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Review

Regulatory signaling network in the tumor microenvironment of prostate cancer bone and visceral organ metastases and the development of novel therapeutics

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Abstract This article describes cell signaling network of metastatic prostate cancer (PCa) to bone and visceral organs in the context of tumor microenvironment and for the development of novel therapeutics. The article focuses on our recent progress in the understanding of: 1) The plasticity and dynamics of tumor–stroma interaction; 2) The significance of epigenetic re-programming in conferring cancer growth, invasion and metastasis; 3) New insights on altered junctional communication affecting PCa bone and brain metastases; 4) Novel strategies to overcome therapeutic resistance to hormonal antagonists and chemotherapy; 5) Genetic-based therapy to co-target tumor and bone stroma; 6) PCa-bone-immune cell interaction and TBX2-WNTprotein signaling in bone metastasis; 7) The roles of monoamine oxidase and reactive oxygen species in PCa growth and bone metastasis; and 8) Characterization of

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imprinting cluster of microRNA, in tumor—stroma interaction. This article provides new approaches and insights of PCa metastases with emphasis on basic science and potential for clinical translation. This article referenced the details of the various approaches and discoveries described herein in peer-reviewed publications. We dedicate this article in our fond memory of Dr. Donald S. Coffey who taught us the spirit of sharing and the importance of focusing basic science discoveries toward translational medicine.

1. Introduction

Cancer metastasis and associated secondary symptoms are the greatest contributors to cancer death. In prostate cancer (PCa), significant mortality and morbidity associated with bone and visceral organ metastases make a better understanding of the biology and therapy of the lethal progression of this disease an important research problem. In this review, we describe our collective approaches inspired by Dr. Donald S. Coffey, who once said that when biochemistry and molecular biology fail, look for structure, function and clinical relevance for a solution. As a group, after decades of research in the biochemistry and molecular biology of PCa, we realized that further advances contributing to new knowledge of PCa must assess an integrated functions of the organ structure in relationship to the host and translational relevance of PCa metastases.

PCa metastasis is controlled by multiple elements including tumor cells and cells in the tumor microenvironment and millions of soluble and insoluble molecules aligned and organized as signaling networks communicating through inter- and intra-cellular- and subcellular organelle, extracellular vesicle (EV) and extracellular matrix (ECM). These interactions ultimately determine the fate of the host's cancer cells. We chose to focus our research on PCa bone and visceral organ metastases because of the well-established clinical manifestations encountered by the PCa patients with advanced metastatic disease, including pain, pathologic bone fractures, spinal compression, restriction of mobility, bone marrow suppression that led to infection, anemia, thrombocytopenia, and/or metabolic imbalances and cachexia in over 85%—100% of the patients diagnosed with advanced PCa. Through innovative research, we collectively could contribute to understanding PCa biology and therapy, with future prospects of improving the mortality and quality of life of PCa patients, particularly those with advanced metastatic and castration-resistant disease. This review focuses on eight selected interrelated concepts in PCa for advancing clinical care through laboratory research. These are: 1) The plasticity and dynamics of PCa—stroma interaction (contributed by Shian-Ying Sung); 2) The molecular basis and significance of epigenetic reprogramming in cancer growth, invasion and metastasis (contributed by Gina Chi-Yi Chu); 3) Evidence of altered junctional communication affecting PCa bone and brain metastases (contributed by Haiyen E. Zhau with support from Lijuan Yin and Qinglong Li); 4) Novel strategies to overcome PCa therapeutic resistance to hormonal antagonists and chemotherapy (contributed by Leland W. K. Chung, Gina Chia-Yi Chu and Ruoxiang Wang with support from Stefan Mrdenovic, Liyuan Yin and Ji Lyu); 5) Genetic-based therapy to co-target tumor and bone stroma (contributed by Chia-Ling Hsieh); 6) Understanding PCa-bone-immune cell interaction and defining the roles of TBX2-WNT signaling in PCa bone metastasis (contributed by Srinivas Nandana); 7) The roles of monoamine oxidase A (MAOA) and reactive oxygen species (ROS) in PCa growth and bone metastasis (contributed by Jason Boyang Wu); and 8) The roles of an imprinting class of microRNA (miRNA) in PCa—stroma interaction (contributed by Sajni Josson and Murali Gururajan). The accumulated knowledge of these different subject matters has been contributed independently by the authors with Leland W. K. Chung as a facilitator. Most rewardingly, the concepts emerging from our laboratory remain strong and active and are now being pursued by the authors of this article. This article is written in fond memory of Dr. Coffey. The spirit of sharing among colleagues, students and co-investigatiors was his trademark, and he has given so much to every one of us!

2. Tumor stromal interaction and co-evolution drive PCa progression: opportunity for developing new PCa biomarkers and identifying new therapeutic targets

The importance of cross-talk between tumor cells and neighboring stroma in the primary tumor and at metastatic bone and visceral organ sites has been demonstrated in the malignant progression and development of therapeutic resistance. In our early work, we proposed that reciprocal tumor—stroma interaction drives phenotypic and genotypic changes in both of these cellular compartments as the molecular basis of co-evolution during malignant PCa progression [1—10]. This concept was confirmed experimentally by genetic studies and gene expression profiling of laser capture microdissected tissue specimens from cancer and its adjacent cells in the tumor microenvironment, including the immune cells, endothelial cells, and fibroblasts [11,12], and validated by several other groups using different tumor models [13—17]. These results are also consistent with the well documented morphologic features of desmoplastic stromal reaction associated with cancers, long recognized by pathologists [18]. In this context, we acknowledge the vision of Dr. Coffey and his students who also noticed morphologic transformation of normal rat

kidney cells when placed on ECM secreted by tumor-derived basement membranes but not those secreted by normal placenta cells [19]. Although the precise molecular mechanism of these morphologic transitions remains to be determined, Hsieh et al. [20] recently made a remarable discovery in which she and colleagues noted stromelysin-1 (or matrix metallopeptidase 3, MMP-3), a key enzyme regulating ECM and expressed by both PCa and bone stroma, is modulated reciprocally by ROS. Mechanistically, ROS downregulates stromal MMP-3 expression in reactive bone stromal cells by activating nuclear factor-κB (NF-κB) through enhanced nuclear translocation, repressing MMP-3 transcription, whereas ROS elevates *MMP-3* gene transcription in PCa epithelial cells through increased miR-128, which depresses the expression an MMP-3 transcription repressor, thrombospondin 2, resulting in overall increase of MMP-3 transcription and expression in PCa cells [20]. It should be noted that decreased of MMP-3 expression in PCa associated reactive bone stromal cells was an unexpected finding in this report, because most previous reports indicated that increased MMP-3 expression in cancer cells drives cancer progression [21,22]. Results of this report, confirmed by tissue staining, support the reduced presence of MMP-3 in the surrounding stroma, but increased expression in PCa, implying that MMP-3 expression is stage and cell-type dependent during cancer progression. Further, this report proposes that stroma is a physical barrier against the advance of carcinogenesis during cancer progression. However, based on the lesson learned from the MMP-3 discovery, the co-evolution and vicious cycle cross-talk concept needs be reconsidered in the larger context of differential, or even opposite responses of PCa and reactive stroma exposed to the same stimuli in the tumor microenvironment. The differential regulation in this case is attributed to the different signaling networks wired within PCa and the reactive stromal cells.

