

# Prostate cancer metastasis: roles of recruitment and reprogramming, cell signal network and three-dimensional growth characteristics

Shabnam Ziaee<sup>1\*</sup>, Gina Chia-Yi Chu<sup>1\*</sup>, Jen-Ming Huang<sup>1\*</sup>, Shirly Sieh<sup>1,2</sup>, Leland W. K. Chung<sup>1,3</sup>

<sup>1</sup>Department of Medicine, Samuel Oschin Comprehensive Cancer Center, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA; <sup>2</sup>Australian Prostate Cancer Research Centre, Brisbane, Queensland 4102, Australia; <sup>3</sup>Department of Surgery, Samuel Oschin Comprehensive Cancer Center, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA

*Contributions:* (I) Conception and design: S Ziaee, GC Chu, JM Huang, LW Chung; (II) Data analysis and interpretation: S Ziaee, S Sieh; (III) Manuscript writing: S Ziaee, GC Chu, JM Huang, LW Chung; (IV) Final approval: All authors.

\*These authors contributed equally to this work.

*Correspondence to:* Leland W. K. Chung, Ph.D. Uro-Oncology Research, Department of Medicine and Surgery, Cedars-Sinai Medical Center, 8750 Beverly Blvd. Atrium 103, Los Angeles, CA 90048, USA. Email: Leland.chung@cshs.org.

**Abstract:** Prostate cancer (PCa) metastasizes to bone and soft tissues, greatly decreasing quality of life, causing bone pain, skeletal complications, and mortality in PCa patients. While new treatment strategies are being developed, the molecular and cellular basis of PCa metastasis and the “cross-talk” between cancer cells and their microenvironment and crucial cell signaling pathways need to be successfully dissected for intervention. In this review, we introduce a new concept of the mechanism of PCa metastasis, the recruitment and reprogramming of bystander and dormant cells (DCs) by a population of metastasis-initiating cells (MICs). We provide evidence that recruited and reprogrammed DCs gain MICs phenotypes and can subsequently metastasize to bone and soft tissues. We show that MICs can also recruit and reprogram circulating tumor cells (CTCs) and this could contribute to cancer cell evolution and the acquisition of therapeutic resistance. We summarize relevant molecular signaling pathways, including androgen receptors (ARs) and their variants and growth factors (GFs) and cytokines that could contribute to the predilection of PCa for homing to bone and soft tissues. To understand the etiology and the biology of PCa and the effectiveness of therapeutic targeting, we briefly summarize the animal and cell models that have been employed. We also report our experience in the use of three-dimensional (3-D) culture and co-culture models to understand cell signaling networks and the use of these attractive tools to conduct drug screening exercises against already-identified molecular targets. Further research into PCa growth and metastasis will improve our ability to target cancer metastasis more effectively and provide better rationales for personalized oncology.

**Keywords:** Metastasis-initiating cells (MICs); dormant cells (DCs); recruitment and reprogramming; cell signaling network in tumor microenvironment; three-dimensional cell culture

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## Introduction

Worldwide, prostate cancer (PCa) ranks third in cancer incidence and sixth in cancer mortality in men (1). PCa has the highest cancer incidence among men in western countries and is the second most common cause of

cancer death after lung cancer (2). The American Cancer Association reported that in 2013 PCa accounted for approximately 28% of new cancers diagnosed in the United States (238,590 new cases) with an overall 10% death rate (29,720 cases) (1). While local and regional PCa cases are curable with a 5-year survival of nearly 100%, 5-year

survival of PCa patients with metastasis to a distant site goes down to 28% (1), making PCa one of the deadliest cancers. New treatments and diagnostic approaches are badly needed.

