NOT INTENDED FOR USE IN HUMAN OR ANIMALS.
Fixed cell pellet and embedded in paraffin

1. Pellet cells by centrifugation 400 ×g for 10 minutes at room temperature.
2. Resuspend cells in 10 ml 10% buffered Formalin, and fix for two hours at room temperature.
3. Pellet cells by centrifugation 400 ×g for 10 minutes at room temperature.
4. Resuspend cells in 300 µl of PBS in a microtube.
5. Prepare 3% low melting point (LMP) agarose in PBS; weigh before microwaving.
6. Melt in microwave and replace lost water.
7. Place melted agarose mixture in water bath at 65 °C. Allow agarose to equilibrate to 65 °C (about 30 minutes).
8. Heat microtube with cells in it to 65 °C for no more than 10 minutes.
9. Working in water bath, add 600 µl of agarose mixture to cells, and mix by pipetting up and down.
10. Immediately transfer microtube to a room temperature microcentrifuge, and spin at 2000 ×g for five minutes.
11. Place microtube on ice, remove gelled pellet, and trim of excess agarose.
12. Wrap pellet in lens paper, place in a paraffin processing cassette, and process to paraffin block.

http://www.umassmed.edu/morphology/protocols/fixcellpellet.aspx
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Cancer Res 54: 2577-2581


Vita

Jung-Kang Jin was born in Keelung, Taiwan, the son of Tzu-Shen Jin and Shiou-Fang Han. He graduated from Er Xin High School in Keelung, Taiwan before serving in the military for two years. He received a Bachelor of Science in botany from National Chung-Hsing University, Taiwan in 2002. For the next two years, he worked as a Research assistant in the Department of Botany at the National Chung-Hsing University, Taiwan. In 2004, he entered San Diego State University, San Diego, California, and then received a Master of Science in molecular and biochemistry science in 2007. Then he worked at Roche Pharmaceuticals, Palo Alto, California as a research associate. In 2008, he entered the Graduate School of Biomedical Sciences at the University of Texas Health Science Center at Houston, Texas.