To elucidate the concept of vicious cycle interaction between PCa and bone stroma experimentally, we adopted the 3-dimensional (3D) co-culture system with the rotary wall vessel (RWV). The rotary cell culture system was established by the National Aeronautic and Space Administration (NASA), and was originally designed to test intercellular communication in space shuttle flight using bioreactors [3]. Using this 3D co-culture model, we showed that not only do reactive bone stromal fibroblasts induce permanent cytogenetic, gene expression and behavior changes in the PCa cells [9], we demonstrated reciprocally that bone stromal cells exposed to inductive influence by PCa assume the morphologic features of myofibroblasts or reactive stroma [23], and gain the ability to drive malignant progression of PCa in xenograft mouse models *in vivo* [10]. We confirmed further that morphologic changes in stromal fibroblasts are accompanied by permanent cytogenetic, gene expression, and behavior changes, after co-culture with PCa cells in the 3D RWV system. Further molecular characterization of the morphologically altered bone stroma cells revealed altered ECM components (osteonectin, tenascin, and versican), chemokines (BDNF, CCL5, CXCL5, CXCL16), and chemokine receptors (CCR5 and CCR7), compared to the control parental bone stromal fibroblasts grown under 3D condition without PCa epithelial cells. Remarkably, we further confirmed that differentially expressed reactive stromal related genes were expressed in the sera specimens obtained from benign prostatic hyperplasia (BPH) and PCa patients, strongly implicating PCa—bone stromal interaction under 3D conditions with the *in situ* biology and gene expression profiles of PCa patients [10]. Understanding the plasticity of tumor—stroma interactions and how their differential responses to a given stimuli or repressors in the tumor microenvironment are modulated by cell signaling networks could lead to the design of more effective targeting strategies for preventing cancer progression and aid our future studies in precision medicine.

Our laboratory also investigated another ROS- and stress response-induced protein, disintegrin and metalloproteinase domain—containing protein 9 (ADAM9), which participates in cell—cell and cell—matrix interaction. This protein was identified by tissue microarray using our cell lineage—derived human LNCaP, C4-2 and C4-2B PCa cells. We noticed a significant increase of ADAM9 mRNA expression during androgen-deprived PCa progression toward castration-resistance. Upon further evaluation of ADAM9 protein expression, we noticed that certain pathophysiologic stress conditions, such as cell crowding, hypoxia and the addition of hydrogen peroxide, could induce a steady-state level of ADAM9 [24]. This increase of ADAM9 expression was confirmed by tissue staining [24,25], and also can be used as a biomarker in patient serum [26]. Interestingly, we observed that blocking ADAM9 expression could trigger the morphologic, biochemical and behavioral transition of PCa cells from mesenchymal to epithelial phenotype, thus reversing therapeutic resistance. These results suggest that ADAM9 could be targeted to provoke mesenchymal to epithelial transition (MET) through modulating epithelial specific characteristics, including increased expression of E-cadherin, specific integrin subtypes and polarization proteins [27]. Increased expression of ADAM9, interestingly, is not limited to PCa; we found that lung cancer cells specifically metastasized to the brain also overexpressed ADAM9 [28]. This finding suggests that ADAM9 not only serves as a determinant for morphogenetic changes of cancer epithelial cells [27], but also enhances cancer motility, invasion and metastasis [28]. Alternatively, ADAM9 may regulate specific proteins or complexes with dual morphogenetic and motility/invasion roles in cancer cells.

To evaluate the biological roles of ADAM9 during cancer progression and whether ADAM9 can be used as a therapeutic target, we permanently knocked down ADAM9 expression by small hairpin RNA (shRNA) [29] and showed that this impeded androgen-independent prostate tumor formation and cancer-induced skeletal osteolysis in a xenograft mouse model. Our results suggest the feasibility of using *in vivo* lentivirus-derived ADAM9 shRNA to reduce tumor burden in mice, confirming our hypothesis that therapeutic targeting of ADAM9 could be a viable option for PCa therapy. Our data support the notion that ADAM9 targeting could depress prostate tumor growth, invasion and metastasis by promoting MET, epithelium polarization and decreased cancer migration. We proposed that ROS-mediated elevation of ADAM9 levels and the associated enhancement of tumor growth and tumor metastases in PCa cells can be effectively targeted by either lowering intracellular ADAM9 or ROS using genetic or pharmacologic inhibitors.

3. Epigenetic mechanisms mediating PCa tumorigenesis and metastasis: recruitment and reprogramming of indolent bystander cells by an aggressive population of metastasis-initiating cells (MICs)

While the published literature overwhelmingly implicates the dominant roles of genetic factors (Dr. Coffey refered these factors as the "hardware" of cancer cells) to determine cancer cell tumorigenesis and metastasis, mounting evidence suggests that epigenetic factors in the form of secreted factors, ECMs or EVs or even subcellular organelles such as mitochondria or lysosomes participate in crucial information transfer between cells to determine the ultimate fate and behaviors of cancer cells. Dr. Coffey referred to these factors as the "software" of cancer cells. By tagging genetically distinct PCa cells with either green fluorescence protein (GFP) or red fluorescence protein (RFP), Chu and Chung [30] revealed the presence of a special population of MICs with a "leader cell" phenotype, appearing at the invasive front of cancer, that "coerces" indolent bystander cells to gain tumorigenic and metastatic potential to bone and soft tissues. This remarkable experimental observation supports the proposal that histopathologically invasive cells, arising from the invasive front of cancer, express a leader cell phenotype with collective invasive properties [31—33], migrating and invading cohesively with follower cells programmed behaviorally by the leader cells to function as a heterogeneous multicellular unit. This concept could also explain the "awakening" of dormant cells at metastatic sites by interaction between the population of intrinsically quiescent dormant cancer cells and the MICs (Fig. 1).

We first identified PCa cells with MICs phenotype in a genetically modified PCa cell population that overexpressed the receptor activator of NF-κB ligand (RANKL). MICs were found to exhibit the ability to recruit and reprogram indolent bystander PCa cells to cooperate in the metastatic process in bone and soft tissues when co-cultured under 3D conditions as chimeric organoids [30]. These RANKL-expressing MICs expressed elevated levels of epithelial to mesenchymal transition (EMT), stem cell, and neuroendocrine (NE) genes through enhanced transactivation by c-Myc/Max and *AP4* transcription factors (TFs) mediated by the receptor activator of NF-κB (RANK) signaling. Aberrant expression of genes represented by cells with MICs phenotype can confer their tumorigenic and metastatic phenotype to the recruited non-tumorigenic bystander cells in the tumor microenvironment, which then acquire MICs properties by expressing elevated EMT, stem cell, and NE genes and gaining tumorigenic and metastatic potential in mice and enhanced migration and invasion ability *in vitro* [34,35]. To validate the MICs phenotype in clinical PCa specimens, we developed a multiplexing quantum dot technology to stain MIC gene profiles at the single cell level. These studies identified MIC-expressing cells in PCa tissues collected from patients and also confirmed that the relative abundance of these cells in pathologic tissue specimens can predict progression to castration resistance and the overall survival of PCa patients [36,37]. In support of the MIC concept, we

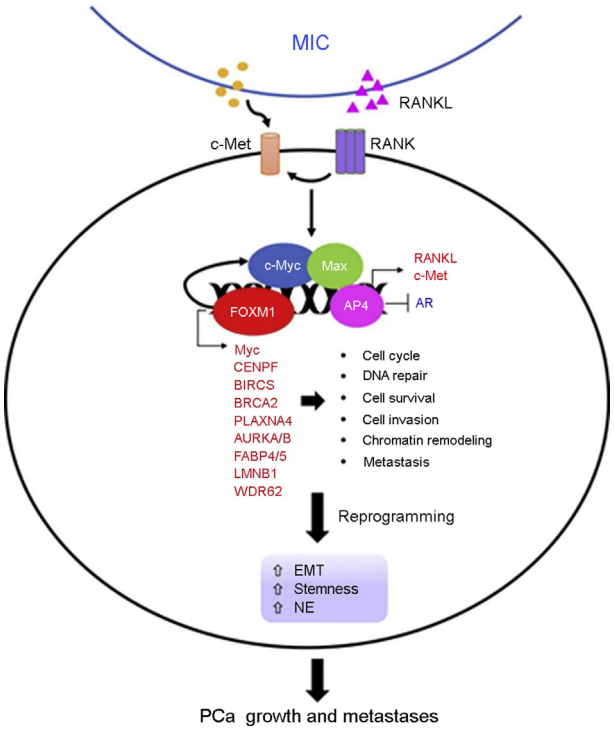

Figure 1 Schematic diagram illustrates the concept of tumor cell recruitment and reprogramming in the tumor microenvironment. Through cell—cell interaction, MIC in the tumor microenvironment has the potential of recruiting resident bystander cells or indolent or dormant PCa cells, resulting in reprogramming these cells to express increased EMT, stemness, and NE phenotypes through RANK and c-Met-mediated activation of FOXM1-c-Myc/Max-AP4 signaling to promote PCa progression and metastases. Results of these series of studies emphasize the importance of tumor microenvironment driving cancer progression and metastasis through epigenetic reprogramming. EMT, epithelial to mesenchymal transition; NE, neuroendocrine; PCa, prostate cancer; RANK, the receptor activator of NF-κB; MIC, metastasis-initiating cells; RANKL, receptor activator of NF-kB ligand; AR, androgen receptor..

characterized two naturally occurring MICs (nMICs) derived from *ex vivo* culture of the ascites fluid of a bone metastatic PCa patient. The nMICs not only expressed MIC signature and exhibited aggressive MIC phenotypes and behaviors [38—40], but also conferred tumorigenic and metastatic phenotype to newly established indolent PCa epithelial cell line as well as the normal prostatic epithelial RWPE-1 cells (unpublished observations). These results support the concept that PCa cells with MIC phenotype are the drivers of PCa progression and metastasis.