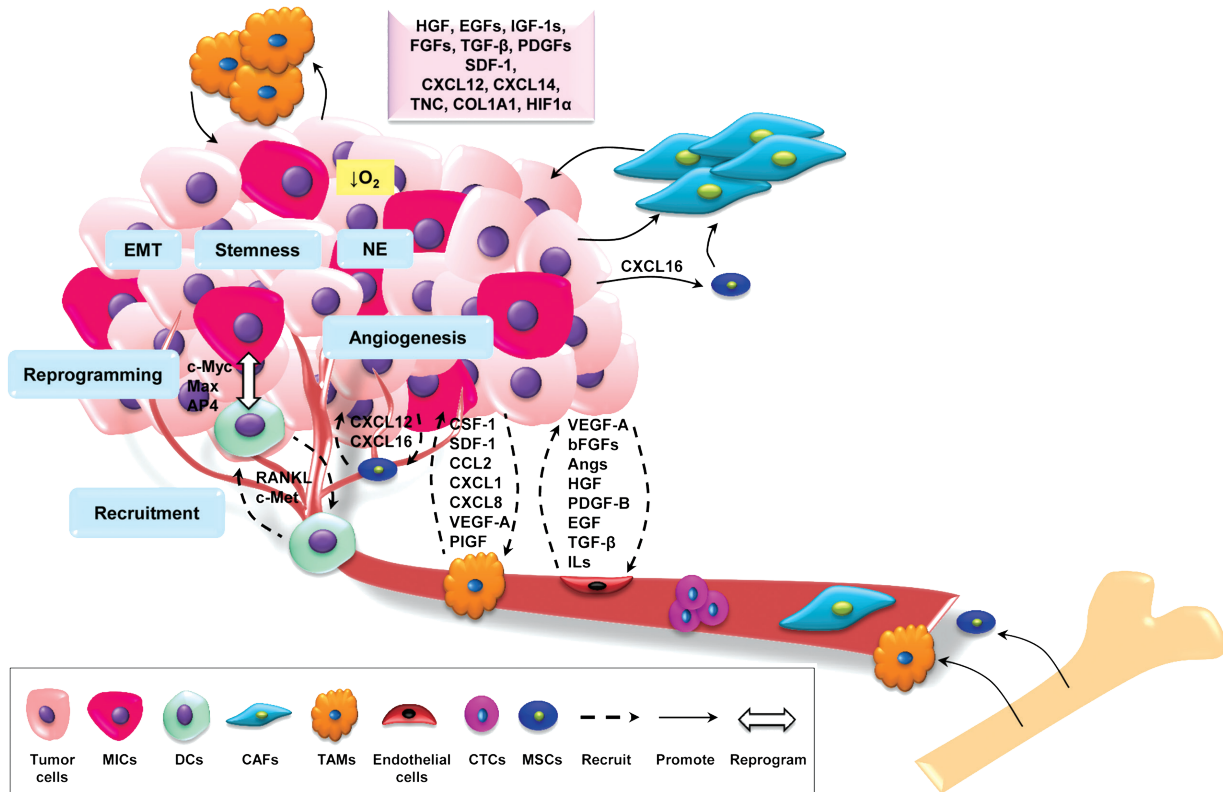
The main clinical complication of PCa is bone metastasis. Previous studies comparing progressive castration-resistance and non-metastatic human samples revealed bone lesion metastasis in >80% of all men who die of PCa (3,4). Despite the high occurrence of skeleton metastasis and major patient mortality (5), the molecular mechanisms behind PCa's prevalence for homing to bone are not well understood. Bone is a dynamic environment, with a balance between bone resorption and bone formation activities. The main players in normal bone remodeling are osteoclast (OC, bone-destructive) and osteoblast (OB, bone-forming) cells (6,7). OC cells express receptor activator of nuclear factor (NF) kappa-B (RANK) and become mature through interaction with their ligand, RANKL, expressed on OB cells located as the membrane-bound form on the bone surface (8,9). The balance between the activities of these cells is controlled by the triad relationship among RANK, RANKL and osteoprotegerin (OPG), a decoy receptor of RANK that binds RANK ligand (RANKL) and blocks RANK-RANKL interaction and subsequent bone resorption (10-12). There are many other players controlling bone development that are also known to affect PCa bone metastasis. The growth factors (GFs) in the bone microenvironment, such as transforming GF- $\beta$  (TGF- $\beta$ ), bone morphogenetic proteins (BMPs) (13), runt-related transcription factor-2 (RUNX2), stromal cell-derived factor 1 (SDF-1/CXCL12) (14) and endothelin-1 (ET1) have roles in bone turnover and cancer metastasis to bone (15). Some of the main factors involved in normal bone remodeling are secreted by cancer cells (16-21) and could serve as predictors of cancer bone metastasis (22). For instance, chemotaxis of cancer cells toward bone sites could be explained in part by the interaction of CXCL12, a soluble stroma-derived factor, and its receptor CXCR4 on the surface of cancer cells (21,23). It has been shown that cancer cells with bone-homing propensity expressed bone-forming factors such as BMP, insulin-like growth factors (IGF-1) and ET1, all of which affect the balance of bone resorption and formation (24). Cancer cells can also secrete soluble factors, RANKL, parathyroid hormone-related protein (PTHrP) (25) and the interleukin (IL)-6 (26-28) to promote bone resorption. Consequently, higher bone resorption increases bone breakdown that releases TGF- $\beta$ , IGF-1, and BMPs, which further promote and

enhance cancer cell proliferation and survival (29). Taken together, tumor cells interact with bone cells by establishing a "vicious cycle" through secreted soluble factors in the bone microenvironment that culminate in enhanced bone metastasis (30).

In this review, we emphasize three areas. (I) We present a new concept based on our finding that a selective population of cancer cells can recruit and reprogram gene expression and cell behaviors of bystander or dormant cells (DCs), conferring their metastatic potential to bone and soft tissues. (II) We discuss how cell signaling networks are activated by GFs, extracellular matrices (ECMs) and androgen receptors (ARs) to govern the metastatic cascade of cancer cells. (III) We summarize cell and animal model systems used to study PCa progression, share our experience with 3-D culture, and discuss how these culture methods could be further expanded to understand the underlying molecular basis of tumor-microenvironment interaction.

### **Possible mechanisms of tumor metastasis: recruitment and reprogramming**

Tumors are not single insular entities; they are complex tissues composed of various distinct cell types that engage in collaborative interactions with one another during tumorigenesis (31,32). Tumor microenvironments shaped by the presence of tumor cells are therefore critical to allow cooperative interaction among tumor cells and their neighboring cells via soluble factors and ECMs, in order to sustain tumor growth, invasion and metastasis. It is increasingly evident that malignant cancer cells are capable of recruiting and transforming normal or non-tumorigenic bystander cells to serve as active collaborators and participate in tumorigenesis, evade immune surveillance and develop distant metastasis (32-35). The supportive cells recruited to the tumor microenvironment include a heterogeneous population of stromal cells, including fibroblasts, immune cells, endothelial cells, and bone marrow-derived mesenchymal stem and progenitor cells (MSCs) (36). The tumor-stroma crosstalk, either by direct cell-cell interaction or by secreted soluble factors or insoluble ECMs, is reciprocal. Through the interaction of tumor cells with their microenvironments, heterotypic signaling between the diverse cellular constituents of the tumor microenvironment promotes tumor growth, survival and metastatic progression (32) (*Figure 1*). Investigation of the interactions between cancer cells and their supporting