Seeking a molecular biological understanding to develop novel therapeutics targeting PCa bone and visceral organ metastases, Chu and collaborators [34] analyzed c-Myc and its downstream regulation of RANKL-RANK signaling governing the MIC phenotype. RNA-sequencing (RNA-seq) analysis identified FOXM1, a proto-oncogene [41], as the most significant transcriptional master regulator governing the gene expression changes in the programmed target cells. Gene Set Enrichment Analysis (GSEA) further revealed

that 511 of FOXM1 target genes are significantly enriched in the target cells after exposure to three different inducing MICs, genetically-transduced indolent LNCaP cells with RANKL (LN^{RANKL}) and two naturally-occurring nMIC-1 and nMIC-2 cells. Because of the much elevated levels of c-Myc and FOXM1 in PCa cells with MIC phenotype and in indolent PCa cells after being programmed by MIC, we performed *in vivo* animal studies confirming that JQ1, a small-molecule inhibitor targeting the amino-terminal bromodomains of BRD4 [42], which is required for the transcription of c-Myc and its downstream targets [43,44], inhibited tumor growth of the programmed target cells in mice. Further, we employed a small molecule inhibitor of FOXM1, thiostrepton, a thiazole antibiotic, as a natural inhibitor of FOXM1 transcription [45], and showed nearly complete abrogation of MIC-instigated *in vitro* recruitment and reprogramming and *in vivo* tumor formation in both the indolent target cells, suggesting that FOXM1 is an upstream regulator of c-Myc in mediating the reprogramming process, and that blocking FOXM1 is sufficient to inhibit downstream c-Myc signaling and activation. FOXM1 is known to be involved in stem or progenitor cell expansion important for cancer initiation [46], and recently FOXM1 was also identified in both human and mouse PCa models as one of the regulatory drivers of PCa malignancy. FOXM1 expression also serves as a robust negative prognostic indicator of PCa survival and metastasis [47]. Importantly, we also identified in clinical specimens that FOXM1 expression is correlated with PCa progression, with increased expression in high-grade and metastatic PCa, and is associated with poor survival of PCa patients (Chu et al., unpublished results). Our ongoing work is focused on identifying soluble factors in the tumor microenvironment and dietary factors such as cholesterol that could specifically regulate the extent of reprogramming of the indolent PCa cells by MIC, with the hope that understanding how MIC regulates PCa progression and metastasis could help us developing countermeasures to slow down, or even deprogram the already-programmed PCa cells at primary and metastatic sites.

Our study of the underlying molecular basis of cancer cell reprogramming pointed in the direction of epigenetic modifications of cellular DNA as important drivers of cancer cell tumorigenicity and metastasis, largely by promoter hypomethylation, histone modification, and chromatin remodeling to ultimately govern gene expression changes conveyed by interactions between TFs and DNA in the context of chromatin [48—50], particularly by changes in methylation status of specific gene promoters. Upon 5-azacytidine treatment to inhibit DNA methylation [51], we examined the expression of MIC genes in indolent target cells and confirmed their gene regulation by changes of DNA methylation status. We further performed methylation array analysis with EPIC, an updated version of the Human Methylation450 BeadChip by Illumina offers an ideal cost-effective solution for epigenome-wide association studies (EWAS), and identified several MIC signaling downstream genes that are hypomethylated at their promoter regions, including vimentin, aldehyde dehydrogenase 1A (ALDH1A), chromogranin A (CHGA), and androgen receptor (AR), reflecting their increased expression in programed target cells. These results will be the basis for evaluating global gene expression changes in MIC subjected to epigenetic control, so we can develop reprogramming interrupters for tumorigenic and metastatic PCa.

4. Modulation of cell junctional communication promotes bone and brain metastases of endocrine-related cancers

Intercellular junctional communication through adherins is pivotal in determining cell adhesion, cell—cell and cell—ECM communication affecting cell motility, growth and survival of normal epithelium and cell proliferation, invasion and metastasis of cancer cells [52—54]. Cell—surface adhesion complexes or plaques are known to link intracellularly with intermediate filaments that interconnect through a number of structural proteins, such as pakaloglobin and desmoplakin complexes, that together determine the phenotype and behavior of cancer cells through the activation of intracellular targeted oncogenic signaling pathways [55,56]. We studied one of the intermediate filament keratins, cytokeratin 13 (KRT13), which is known to be expressed by many non-cornified developing epithelial tissues including fetal prostate glands and is associated with the tubule initiating activity of prostate and urinary bladder cells. In adult tissues, KRT13 is expressed in megakaryocytes and also in bone metastatic PCa [57,58], but the functions of KRT13 are largely unknown.

We took a genetic approach, overexpressing KRT13 in a broad spectrum of indolent and aggressive human PCa and breast cancer (BCa) cell lines. We observed that overexpressing KRT13 greatly enhanced the malignant potential of all PCa and BCa cell models from indolent to moderately aggressive to exaggeratedly aggressive and metastatic phenotypes in bone, brain and/or other soft tissues [57]. We performed confirmatory studies by knocking down KRT13 in triple-negative BCa cells overexpressing KRT13, and observed their decreased malignant potential. BCa models allow us to either overexpress or knock down KRT13, depending on their intrinsic expression levels, to understand the function of this intermediate filament in endocrine-responsive cancers. We observed consistently that KRT13 is a driver for enhanced PCa and BCa growth and bone metastasis in mice. In contrast to PCa, KRT13 drove BCa to lung and liver but not brain metastasis. To understand the intracellular molecular switch by which KRT13 exerts its mode of action, we conducted an antibody pool-down assay and observed that KRT13 antibody co-immunoprecipated KRT13 with desmoplakin and plakoglobin complexes. KRT13 has higher affinity with desmoplakin, disrupts its complex with plakoglobin, and releases the suppression of plakoglobin on c-Myc oncoprotein and enhances metastasis of BCa cells to bone and soft tissues. We conducted a series of gene expression screenings of both PCa and BCa models using quantitative reverse transcription coupled polymerase chain reaction (qRT-PCR), Western blot and immunohistochemical stain (IHC) and found that KRT13 drives the expression of EMT, stemness and NE phenotype in PCa and BCa cells [59]. We observed in PCa and BCa cells that KRT13 promotes both osteomimicry and neuromimicry. In these studies, interestingly, although KRT13 overexpression drives PCa and BCa to mouse

skeleton, there was no alteration of RANKL, RANK and osteoprotegerin (OPG) expression in these cells, suggesting that KRT13 action is limited to interference with intercellular junctional communication and is not related to RANKL-RANK mediated cell signaling. These results allow us to speculate that developing RANKL-independent, but junctional communication-dependent agents as interfering therapeutics may prove to be an effective treatment for PCa and BCa bone and brain metastases.

5. Targeting mitochondrial and lysosomal organelles to overcome PCa therapeutic resistance

Therapeutic resistance is a stumbling block preventing the survival and worsening the quality of life of patients with PCa and other cancers. Numerous strategies have been attempted to overcome therapeutic resistance, including the classic approach of targeting multiple drug resistant genes responsible for drug transport and accumulation in cancer cells [60], targeting mechanistically alternative signaling pathways [61], or designing stronger antagonists bypassing the resistance mechanism [62], identifying the recurrence-initiating stem cells in tumors and designing specific agents to target this resistance-related population [63], and characterizing and modulating activated non-cancer cells in the tumor microenvironment including cancer associated fibroblasts, myeloid, immune and endothelial cells and mesenchymal stem cells in a broad-spectrum of cancer types to prevent their ability to confer therapeutic resistance [64,65]. Castration-resistant PCa (CRPC) and metastatic CRPC (mCRPC) are the lethal forms of PCa, often treated by androgen antagonists, abiraterone acetate, and enzalutamide or microtubular inhibitor taxanes [66]. Resistance inevitably develops after prolonged treatment. We need strategies to understand the mechanisms of development, and new therapeutic approaches to overcome therapeutic resistance. We tested a new class of tumor-cell specific heptamethine cabocyanine dye (HMCD)-based targeting agents [67—71]. HMCD was chemically conjugated with therapeutic drugs, such as cytotoxic docetaxel, gemcitabine, cisplatin, or targeted therapeutics against specific cellular regulatory proteins such as monoamine oxidase (MAO) or mTOR [70,72—75]. The molecular basis of tumor-specific targeting by HMCD was discovered by our laboratory over a decade ago. We recently expanded this study by designing new targeting drugs to overcome therapeutic resistance. The unique features of this class of new agents are: 1) HMCD is a tumor-specific small molecule that enters cancer but not normal cells by binding to a family of organic anion transporting polypeptides (OATPs); these membrane carriers are elevated in cancer compared to normal cells, due to higher intrinsic OATPs expression and activity in cancer compared to normal cells [70]. OATPs gene transcription is further enhanced by hypoxia, one of the hallmarks of cancer [73]. 2) In comparison to most existing chemotherapeutics, HMCD—drug conjugates kill cancer cells quickly and efficiently. 3) HMCD—drug conjugates induce programmed cell death through their interactions with mitochondria and lysosomes (see below). We and others have observed that HMCD—drug conjugates are not toxic, specifically deliver the drug payload to tumor but not normal cells, and can deliver a sufficient dose of drug to inhibit tumor growth in cultured cells and in tumor xenografts in mice. An extensive survey of how these drugs target the growth of tumors revealed that both tumor mitochondria and lysosomes seem to be the key targets of HMCD—drug conjugates. Molecular profiling supported the conclusion that all HMCD—drug conjugates seemed to share some common and overlapping downstream targets, such as inducing caspases-mediated programmed cell death, depressing mitochondrial oxidative phosphorylation, downregulating anaerobic glycolysis, inducing endoplasmic reticulum stress responses resulting in an accumulation of unfolded protein responses, and blockade of proteosome degradation despite increased protein ubiquitination activity. Many of these effects are believed to be triggered by the specific accumulation of these HMCD—drug conjugates in the mitochondrial and lysosomal compartments of cancer cells to trigger complex programmed cell death involving but not limited to compromised mitochondrial membrane potential and caspases activation. We are presently resolving the molecular mechanisms of this class of new agents, and asking the crucial question how the interactions of HMCD—drug conjugates could unequivocally reverse the therapeutic resistance of three mechanism-unrelated cytotoxic agents: androgen antagonists, abiraterone acetate, and enzalutamide and microtubular inhibitor taxanes, which have three distinct mechanisms of action against the growth of CRPC and mCRPC in patients. It is fascinating that resistance to all three can be reversed by a single HMCD—drug conjugate.

6. Co-targeting tumor—stromal interaction in mCRPC

It is well accepted that cancer growth, progression, and metastasis is intimately affected by its interaction with the host microenvironment [76]. Strong experimental evidence, validated by clinical specimens, suggests that cancer epithelial cells can induce permanent genotypic and phenotypic changes in stromal cellular components, including the resident stromal fibroblasts at the primary, osteoblasts and marrow stromal cells at distant metastatic sites, and infiltrating mesenchymal stem cells or immune cells, all of which ultimately contribute to PCa tumorigenesis and metastasis [10,77—79]. Conversely, reciprocal stromal induction of epithelial genotypic and behavioral changes also occurred, mediated by cell adhesion molecules, soluble factors and ECM components in the tumor microenvironment. This reciprocal tumor—stroma interaction presents an attractive opportunity to test the co-targeting concept for therapeutic intervention [80]. We tested this concept by developing gene therapeutics co-targeting PCa and bone stroma, using tissue-specific and tumor-restrictive promoters directing replication-deficient adenoviral gene therapy to drive the expression of therapeutic genes in both PCa and bone stroma. We selected the promoters of non-collagenous bone matrix proteins such as osteopontin (OPN), osteocalcin (OC), bone sialoprotein (BSP) and osteonectin (ON), as the potential drivers of therapeutic

genes expressed in both bone stromal and PCa epithelial cellular compartments. It has been shown that during osteoblastic differentiation for the maintenance of bone mass, high levels of OPN, OC, BSP and ON are co-expressed in the stromal and epithelial compartments of metastatic PCa specimens [81—84]. We hypothesized that in order to thrive and grow in the bone environment, PCa cells must acquire osteomimetic or bone-like properties while in the primary and at the metastatic environment [85]. Because 20% of PCa patients do not have elevated level of prostate-specific antigen (PSA) despite the detection and progression of the disease [86], the promoters of osteogenic proteins could be used as tissue-specific and tumor-restrictive promoters to drive the expression of therapeutic genes in prostate tumors, irrespective of their basal level of AR and PSA expression. Also, targeting both the tumor epithelial cells and the supporting bone stromal cells using the same promoter could achieve either an additive or a synergistic effect in maximizing cell-kill. This hypothesis was initially proven in our previous studies [87] using an adenoviral vector carrying OC promoter-driven herpes simplex virus thymidine kinase (TK). The Ad-OC-TK construct was originally designed for osteosarcoma gene therapy [88], which achieved greater growth inhibition than Ad-PSA-TK (a vector carrying human PSA promoter-driven TK transgene) when combined with appropriate prodrug ganciclovir (GCV) or acyclovir in a co-culture model consisting of PCa cells and osteoblasts [89]. With these promising results from preclinical animal studies [90] where Ad-OC-TK plus GCV effectively eliminated human prostate tumor growth in immunocompromised mice at both subcutaneous and bone sites, Ad-OC-TK/prodrug therapy was implemented for phase I/II clinical trials for metastatic or locally recurrent PCa [91,92]. Similarly, a novel human osteonectin promoter (hON-522E) was recently identified and constructed into adenoviral vectors to direct the therapeutic suicide gene toward both androgen-independent PCa cells and cancer-associated fibroblasts at both primary and metastatic sites, for potential treatment of hormone refractory and bone metastatic PCa [79].

6.1. Oncolytic viruses to co-target tumor and bone stroma

It has been shown that replication-competent adenoviruses could have delivery advantages over targeting agents to tumors directly. These oncolytic viruses rely on the natural ability of viruses to infect, replicate within, and ultimately lyse the host tumor cells and could be an promising approach to tumor-specific targeting. To extend our efforts to develop co-targeting adenoviral gene therapy for better treatment outcomes, we generated a conditionally replication-competent adenoviral vector, Ad-OC-E1a, whose viral replication was controlled by mouse OC promoter-directed viral early E1a gene expression. Unlike replication-deficient adenoviral therapy, which requires repeated treatment, a single dose of Ad-OC-E1a was demonstrated to suppress the intraosseous growth of human PCa xenografts [87]. Because both the *E1A* and *E1B* genes are essential for adenovirus replication, Ad-hOC-E1, an analog of Ad-OC-E1a that contains a vitamin D3 inducible human OC (hOC) promoter to drive *E1A* and *E1B* genes in a bi-directional manner, showed high specificity and efficacy controlling AR- and PSA-negative PCa growth in mice through systemic administration, either as monotherapy [93] or combination therapy with other modalities such as anti-angiogenesis therapy [94]. The unique features, including stringent control of both the E1A and E1B gene by a single promoter but with heterologous sequences to prevent homologous recombination, caused transgenes deletion and ligand (vitamin D3) dependent activation of OC transcription leading to a rapid induction kinetics and refined the ability of OC promoter to modulate the viral product to reach the therapeutic range for selective cytotoxicity in PCa irrespective of the basal AR and PSA status (Fig. 2). Significantly, this approach co-targeting tumor and bone stromal components makes Ad-hOC-E1 a superior vector to the current PSA-dependent oncolytic adenoviruses such as CN706 [95] and CN787 [96], which are under clinical trials as PSA-dependent oncolytic adenoviruses for the treatment of men with CRPC.