**Figure 1** Recruitment and reprogramming of bystander and dormant cells (DCs) by metastasis-initiating cells (MICs) in the tumor microenvironment. Tumor cells recruit various distinct cell types, including fibroblasts, immune cells, endothelial cells, bone marrow stromal cells, and bystander DCs to the microenvironment via soluble factors to allow cooperative interactions promoting tumor growth, survival, and metastatic progression. Tumor-stroma crosstalk, either by direct cell-cell interaction or by secreted soluble factors (cytokines, chemokines, and growth factors) or insoluble ECMs, is reciprocal. A unique population of MICs are capable of recruiting normal or non-metastatic DCs or circulating tumor cells (CTCs) and reprogram them to undergo genetic transformation to display MIC phenotypes with acquisition of EMT, stem cell and neuroendocrine properties through transactivation of c-Myc/Max and AP4 via RANK-mediated signaling, thereby gaining the ability to migrate, invade, and metastasize to bone and soft tissues. CSF-1, colony-stimulating factor 1; SDF-1/CXCL12, stromal cell-derived factor 1; VEGF-A, vascular endothelial growth factor A; PIGF, placental growth factor; bFGFs, basic fibroblast growth factors; HGF, hepatocyte growth factor; EGF, endothelial growth factor; TGF-β, transforming growth factor-β; IL, the interleukin.

“co-conspirators” during tumorigenesis and metastasis provides a crucial understanding of cancer pathogenesis, leading to the development of novel and effective therapeutic strategies.

**Cancer-associated fibroblastic cells**

Since fibroblasts are associated with wound healing, tumors with aberrant wound healing and tissue repair mechanisms were found to recruit a variety of fibroblastic cells. In particular, both local and bone marrow derived MSCs are

recruited and activated to become carcinoma-associated fibroblasts (CAFs) or myofibroblasts (34), characterized by the production of α smooth muscle actin (αSMA) (37). Different CAF subpopulations can contribute to a variety of tumor-promoting and differentiation functions with the production of different stimulatory or differentiation factors, whose functions are significantly influenced by the adjacent heterogeneous tumor epithelial cells and other tumor-associated cellular compartments (38). CAFs within prostate tumors secrete high levels of mitogenic GFs and chemokines, including hepatocyte growth factor (HGF),

EGF family members, IGF-1s, fibroblast growth factors (FGFs), TGF- $\beta$ , SDF-1/CXCL12, CXCL14, tenascin-C, collagen 1, and hypoxia inducible factor 1 (HIF1 $\alpha$ ) that together stimulate cell proliferation and promote epithelial-to-mesenchyme-transition (EMT) (39-42). One of the potential mechanisms underlying PCa progression and metastasis by the recruitment of MSCs is mediated by the CXCL16-CXCR6 signaling axis as recently demonstrated by Jung *et al.* They found that tumor-derived CXCL16 recruits and interacts with CXCR6-positive MSCs, leading to the transformation of MSCs to CAFs, which produce high levels of SDF-1/CXCL12, further promoting EMT progression and metastasis by up-regulation of CXCR4 in PCa cells (43).

### *Infiltrating immune cells*

The relationship between inflammation and tumorigenesis was first proposed in 1,863 by Rudolf Virchow based on the observation of unusual numbers of infiltrating leukocytes in neoplastic tissues (44). Since then, a plethora of studies have documented that human tumors are infiltrated with heterogeneous populations of inflammatory cells (44-46), in particular macrophages, or tumor-associated macrophages (TAMs). TAMs are derived from monocytes actively recruited to tumors from the blood in response to chemokine cues in the tumor microenvironment (47,48). TAMs can constitute up to 50% of the tumor mass and the high numbers of TAMs present in solid tumors are strongly implicated in cancer progression and metastasis (36,49,50) and correlate with poor patient prognosis (51). TAMs have multifaceted roles; even though TAMs recruited to tumor sites represent the first line of defense as part of an innate immune response, it is well documented that TAMs also support multiple aspects of tumor progression (36,52,53). TAMs produce high levels of reactive oxygen and nitrogen species that can induce DNA damage and mutations of the surrounding epithelium (54). TAMs also secrete several GFs, such as HGF, FGFs, EGF, platelet-derived growth factor (PDGF), and TGF- $\beta$  that are capable of promoting tumor growth and EMT, leading to metastatic progression (55). Colony-stimulating factor 1 (CSF-1) is one of the key factors that promotes monocyte recruitment and macrophage differentiation, survival, and proliferation (36,51). In a breast cancer model, CSF-1 deficient mice have impaired macrophage differentiation and survival, leading to significantly lower incidence of tumor formation and metastasis (54). Further studies have identified other

chemokines, such as CCL2 (56,57), CX3CL1 (58), CXCL8, and SDF-1 (57), and GFs such as vascular endothelial GF A (VEGF-A) and placental GF (PIGF) (59,60) that are expressed by tumor cells, fibroblasts, endothelial cells, or TAMs that can induce monocyte/macrophage recruitment into specific tumor microenvironments (36). Furthermore, TAMs tend to accumulated in the hypoxic regions of tumors, mediated by the chemoattractants endothelin-2 and VEGF regulated by hypoxia-inducible factor-1 (HIF-1) (47,60). TAM recruitment and accumulation in hypoxia regions results in angiogenesis and the progression of tumor cells to a more invasive phenotype (60).