6.2. Genetic targeting of adhesion processes in the metastatic cascade by RNA interference

The propensity for metastasis has recently been linked to the accumulation of certain ECM proteins within the metastatic niche. While it is well established that integrin expression by endothelial cells during the process of angiogenesis facilitates the spread of cancer cells to other tissues, the function of integrin on the cell surface of tumor cells to directly bind ECM ligands and trigger the signaling pathways necessary for cell motility, invasion, proliferation and survival is still being interrogated [97,98]. Expression profiling correlated the upregulated αv integrins with prostate tumor progression to advanced stage [99], and this phenotype is also correlated with the co-expression of a metastatic stem/progenitor cell phenotype [100]. In bone metastatic PCa cells, active $\alpha v\beta 3$ and $\alpha v\beta 5$ are necessary for tumor cell adherence and migration along the gradient of bone matrix proteins such as OPN and vitronectin, to support tumor cell homing and colonization at metastatic niche [101]. Taking the unique features of small interfering RNA (siRNA) for sequence-specific gene silencing, we designed siRNAs targeting the integrin αv subunit to interfere with the integrin-ECM adhesion process [102]. The *in vitro* data confirm the ability of integrin αv-targeted siRNAs to reduce the prometastatic phenotypes of PCa cells in response to the bone matrix cue, vitronectin. Intratumoral delivery of αv-targeted siRNAs significantly regressed prostate tumor xenografts in mouse bone but not in subcutaneous sites, thus implicating the critical role of αv-containing integrin receptor for prostate tumor survival in the bone microenvironment and the feasibility of constructing αv-mediated siRNAs for PCa bone metastasis therapeutics.

Besides cell—matrix interaction, cell junctional communication via cell adhesion and intermediate filament complex presents another attractive therapeutic target for metastatic PCa. The L1 cell adhesion molecule (L1CAM), previously implicated in the development and plasticity of the nervous system, has been reported for aberrant

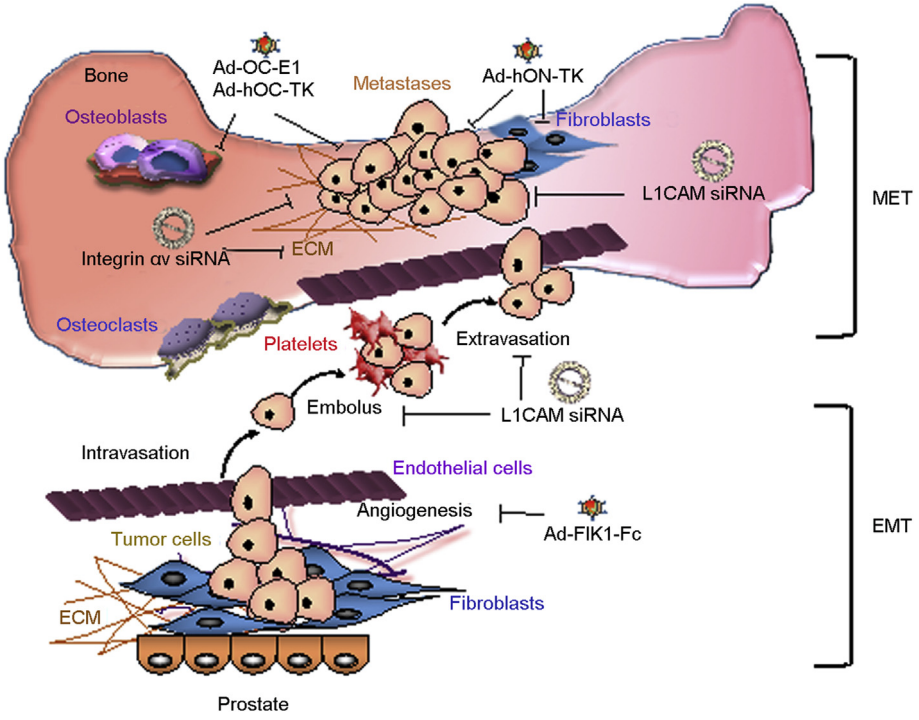

Figure 2 Gene-based therapies for mCRPC demonstrated in our studies by dually targeting tumor—stromal interactions in multiple steps of the metastatic cascade. Therapeutic targets in primary and metastatic sites against both PCa and cancer-associated stromal cells are outlined. This figure summarizes our concept of therapeutic co-targeting of tumor and stroma using gene therapeutic approaches. This concept is based on our observation that PCa cells, upon progression to develop bone metastatic potential, expressed high levels of genes mimicking the bone, or exhibiting osteomimicry phenotype, such as expressing non-collagenous bone-like proteins, osteocalcin (OC), osteopontin (OPN), osteonectin (ON) and bone sialoprotein (BSP). We constructed both replication-deficient and -competent adenoviral vectors using the bone-like protein promoters driving either therapeutic gene, thymidine kinase (TK), or E1, that render the viruses to become replication competent, to co-target both tumor and bone compartments, and observed cytotoxic effects against tumor growth in mouse skeleton. We have also observed beneficial effects in PCa tumor regression in mice at various anatomical sites by targeting cell—matrice interactions. We observed ECM—integrin interaction via $\alpha v\beta 3$ and $\alpha v\beta 5$, or interaction of PCa—cell adhesion-intermediate filament complex, through L1 cell adhesion molecule (L1CAM), can be effectively targeted by target-specific siRNA constructs. EMT, epithelial to mesenchymal transition; MET, mesenchymal to epithelial transition; mCRPC, metastatic castration-resistant prostate cancer; PCa, prostate cancer; ECM, extracellular matrix.

expression and involvement in cancer progression [103]. L1CAM-mediated cell—cell interaction can occur through homophilic ligation or heterophilic binding with different partners, including integrins, receptor tyrosine kinases, CD24, neurocan, neuropilin-1 and other members of the neural cell adhesion family [104]. Adhesion between cancer cells and endothelial cells, mediated by the L1CAM-ligand interaction, has been implicated in tumor cell extravasation during metastatic dissemination [105]. Therefore, L1CAM association with poor outcome in patients is expected from the aforementioned functions of this protein due to its role in promoting cancer cells to undergo EMT, detach from the primary tumor, invade surrounding tumor microenvironment, gain tumor-initiating capacity, resistance to treatment, and ultimately tumor metastasis and recurrence [106]. Apart from the EMT pathway during the early stage of metastasis, our study provided the first link between L1CAM and MET, a process responsible for cancer colonization and metastasis by increasing the adhesiveness and bulkiness of the cell aggregates when tumor cells reach the distant sites [107]. The involvement of L1CAM in almost the full spectrum of the metastatic cascade makes it an attractive target for therapeutic intervention. In our study, L1CAM-siRNAs efficiently inhibited PCa cell metastasis and suppressed the growth of bone metastases in preclinical mouse models [107]. Collectively, these results strongly support our concept that siRNA-based therapeutic strategies targeting EMT and/or MET would have clinical benefit for patients with mCRPC (Fig. 2).

7. Assessment of a triad relationship between tumor—bone—immune cell interaction in PCa metastasis

Several studies have shed light on the myriad facets through which immune cells are involved in tumor cell interactions with the surrounding microenvironment in their journey to distant metastatic sites. It is now known that specific types of immune cells play discrete roles either abetting or hindering the tumor cell's complex and inefficient journey at specific steps of the metastatic cascade,

involving invasion, intravasation, extravasation, colonization and growth in the metastatic microenvironment [108,109]. We designed studies to test the hypothesis that the immune microenvironment plays a critical role in the metastatic process by influencing the tumor cells (seed), or their site of metastasis (soil), or both. This question was addressed by creating a novel syngeneic pre-clinical mouse model to investigate the biology of PCa bone metastasis in an immune intact mouse model.

Though transgenic mouse models offer the inherent advantage of an intact immune system, PCa transgenic mice that develop bone metastasis at a frequency adequate for studying the biology and therapy of PCa bone metastasis are very rare. For this reason, we investigated whether genetically engineering mouse PCa cells could promote their bone homing potential. We transduced mouse PCa cells with RANKL, a member of the tumor necrosis factor (TNF) family and a crucial mediator of PCa/osteoblasts and osteoclasts. RANKL/RANK signaling leads to the continuous activation and survival of osteoclasts and therefore allows unchecked bone resorption [110]. Studies using osteoprotegerin (OPG), a soluble decoy receptor that blocks all the effects of RANKL [111], found that RANKL is a mediator of human PCa-induced bone destruction, and that human PCa bone metastases express significantly higher levels of RANKL compared to non-osseous metastases or primary prostate tumors [34,112,113]. Because RANKL overexpression in human PCa cells drives PCa bone metastasis in immune-deficient mice, we tested whether RANKL overexpression in mouse PCa cell lines could also drive bone colonization. Results of these studies confirmed that RANKL did drive mouse PCa cells to bone colonization. Control *neo*-transfected MPC3 mouse PCa cells failed to develop bone metastasis in C57/B6 mice, whereas RANKL overexpression promoted the development of PCa bone metastasis in about 40% of the syngeneic hosts. We observed that the CXCL12/CXCR4 axis is dramatically upregulated during bone metastasis in the syngeneic mouse model. Our study provides strong evidence that to develop successful bone metastasis, PCa cells rely on secretion of specific chemokines inducing directional migration of cancer cells towards the bone site, thus supporting an intimate interaction between PCa cells and bone cells within the bone niche (Fig. 3).

Several studies have determined the pivotal role of the CXCL12/CXCR4 chemokine axis in the homing of PCa cells to the bone marrow [114—117]. CXCR4 expression is elevated in PCa cell lines [118], and CXCR4 is an independent prognostic factor for overall survival in PCa patients [119]. Further, PCa cells home to the bone marrow through the CXCL12/CXCR4 axis by competing with the hematopoietic stem cells, and the mobilization of both types of cells can be blocked by CXCR4 inhibition [120]. Importantly, CXCR4 is also reported to regulate the tumor immune microenvironment through its ability to decrease intra-tumoral regulatory T-cells [121]. Further, studies have shown that CXCL12/CXCR4 and RANKL/RANK signaling independently impinge upon PI3KAKTNF-κB, a convergent pathway that regulates cell survival, proliferation, metastasis, stemness and immune response [122—126]. Thus, blocking RANKL is thought to affect the immune system, since RANKL is expressed in immune cells in addition to bone cells, and is

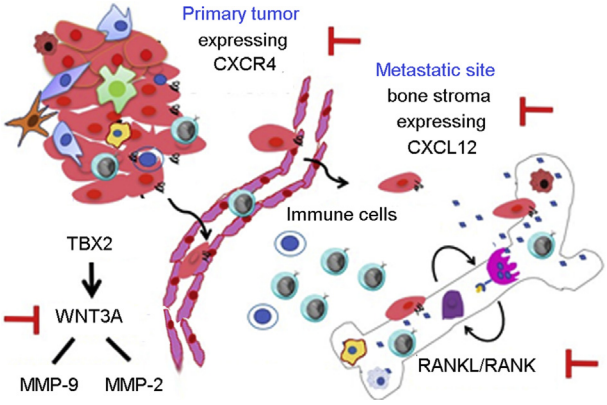

Figure 3 Factors modulating co-evolution of PCa and cancer-associated stromal cells. Diagram depicting the interaction of factors in the prostate primary tumors (seed) and the bone metastatic site (soil) with components of the immune microenvironment during the progression of the metastatic PCa. We identified additional molecular targets that can be manipulated in PCa local tumor growth and PCa tumor growth in bone. This includes inhibition of CXCR4 receptor and Wnt 3a in the primary PCa, and CXCL12 and RANKL-RANK communication in bone metastatic PCa. RANKL, receptor activator of NF-κB ligand; RANK, receptor activator of NF-κB; PCa, prostate cancer; CXCR4, CXC chemokine receptor 4; MMP-9, metalloproteinases-9; MMP-2, metalloproteinases-2.

essential for the development and activation of T cells and dendritic cells [111,127]. These findings clearly point to the indispensable need for an intact host immune system to investigate CXCL12/CXCR4 and RANKL/RANK signaling convergence. Denosumab, a fully humanized monoclonal antibody that blocks RANKL, has been approved for patients with PCa bone metastases. Clinical trials have shown that denosumab significantly increases bone metastasis-free survival in patients with non-metastatic CRPC [128,129]. However, the impact of denosumab on overall patient survival, pain metrics and other indices of quality of life remains equivocal [130].

Plerixafor (AMD3100), a small molecule CXCR4 inhibitor, is currently being examined in clinical trials in combination with other inhibitors of pro-oncogenic pathways [131], and is currently being tested in clinical trials against metastatic PCa. Additionally, CXCL12 is increased in the circulation of PCa patients after hormone therapy compared to control patients with surgery or no treatment [132]. Given that CRPC has a striking preference for skeletal localization, these findings emphasize the relevance of targeting the CXCL12/CXCR4 axis in advanced PCa since the CXCL12/CXCR4 axis is associated with both of the crucial aspects of the disease, *i.e.*, progression to CRPC and homing to bone.

In sum, due to the lack of suitable mouse models to study human PCa bone metastasis, we developed a syngeneic mouse model of PCa bone metastasis that allows us to study the twin steps of 1) homing to the bone, and 2) growth in the bone microenvironment in an immune-intact host. Our model suggests that there is interplay between CXCL12/CXCR4 and RANKL/RANK pathways in the manifestation of PCa bone metastasis. In addition, studies have shown independently that CXCL12/CXCR4 and RANKL/RANK

signaling regulate a common pathway PI3KAKTNF-κB that mediates tumorigenesis and the metastatic cascade [122—126]. The CXCL12/CXCR4 axis is critical for the homing of PCa cells to the bone and the RANKL/RANK pathway plays a crucial role in the subsequent interaction and growth of PCa cells within the bone microenvironment. These two pathways control two discrete stages in the manifestation of PCa bone metastasis.

Our work has also led to a further understanding of the T-box family of transcription factor TBX2 with canonical WNT signaling axis as a potential novel therapeutic target for PCa bone metastasis. In our study we co-ordinated signaling between TBX2 and WNT3A, in which the T-box family of transcription factors, including TBX2 known to play critical roles during embryonic development [133—135], and the WNT signaling pathway documented to mediate a plethora of biological processes including cell proliferation and survival. Additional recent reports have implicated TBX2 in the transcriptional repression of $p14^{ARF}$/$p19^{ARF}$ [136,137] and p21 expression [138]. Intriguingly, a report showed that the 17q23 amplicon that harbors TBX2 is amplified in 46% of late stage hormone refractory adenocarcinomas and 31% of metastases [139]. Likewise, canonical WNT signaling culminates in the stabilization and translocation of β-catenin to the nucleus and resultant activation of downstream targets linked with increased PCa tumorigenesis and metastasis.

Using a combination of *in vitro* assays and *in vivo* xenograft approaches, we investigated the specific role of TBX2 in PCa progression, with an emphasis on invasion and metastasis. Utilizing various pre-clinical mouse models, we asked if TBX2 mediates local invasion to lymph nodes, metastasis to bone, and subsequent bone remodeling events that ensure PCa cell colonization and growth in the bone microenvironment. Our data demonstrated that blocking endogenous TBX2 reduced PCa cell proliferation and invasion *in vitro*, abrogated PCa metastasis to the bone, and greatly reduced the *in vivo* ability of PCa cells to grow within the bone microenvironment. We further found that WNT3A signaling is a crucial mediator for TBX2 in PCa metastasis, thereby raising the possibility of targeting WNT3A for the clinical management of men with locally invasive and metastatic PCa. We believe this newly unravelled TBX2-WNT3A signaling pathway is an exciting opportunity to develop novel therapies targeting PCa bone metastasis [140].