### *Endothelial cells*

Many different cytokines and GFs produced by tumor cells, infiltrating immune cells, and fibroblasts are involved in the recruitment of endothelial cells during angiogenesis, including VEGF, basic FGF (bFGF), angiopoietins, HGF, PDGF-B, EGF, TGF- $\beta$ , and interleukins (61). These proangiogenic factors can further activate matrix metalloproteases such as matrix metalloproteinase (MMP)-1 and MMP-9 and urokinase-type plasminogen activators (uPA) to break down ECMs and facilitate endothelial cell and pericyte invasion (62) and vascular remodeling (36,63). Recently, Png *et al.* demonstrated that tumor suppressor miR-126, a miRNA silenced in a variety of common human cancers, non-cell-autonomously suppresses endothelial cell recruitment, metastatic angiogenesis, and metastatic colonization of breast cancer cells *in vitro* and *in vivo*. It coordinates the targeting of insulin-like GF binding protein 2 (IGFBP2), phosphatidylinositol transfer protein, cytoplasmic 1 and c-Mer tyrosine kinase, which are novel pro-angiogenic genes and biomarkers of human cancer metastasis (64). Endothelial cells provide blood flow to tumors and they also clearly signal and facilitate cancer cells to metastasize and colonize at distant sites.

### *Non-tumorigenic and -metastatic bystander and DCs*

Using the RANKL-overexpressing PCa cell model, we identified a population of metastasis-initiating cells (MICs) that can recruit bystander non-metastatic cancer cells, in this case red fluorescent protein (RFP)-tagged LNCaP cells, to metastatic sites to participate in skeletal and soft tissue metastasis (65). We also observed that the bystander non-metastatic cancer cells (65,66) can be reprogrammed by the MICs to undergo cytogenetic and gene expression changes,

subsequently displaying MIC phenotypes such as EMT, stem cell, neuroendocrine cell and bone-like properties through transactivation of c-Myc/Max and AP4 via RANK-mediated signaling, thereby gaining the ability to migrate, invade, and metastasize to bone and soft tissues (33). Similarly, we found that these genetically modified MICs can recruit and reprogram DCs established from primary PCa tumor tissue and also human circulating tumor cells (hCTCs) freshly obtained from patients by *ex vivo* culture under a 3-D organoid co-culture system. In this setting, DCs and hCTCs were reprogrammed by the experimental MICs to express elevated genes related to an increased RANKL-RANK signaling. In another study, we observed that experimental PCa epithelial cells can transform normal fibroblastic cells to gain extensive and consistent cytogenetic changes notably found in tumor cells (67). Recently, we extended our work to define two naturally occurring MICs (nMICs) that were isolated by *ex vivo* culture of the ascites fluid of a bone metastatic PCa patient (68-70), expressing MIC signature genes and displaying MIC properties, including aggressively forming metastatic bone tumors when inoculated intracardially in mice. We demonstrated that, similar to the RANKL-overexpression genetic model of MICs, the nMICs, are capable of recruiting and promoting the growth of DCs in 3-D organoid co-culture. These recruited DCs were shown to be reprogrammed to mimic nMICs with enhanced expression of RANKL-RANK downstream target genes, including the mesenchymal, the stem cells, the neuroendocrine and the osteomimicry biomarkers. We believe CTCs and DCs once recruited and reprogrammed by MICs undergo permanent changes sustained possibly by genetic mutation, cytogenetic changes (e.g., chromosomal rearrangement and translocations) (35) or epigenetic modification by methylation of specific promoters whose status may be controlled by the reciprocal cellular interaction between nMICs and CTCs or DCs (71-75). Understanding the underlying mechanisms of the recruitment and reprogramming of DCs or CTCs by either experimental MICs or nMICs will provide significant insights into cancer evolution, progression, and metastasis with major implications for both basic biology and clinical medicine.

### **Intracellular cell signaling pathways governing the metastatic cascade**

Cytokines also have strong pro-tumorigenic activities on host cells in the tumor microenvironment. For example, RANKL exerts multiple effects on bone turnover and

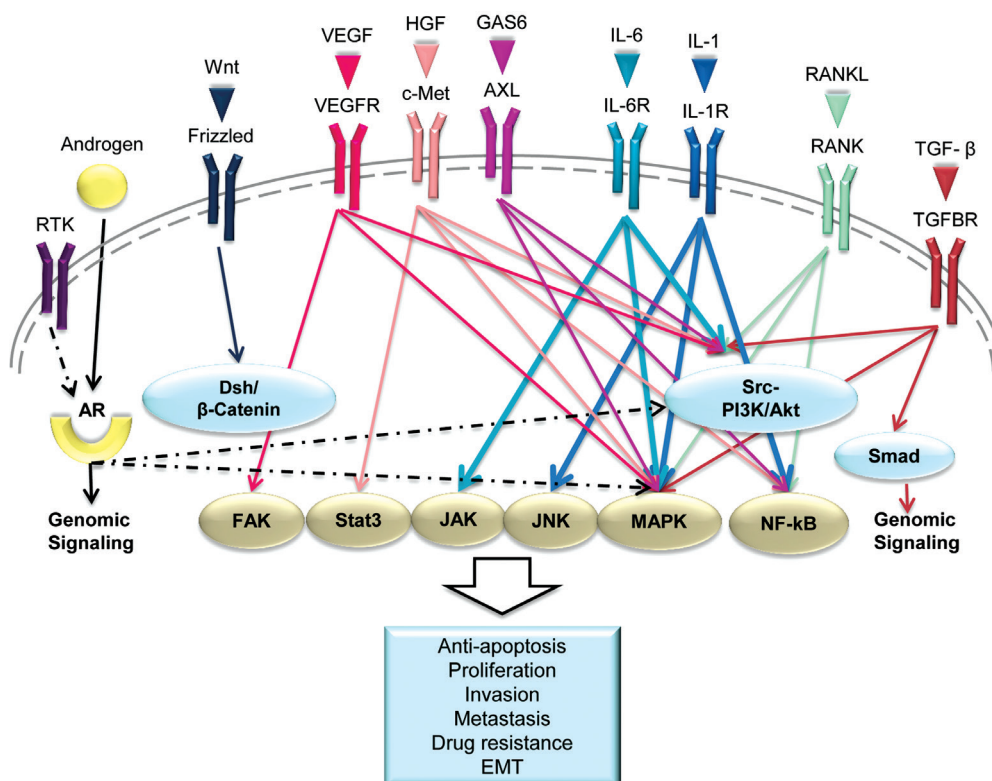
metabolism, stem cell renewal, tumor cell proliferation and survival, angiogenesis, and inflammation. These effects are mediated by several common intracellular signaling pathways. We highlight a few examples of specific pathways that are induced by cytokines or their receptors, with emphasis on pathways that offer possibility as potential therapeutic targets (*Figure 2*).

#### ***Androgen receptor (AR)***

Molecular modeling of PCa has demonstrated that androgens have critical roles in PCa development and progression at all stages of disease, with their actions mediated by AR (76). AR, a large ~110 kDa transcription factor of the steroid nuclear receptor family, can be activated in target organs in a ligand-dependent or -independent manner to support the growth and differentiation of the normal prostate gland as well as the malignant growth and progression of PCa (76). The AR signaling axis is intricately regulated by its interactive factors and converges with a large number of other signaling pathways (77,78). For example, AR was shown to be responsible for metastatic progression of PCa through downstream signal convergence with chemokine receptor/ligand function and G-protein coupled receptors (79). Altered AR functions were observed in cells that expressed tumor-associated AR cofactors (e.g., FOXA1) (80), or formation of AR-dependent gene fusions (e.g., TMPRSS2-ERG) (81) and downstream effectors (e.g., SOX9) (82). AR mutations at the c-terminal ligand-binding domain (83) or deletion of the c-terminal domain to yield truncated AR variants (ARs) (84) have been shown to produce, respectively, promiscuous AR that can be activated by a broad spectrum of steroidal and non-steroidal ligands or a constitutively activated AR without the requirement of steroid ligands (85). AR mutations and ARs have been implicated in the development of CRPC and the resistance of PCa to current androgen antagonist therapies such as enzalutamide or abiraterone acetate (86,87).

#### ***RANK ligand (RANKL)***

RANKL-RANK signaling has many crucial physiological roles in bone and peripheral soft tissues. Aberrant RANKL-RANK signaling in cancer and bone cells affects cancer bone colonization (87,88). Our group previously reported that RANKL-expressing PCa cells can recruit and reprogram neighboring non-metastatic bystander or DCs via specifically activating transcription factors through the



**Figure 2** Schematic cytokine-mediated intracellular pathways. MAPK, Akt, and NF-κB pathways are commonly activated by many converging signaling axes of cytokines and their receptors. The FAK, JAK, and JNK signaling pathways are rarely active through separate mediation of VEGF, IL-6, and IL-1. AR, Smad family, and STAT-3 are the transcription factors directly binding to the promoters of their target genes in response to cytokines, or androgen, or through the TGF-β or c-Met receptors. Wnt/Frizzled mainly activates the β-catenin cascade.

RANK-mediated downstream signaling network *in vitro* and *in vivo* (65). Significantly, RANKL and its downstream signaling network in primary human PCa tissues predict patient survival (89). There are two TRAF6-mediated intracellular cascades induced by RANKL-RANK signaling: the classic and non-canonical NF-κB pathways (90,91) and the c-Src-mediated Akt and MAPK protein kinases pathways, enhancing cancer cell migration and survival (92-94).

**The interleukin (IL) family**

IL-1 and IL-6 activate OBs and OCs, and disrupt the homeostatic balance that controls bone formation and resorption.

**IL-1**

One of the IL-1 receptors, IL-1β, was recently shown to be overexpressed in non-metastatic cancer cells to promote the growth of large skeletal lesions in mice,

whereas the knockdown of IL-1β significantly impaired the bone metastatic progression of a highly metastatic cancer cell line (95). Human PCa tissue specimens isolated from skeletal metastatic patients, while not expressing PSA, were positive for both IL-1β and synaptophysin, a neuroendocrine differentiation marker (95). Like RANKL, IL-1 also initiates a TRAF6-mediated signaling cascade to activate the downstream NF-κB-, JNK-, and MAPK-mediated signaling pathways (96,97).

**IL-6**

It has been reported that human PCa cells produce IL-6 and that serum levels of IL-6 are elevated in patients with PCa, including serum isolated from patients with advanced hormone-refractory disease. IL-6 has been suggested as a prostate exocrine gene product and a candidate mediator of PCa intra- and inter-cellular communication (98). Binding of IL-6 to its receptor, IL-6R, activates the JAK kinase family (JAK1, JAK2, and TYK2) (99). These kinases

phosphorylate STAT-3, promoting its nuclear translocation and transcriptional function (100). IL-6 also activates Ras and promotes its translocation to the plasma membrane where it activates the Raf-MEK pathway. Finally, a third pathway activated by IL-6 is the PI3K-Akt pathway (101,102).

### *The Wnt pathway*

In PCa bone metastasis, Wnt production stimulates osteoblast differentiation and exerts autocrine effects on cancer proliferation (103). Wnt binds to cell surface receptors of the Frizzled family, and activates the members of the Dishevelled family of proteins, such as Dickkopf-related protein 1 (DKK1). The Wnt/Frizzled family subsequently stabilizes  $\beta$ -catenin, which then translocates to the nucleus and promotes multiple downstream signaling to promote bone formation. Wnt/ $\beta$ -catenin signaling also induces OB differentiation through BMP-dependent and -independent signaling pathways. Wnt-mediated signaling also plays a role in the development of invasive CRPC in both mouse and man; Wnt was found to be upregulated in prostate stromal cells when TGF $\beta$ , signaling was attenuated (104). The activity of Wnt is controlled by soluble extracellular antagonists including secreted Frizzled-related proteins, WIF-1, Cerberus, and DKK1. This identifies Wnt as a potential therapeutic target to interrupt both autocrine and paracrine interactions between PCa cells and the interactions between PCa-OB and PCa-stromal cells (105).