8. MAOA-ROS signaling-dependent tumor-stromal interaction promotes PCa metastasis

Reactive oxygen species (ROS), mainly consisting of superoxide anion radical ($O_2^{\cdot-}$), singlet oxygen, hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical, are vital for nearly every stage of PCa development and progression. Increased ROS generation was observed in PCa cells compared with normal prostate epithelial cells, and this increment continues during disease progression to more aggressive phenotypes, such as metastatic PCa [141]. A recent meta-analysis of 23 independent case-control studies with 6439 participants in total also revealed that oxidative stress, using plasma malondialdehyde levels as an indicator, was significantly higher in PCa patients compared with control groups [142]. PCa cells produce persistently high levels of ROS after undergoing oncogenic transformation, including genetic, metabolic and tumor microenvironment alterations. In turn, ROS likely promotes PCa by ROS reactivity towards such key cellular components as nucleic acids, proteins and lipids [143]. ROS may also be a signaling molecule mediating multiple signaling pathways critical for cell growth, survival, migration and invasion. For example, ROS was shown to activate the RAS-MAPK/ERK and PI3K/AKT/mTOR pathways, promote the production of prostaglandin E2 and matrix metalloproteinases, and stimulate the expression of perlecan/heparin sulfate proteoglycan augmenting PCa cell growth in bone microenvironment [144,145]. These studies suggest that therapies reducing ROS production may be effective against currently incurable advanced and metastatic PCa.

The mitochondrial respiratory chain contributes the maximum in ROS generation in cancer cells through consumption of approximately 80% of molecular oxygen during oxidative phosphorylation [143]. Recently, an additional mitochondrial protein, MAO, emerged as a major ROS source with potential pathophysiological relevance to a variety of types of cancers, including PCa [146,147]. MAO is a ubiquitously-expressed flavoenzyme localized at the outer mitochondrial membrane that exists in two isoforms, MAOA and MAOB, which differ in tissue distribution, substrate selectively and inhibitor specificity. MAOA degrades a number of biogenic and dietary amines, including monoamine neurotransmitters such as serotonin, norepinephrine and dopamine. By catalyzing the electron transfer from amines to molecular oxygen, aldehydes, ammonia and H_2O_2 are formed as by-products, with H_2O_2 constituting a major form of ROS. MAOA has been most widely studied in the brain, where aberrant activity is associated with aggression, depression and other neuropsychological disorders. MAO inhibitors (MAOIs) are currently used in the clinic as antidepressants [148].

Since the initial discovery associating high levels of MAOA expression with poorly differentiated PCa [147], our appreciation for this old protein's new function in PCa has increased dramatically over the past few years. In our first report of this series of investigations, we demonstrated that knockdown of MAOA significantly reduced or even eliminated prostate tumor growth and metastasis in PCa xenograft mouse models. High MAOA expression in PCa tissues correlates with worse clinical outcomes, including metastasis (to lymph node, bone and soft tissues), seminal vesicle invasion, biochemical recurrence and decreased survival, in multiple independent PCa patient cohorts. Mechanistically, MAOA induces EMT via the generation of ROS to impair PHD3 activity and stabilize hypoxia inducible factor 1α (HIF-1α) in PCa cells. After augmenting hypoxia, MAOA activates vascular endothelial growth factor (VEGF) and its co-receptor neuropilin-1 (NRP1) to promote AKT/FOXO1 signaling. We showed that FOXO1 is a transcription repressor of Twist1, a known master regulator of EMT, and that phosphorylation of FOXO1 enables its nuclear export, resulting in increased expression of Twist1 to induce an EMT program in PCa cells. Importantly, the novel MAOA-dependent HIF-1α/VEGF/FOXO1/Twist1 signaling pathway is manifested in high-grade PCa specimens. By converging the functional interplay among EMT, hypoxia and oxidative

stress, MAOA stimulates PCa cell growth, invasiveness and metastasis [149].

We next conducted a sequel study to attempt to define MAOA's role specifically in PCa metastasis, especially to bone, the predominant site for harboring metastatic deposits of PCa, with emphasis on dissecting the reciprocal interactions mediated by MAOA between PCa cells and surrounding stromal cells. We found that MAOA expression increases with PCa metastasis in clinical specimens as well as established lineage-related human PCa cell lines with differential metastatic potential (C4-2 *vs.* C4-2B, PC-3 *vs.* PC-3M, and $ARCaP_M$ *vs.* $ARCaP_{BMC2}$). Using an intracardiac xenograft model to rapidly develop distant metastasis in mice, we showed that MAOA promotes both PCa bone and visceral metastases with higher incidence, more circulating tumor cells, and greater tumor burden and osteolytic lesions when MAOA is overexpressed in PC-3 cells. Histologic evidence reveals a heterogeneous mixture of osteolytic and osteoblastic lesions in PCa bone metastasis, with osteoblastic lesions often characterized radiographically as the dominant type of lesion [150,151]. Although osteolytic lesions were mainly observed in our mouse models by X-ray and microCT examinations, we showed that MAOA is capable of affecting the osteoblastic features of bone metastases by reduced expression of a panel of markers of osteoblastic metastases, including OPG, phosphoglycerate kinase 1 (PGK1), preportachykinin (TAC1) and elastin microfibril interfase (EMI) domain containing 1 (EMID1), in MAOA-knockdown bone metastases formed by C4-2 and $ARCaP_M$ cells with known mixed osteolytic/osteoblastic phenotype [152,153]. We further confirmed this in tumor-bone cell *in vitro* cell co-cultures where tumor MAOA promotes osteoclast maturation and osteoblast differentiation and mineralization. These results suggest that MAOA may drive the development of osteoblastic lesions after initiating a metastatic niche via osteoclastic resorption. We screened a short list of known mediators of tumor-stromal interactions and determined that sonic hedgehog (Shh) is upregulated by MAOA in PCa cells. MAOA induces Shh through direct binding of Twist1 to an E-box element in the Shh promoter. Having established a MAOA-Twist1-Shh regulatory circuit within PCa cells to engage paracrine Shh signaling in tumor—stromal crosstalk, MAOA confers PCa cells with growth advantages in the bone microenvironment by stimulating inerleukin 6 (IL-6) release from osteoblasts through Gli1/Gli2-dependent transcriptional activation of the *IL-6* promoter. In coordination, MAOA triggers skeletal colonization by activating osteoclastogenesis through osteoblast production of RANKL and IL-6, which are both mediated by Shh paracrine signaling. These mechanistic insights outline the MAOA-driven Shh-IL-6-RANKL signaling network, which promotes tumor seeding and colonization to bone and visceral organs by creating a vicious cycle between PCa cells and stromal cells. Finally, we demonstrated that MAOA inhibitor treatment effectively restricts metastasis and prolongs mouse survival by disengaging the Shh-IL-6-RANKL signaling network in stromal cells in the tumor microenvironment. This is a strong pre-clinical rationale for targeting MAOA and its associate molecules to treat PCa metastasis [154].

Collectively, our recent work demonstrated a MAOA-mediated complex triad of cell—cell interactions regulating PCa and osteolytic/osteoblastic microenvironment phenotypes. Blocking MAOA or co-interrupting one or a small number of the MAOA downstream key signaling node(s) may uncouple the vicious cycle driving tumor cell growth and dissemination to bone and other visceral sites. Using the ROS-generating enzyme MAOA as an example, our findings also underscore the functional and mechanistic importance of ROS in activating several signaling cascades underlying tumor—stromal interactions in PCa metastasis. Of translational importance, our work broadens current treatment regimens for metastatic PCa by adding a promising option to repurpose approved MAOIs for the treatment or prevention of PCa metastasis, which can benefit PCa patients suffering from incurable disease.