### *TGF- $\beta$ -mediated pathway*

TGF- $\beta$  plays a tumor suppressive role in normal and pre-neoplastic epithelia, but paradoxically promotes motility and resistance to cell death in transformed epithelia (106). Activated TGF- $\beta$  signaling induces EMT in PCa, suppresses host immune surveillance and drives cancer metastasis (107). Increased TGF- $\beta_1$  expression by tumor cells also correlates with tumor progression in lung, colorectal, and gastric cancers (108). Mechanistically, TGF- $\beta$  binds to heterodimeric receptors (type I and type II TGF- $\beta$  receptors) and can activate the canonical Smads signaling pathway or Smad-independent pathways through PI3K, MAPK, and Akt (109). TGF- $\beta$  mediates both autocrine and paracrine signaling during PCa progression.

### *Receptor of tyrosine kinase family*

Although receptor tyrosine kinases (RTKs) are important in

normal physiology, dysregulation of some RTKs has been implicated in tumor development and progression (110). Because of cross-talk between RTKs, it has redundant functions and can converge with common downstream cell signaling networks, making it difficult to target independently without the concerns of off-target effects. Cancer cells can acquire RTKs resistance after treatment. Therefore, development of inhibitors for multiple RTKs is an active area of pursuit.

### *c-Met*

The expression of c-Met and its ligand, HGF, correlate with PCa metastasis and disease recurrence, with the highest c-Met levels in bone metastases compared with soft tissue and lymph node metastases (111,112). The major signaling networks linking HGF/c-Met are the MAPK, Akt, STAT-3, RANKL-RANK, and NF- $\kappa$ B signaling cascades (113-116).

### *VEGF receptors (VEGFR)*

VEGF and its receptor (VEGFR) are well known to be potent stimulators of angiogenesis in both physiological and pathological conditions and are highly expressed in most solid tumors, including PCa (117). Like other RTKs, VEGF/VEGFR can trigger the MAPK and Akt-dependent axes for proliferation and survival (118,119). VEGF has also been shown to activate FAK and associated proteins for maintenance of survival signals in endothelial cells (119,120).

### *Axl*

Axl has recently been identified as a critical factor driving tumor cell invasion, migration, pro-inflammatory cytokine production, anti-apoptosis, and proliferation. The ligand growth arrest specific gene-6 (GAS6) is the only known ligand for Axl (121). GAS6/Axl activates the Akt pathway to protect cells from apoptosis via multiple mechanisms. In particular, Akt activates the mTOR pathway, inhibits pro-apoptotic caspase 3, and phosphorylates NF- $\kappa$ B, which up-regulates the anti-apoptotic proteins Bcl-2 and Bcl-xL (122,123). In some cell types, Axl also activates the MAPK pathway and contributes to cancer invasion (124).

In summary, this section reviewed selected cell signaling network driving by soluble factors and their receptors that governs PCa growth, invasion and metastasis. Data collected in this section are derived from cell lines, animal models and some are confirmed by clinical specimens.

The utility to target effectively cell signaling network and its translation await additional future studies and critical analyses of experimental data collected thus far by systems biology methods to overcome potential converging and redundant cell signaling pathways that hinder cancer cure at the bedside.

## Current PCa models and their limitations

### *Human PCa cell lines*

Extensive use of immortalized cell lines has greatly increased our understanding of the biology of PCa and its development through gene deregulation. In PCa research, utilizing cell lines have given us an understanding of crucial molecular mechanisms that lead to bone and soft tissue metastases such as PCa androgen-independent progression (69,125-127), the roles of ARs and their ARs in promoting cell signaling networks in the castration-resistant state (128) and the convergence of cell signaling pathways that explains how cancer cells gain therapeutic resistance (129-131). A detailed characterization of PCa cell lines was reviewed by Sobel *et al.* (132) and Russell *et al.* (133).

Despite the knowledge we have gained about PCa using 2-D tissue culture, many disadvantages limit the extent of our understanding and more cell lines with diverse phenotypes are needed. Since most PCa cell lines are derived from a metastatic site, new cell lines from early carcinogenic events could help us to understand the initial steps of PCa transformation and progression from normal tissues. Furthermore, PCa is a heterogeneous disease, samples from same person or different locations in the metastatic cascade were found to behave differently. MDA PCa 2a and 2b, for instance, were derived from bone metastatic specimen of one patient, yet their morphologies and behaviors are quite different (134,135). Therefore, studying different aspects of PCa in the limited number of available cell lines can address only in part the complexity of the disease. More importantly, current monolayer cultures also recapitulate in part the vital PCa cellular interactions with cellular factors including epithelial and stromal cells or non-cellular factors such as ECMs within the context of the tumor microenvironment.

### *Mouse models of PCa*

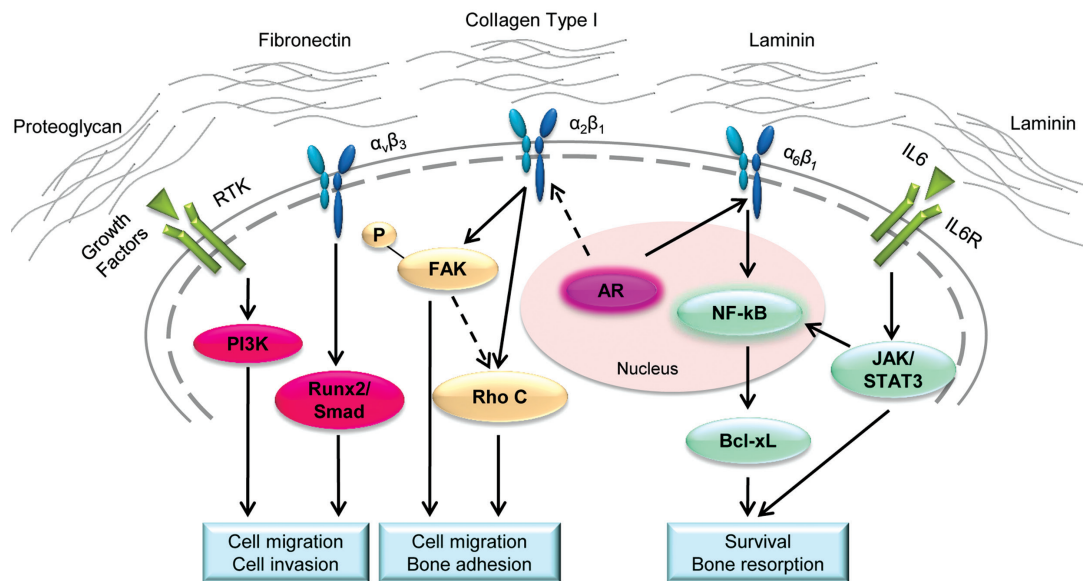
Given the limitations of tissue culture, *in vivo* models are often used to better mimic the natural history and the

complexity of this disease. Mouse models in PCa research include xenograft and patient-derived xenograft models and genetically engineered models (GEM), detailed reviewed by Ittmann *et al.* (136) and Valkenburg and Williams (137). In addition, Goldstein and Witte (138) and Shen and Abate-Shen (139) emphasized the importance of tumor microenvironment interactions that promote PCa development in murine models. Mice are valuable models in PCa biology because, like humans, they are susceptible to cancer development when introduced with oncogenes or carcinogens. Mouse genome shares 95% homology to the human genome and they are relatively easy to manipulate genetically (140,141). However, mouse models have distinct limitations. First and foremost, the mouse and human anatomy are different. Unlike the human prostate, the mouse prostate has four lobes (142) and no clear analog has been delineated between mouse prostate lobes and the human peripheral zone where human PCa arises. In addition to anatomic dissimilarity and the size difference between human and mice prostate glands, dietary, hormonal, age and strain, and gene-environment interactions need to be considered when a mouse model is being used to understand histopathologic changes, etiologic and environmental factors and drug screening studies in human PCa (140,143). Some of the main concerns about using mouse models are: (I) spontaneous PCa in mice is very uncommon and distant metastasis to bone is even more rare, despite forced genetic manipulation (47); (II) age is the main known risk factor for PCa and mice have a 30-50 times shorter lifespan than humans (144); and (III) the most-used mouse models are immune compromised. This combination of drawbacks presents formidable challenges when analyzing mouse models of human PCa (136). To study human immune and drug responses to cancer cell growth, conventional nude and severe combined immunodeficiency (SCID) mouse models are being replaced by immunodeficient mice bearing a mutated IL-2 receptor gamma chain. These highly immunodeficient mice, NOG (145) and NSG (146), allow development of human immune systems, including T and B cells through hematopoietic stem cell transplantation. These models successfully permit *in vivo* investigation of the human immune response to primary human cancer and malignant MSCs (147).

### *Three-dimensional (3-D) in vitro models to mimic PCa progression and metastasis*

Over time, it has become evident that PCa is not a single





**Figure 3** Metastatic cell behavior signaling stimulated downstream from ECM-integrin interaction. The ECM proteins such as collagens, proteoglycans, hyaluronan and laminin modulate cell behaviors by activating cell surface integrins, the ECM receptors [e.g.,  $\alpha_v\beta_3$  (168),  $\alpha_2\beta_1$  (169,170),  $\alpha_6\beta_1$  (171)], receptors for tyrosine kinases or cytokine receptors [e.g., RTK (172), IL-6R (173)] or their ligands. These receptors initiate a variety of downstream signaling cascades such as JAK, NF- $\kappa$ B, FAK, ROCK, and PI3K that lead to enhanced cell survival, motility and bone adhesion and resorption. These ECM-dependent increases of integrins and ligands were shown to be sensitive to environmental cues and potentially responsible for enhancement of PCa growth, invasion, and migration and bone metastasis (solid arrows, promoting pathways; dotted arrows, potential promoting pathways). RTK, receptor tyrosine kinase; ECM, extracellular matrix; IL, the interleukin.

cell disease. Besides the heterogeneity within PCa tumors, there are many factors, including the contributory roles of host microenvironment stromal cells, immune cells, endothelial cells, growth regulatory factors and ECMs that play crucial roles regulating PCa progression and ultimate metastatic behavior (148,149). So far the best way to study and recapitulate these interactions is the use of animal models though as discussed above, animal models also have their own limitations. An alternative approach is the use of *in vitro* 3-D models which provide simplified and more economical systems to mimic tumor-host microenvironment interactions *in vivo* (150-156). Under 3-D growth conditions, tissues, cells, GFs and ECM scan are prepared as physical scaffolding for defined biomechanical strength and composition resembling the pathophysiological conditions of tumors *in vivo*. With respect to ECM, 3-D growth provides physical support of tissues and cells and mediates biological and physical cues from the cell external to various pathophysiological commands, which results in changes in cell morphology (157), proliferation, survival (158), migration and adhesion (159,160) through interaction of cell surface  $\alpha$ - and  $\beta$ -integrin subunits to specific ECMs

(161,162). Dimerization of each  $\alpha\beta$  subunit on cells dictates binding to specific ECM molecules and determines the activation of downstream signaling and ultimately cell fate (163).