9. Roles of an imprinting class of miRNA in tumor—stroma interaction

To understand the mechanism of PCa bone metastasis, our laboratory established several lineage-related and translationally relevant tumor and stromal models [40,155]. These models have been used for several decades to understand the development of PCa and for drug discovery. These models provide highly valuable insights into molecular alterations associated with PCa progression. Using the LNCaP and ARCaP lineage-related PCa cell lines and the cancer-associated fibroblast (CAF) models, we performed a whole genome miRNA analysis. We identified a cluster of miRNA in the maternally imprinted gene cluster that are upregulated in PCa with high Gleason score, and in CAF in metastatic PCa [156—158].

9.1. miRNA in the tumor compartment

These include miR-409-3p/-5p, miR-154* and miR-379, which are activated in the mesenchymal compartment of the tumor microenvironment. These miRNAs are turned on in early embryogenesis and silenced thereafter [159,160]. However, during tumor development and progression these miRNAs are re-activated to develop a highly aggressive form of cancer. miR-409-3p expression in PCa patients was specific to PC aspecimens and its elevated levels correlated with poor prognosis [157]. We demonstrated that overexpression of miR-409-3p/-5p in PCa cells induced EMT. Remarkably, direct introduction of miR-409-3p/-5p, as viral particles directly to the dorsolateral lobe of the mouse prostate gland, promoted oncogenic transformation and the development of large tumors. miR-409-3p blocks tumor suppressors, such as Ras suppressor protein 1 (RSU1), polyhomeotic complex 3 (PHC3) and Von-Hippel Lindau protein (VHL). Inhibition of these genes results in the activation of oncogenes such as the Ras/MAPK pathway, epigenetic reprogramming and hypoxia-mediated signaling [157]. Overexpression of miR-409-5p led to a decrease of stromal antigen 2 (STAG2), which results in deregulation of the cohesion complex [161,162], aneuploidy and increased DNA damage. Increases in miR-409-5p could result in more DNA damage in cancer cells giving rise to a more aggressive phenotype. Inhibition of miR-409-5p in $ARCaP_M$ PCa cells inhibited tumor formation and tumor metastasis [157]. miR-154* and miR-379 are other miRNA increased in prostate

tumor tissues [156]. Elevated expression of miR-379 in PCa patients correlated with poor prognosis [156]. Interestingly, miR-154* and miR-379 were also elevated in response to radiation treatment [163]. Activation of these miRNAs may lead to resistance to radiation treatment. Inhibition of miR-154* in $ARCaP_M$ PCa cells resulted in reduced tumor growth and bone metastasis. MiR-154* also targets STAG2 and SMAD7 [156]. SMAD7 plays a critical role in transforming growth factor β (TGFβ) pathway inhibition. These miRNAs in the megacluster activate oncogenic as Ras, HIF-1α, AKT-signaling and genes involved in EMT, stemness and resistance to treatment, leading to metastatic cancer. These miRNAs are strong predictors of PCa progression and metastasis.

9.2. Cancer—microenvironment crosstalk

To tease out the stroma-mediated effects on cancer, we performed a genome-wide miRNA analysis of CAF obtained from PCa patients using laser capture microscopy and also from bone stromal models grown adjacently with PCa. We found few of the miRNAs in the DLK1-DIO3 megacluster were activated in the CAF or bone stroma, including miR-409-3p/-5p, miR-154* and miR-379 [158]. Using the more sensitive *in situ* hybridization and quantum dots (ISH-QD) methods, we detected that miR-409-3p was significantly elevated in the stroma of PCa patients with high Gleason score (≥7). Ectopic expression of miR-409 in normal stroma induced a cancer-associated fibroblast-like phenotype with dramatic increases in vimentin, β2-microglobulin (β2-M), and smooth muscle α-actin. Increases in miR-409 resulted in a decrease in targets of miR-409-3p, such as RSU1, PHC3, and miR-409-5p targets like STAG2 and retinoblastoma-like 2 (RBL2) [158]. To better understand the crosstalk between the stromal and tumor compartments, we determined the levels of miR-409-3p/-5p in EV in normal stromal cells expressing miR-409 compared to normal stromal cells. miR-409-expressing stromal cells had high levels of miR-409-3p and moderate levels of miR-409-5p. Interestingly, miR-409-expressing stromal cells had increased blebbing and secretion of EVs compared to their normal counterpart. This secretory phenotype released large amounts of miR-409 from EVs into the surrounding microenvironment. We showed that stromal EVs with miR-409 were taken up by cancer cells not expressing miR-409, such as $ARCaP_E$ cells. Exposing cancer cells to stromal EVs resulted in increased miR-409 expression levels in 2 weeks, and stabilized miR-409 expression in cancer cells. The expression of miR-409 in cancer cells drove them to undergo EMT. C4-2 cells not expressing miR-409 were injected into mice with either normal stroma (without miR-409) or with normal stroma expressing miR-409. The expression of miR-409 in the stroma drove the C4-2 cells to form explosive tumors [158]. The other groups did not develop tumors at a 3-week time point. miR-409-3p/-5p was stained in these tumors using ISH-QD analysis. These miRNAs were not only found in the stromal compartment but also in the tumor compartment, suggesting that EVs from the stroma containing miR-409 were taken up by adjacent tumor cells and activated oncogenic Ras/MAPK and STAG2 pathways, EMT genes like *vimentin* and *β2-M* and embryonic markers such as SOX2 and NANOG4. These studies define the role of reactive stroma in inducing tumor growth, EMT, bone metastatic ability and reactivation of embryonic pathways to form aggressive treatment-resistant tumors. miR-409-3p, miR-154* and miR-379 were found to be elevated in the serum of patients with PCa [164], BCa [165] and lung adenocarcinoma [166] and these miRNAs could be used as predictive biomarkers for rational choices in drug treatment.

10. Final thoughts

Looking into the future, based on our collective wisdom of understanding the biology and therapy of tumor microenvironment, we suggest the following areas of investigation could be considered: 1) To define and prioritize key tumor microenvironmental factors that are the drivers for cancer cell invasion, migration and metastasis and the molecular underpinnings of why cancer cells develop therapeutic resistance; 2) To initiate new approaches by understanding how epigenetic mechanisms could profoundly affect cancer cell behaviors, including invasion, metastasis, and therapeutic resistance; 3) To employ new and high throughput technologies to enumerate multiple measurements of cells, tumors, molecules and cells in the tumor microenvironment, subjecting to experimental manipultions that are relevant, and closely mimic the behaviors of cancer cells in the patients; 4) To analyze big data collected from laboratory measurements using computational approaches and to validate such data using well-standardized model systems; and 5) To accelerate drug development plan by improving drug delivery, repurposing existing drugs, rational combination of drugs, and new technologies uncovering biomarkers or through effective imaging of small size tumor lesions for early rapid surgical or therapeutic interventions.

As students with the good fortune to be mentored either directly or indirectly by Dr. Coffey, we experienced the excitement of science beyond the publication of high-impact papers. In the laboratory, we do not follow "rules or hypotheses" laid down by Dr. Coffey, because there was none. Dr. Coffey taught us to love science and knowledge with passion, but challenged us to think beyond existing authorities using our own reasoning, and to test new hypotheses with well-designed studies. He always encouraged us to ask the question, "If our observations are true then what do they imply?" We put Dr. Coffey's teachings to use for common sense solutions to the problems outlined in this article, drafted by Leland and his students and colleagues for the readers to evaluate and hopefully to enjoy. This article serves as examples of several interrelated principles of cancer biology and therapy of PCa research we have engaged in the past, as eloquently summarized by the contributors of this article, whose scientific reasoning undoubtedly benefited from the original teachings of Dr. Coffey.

Author contributions

Study design: Gina Chia-Yi Chu, Leland W. K. Chung, Murali Gururajan, Chia-Ling Hsieh, Sajni Josson, Srinivas Nandana,

Shian-Ying Sung, Ruoxiang Wang, Jason Boyang Wu, Haiyen E. Zhau.
Data acquisition: All authors listed above.
Data analysis: All authors listed above.
Drafting of manuscript: All authors listed above.
Critical revision of the manuscript: Leland W. K. Chung.

Conflicts of interest

The authors declare no conflict of interest.

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