Besides ECM's direct effect on cell behavior, mainly through integrins, ECM also has a significant role in regulating cell surface GF signaling (164). ECM has binding sites for GFs such as FGFs and VEGFs (165) and they can be cleaved and released as soluble factors under certain pathophysiological conditions. Additionally, integrins on the cell surface can also contribute to GF activation or degradation. For instance, a study of epidermoid carcinoma has shown that  $\alpha_2\beta_1$  integrin co-localization with EGFR is required for further activation of Akt and Rho GTPases (166). In a separate study, Caswell *et al.* showed that  $\alpha_3\beta_1$  integrin can form a complex with EGFR1. Consequently, the membrane bound complex regulates protein kinase B (PKB) signaling and enhances cancer invasion (167). *Figure 3* illustrates common known signaling pathways enhancing PCa bone metastasis that are stimulated by ECMs.

Given the broad influence of ECM in cell biological

**Table 1** Comparison of commonly used 3-D models. Summary of pros and cons of the various 3-D models that are frequently used in research and drug screening studies

3-D model name	Pros	Cons
Spontaneous cell-aggregation (suspension)	<ul style="list-style-type: none"> <li>• Study cell-cell interaction</li> <li>• Potential study of cell-ECMs</li> <li>• Large-scale production</li> <li>• Spheroids products are easily accessible</li> </ul>	<ul style="list-style-type: none"> <li>• Require special equipment</li> <li>• Labor intensive</li> </ul>
Matrix-embedded cells		
Matrices (matrigel etc.)	<ul style="list-style-type: none"> <li>• Provides 3-D physical support</li> <li>• Allows study of cell-ECM interaction</li> </ul>	<ul style="list-style-type: none"> <li>• Components are not well defined</li> <li>• Components cannot be modified</li> </ul>
Natural scaffolds (collagen I and hydrogel etc.)	<ul style="list-style-type: none"> <li>• Components better defined</li> </ul>	<ul style="list-style-type: none"> <li>• Commercial batch to batch differences</li> </ul>
Synthetic scaffold from polymers	<ul style="list-style-type: none"> <li>• Reproducible results</li> <li>• Controllable characteristics</li> <li>• Potential for modification for receptors</li> <li>• Co-culture studies</li> </ul>	<ul style="list-style-type: none"> <li>• Absence of essential receptors and motifs compared to natural scaffolds</li> <li>• Cell imaging</li> <li>• Difficulty to analyze single spheroid and live imaging</li> <li>• Limitation to produce large-scale samples</li> </ul>
Microfluid culture (organ-on-chips)	<ul style="list-style-type: none"> <li>• Recapitulating the biological mechanically active microenvironment</li> <li>• Mimic organ-specific microarchitecture</li> <li>• Responsive to inflammatory stimuli biochemical, and functionality</li> </ul>	<ul style="list-style-type: none"> <li>• Costly</li> <li>• Not yet available commercially</li> </ul>

functions, ECM protein abnormality potentially leads to various human diseases related to skeleton development and remodeling, stem cell differentiation, and inflammation, such as multiple sclerosis or osteoarthritis as well as cancer initiation and progression (174-176). Therefore, the detailed study of the role of ECM in cancer progression and metastasis is crucial and requires proper models.

Since cells grown in 2-D and 3-D cultures differ in their physical contacts with ECM scaffolds, and biological and behavioral changes caused by cell-cell and cell-ECM interaction in the microenvironment, the importance of 3-D cultures in cancer biology studies is now well accepted (176-179). Wang *et al.* compared the growth of non-malignant HMT-3522 breast cells with those of the malignant HMT-3522 subline and documented the critical functional differences of  $\beta_1$  integrin in breast cancer cells. They showed that in cells grown as 3-D, but not 2-D, anti- $\beta_1$  integrin antibody treatment restored malignant but not non-malignant breast cancer cells to normalcy (180). In regard to PCa, published studies show restored AR expression in the PC3 and LNCaP<sup>RANKL</sup> cell lines after

growth in matrigel and 3-D suspension culture, respectively (169,181). These results raise the question of whether 3-D culture and co-culture models mimic better the pathophysiology of PCa when considering the screening of AR antagonists against the growth of CRPC.

3-D models were utilized for the first time by Miller in 1985, who studied the drug resistance of mouse mammary tumors in a 3-D collagen gel culture (182). Since then, different types of 3-D models have been developed and many improvements have been made. Current 3D models can be classified into: (I) spontaneous cell aggregation; (II) matrix-embedded cells; and (III) tissue-engineered scaffolds. A detailed discussion of the available 3-D models is beyond the scope of this review. We have summarized the pros and cons of the most commonly used 3-D models in *Table 1*.

Despite the advantages of 3-D over 2-D models in mimicking the growth of cancer *in vivo*, there remain technical difficulties that need to overcome. These include capturing high resolution 3-D imaging and real-time cell tracking. Also, with matrix-embedded cells there is limited access to the cells for DNA/RNA, protein and immunoassay

studies. The available 3-D models and techniques are rapidly improving through multidisciplinary collaborations between biologists and biomaterial engineers, and many limitations have been resolved. For example, Gao *et al.* (183) employed improved GF reduced Matrigel as scaffold for 3-D organoid culture and have successfully established long term culture of freshly harvested human PCa and CTCs. More detailed studies and further optimization are required to develop reliable 3-D models with reproducible data before they can be widely used to bridge the gap between the traditional 2-D cultures and the complex *in vivo* models.

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### Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

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