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*Review Article*

# Role of prostate and bone stromal cells on prostate cancer progression

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**Abstract:** Prostate cancer is a highly heterogeneous disease, often manifesting in a metastatic form to the bone. Complex tumour cells and surrounding microenvironment interactions are important determinants of disease progression and therapy resistance. Here, we provide an overview of some of the early studies that recognized the pro-tumourigenic role of prostate stroma, particularly fibroblasts, bone stromal components, and its permissive tumour properties. This article is written in memory of Prof. Dr LWK Chung and his contributions. Prostate and bone metastasis stroma concepts emerging from his work are now more actively being pursued by the authors of this article and others in the field.

**Keywords:** Prostate cancer, stroma, tumour microenvironment, bone metastasis, organoids, LNCaP

## Introduction

Prostate cancer (PCa) is the third leading cause of cancer-related death among men in Europe [1, 2]. Among primary PCa cases, 10% of patients are diagnosed with metastases [3], of which bone is a common metastatic site with limited therapeutic options at that stage. The prostate gland is hormonally regulated and growth-stimulated by androgens, leading to androgen hormone deprivation therapy (ADT) and blocking the androgen receptor axis, one of the most standard treatment options. However, progressive resistance to ADT and disease progression to castration-resistant PCa (CRPC) and advanced bone metastasis are frequently inevitable [4].

Hallmarks of PCa biology, such as tumour microenvironment (reactive stroma), genomics, multi-omic gene expression patterns, and metabolism, have been thoroughly investigated in the past decades, increasing our knowledge of the various disease mechanisms. Tumour microenvironment and tumour cells are reciprocally and dynamically influencing each other, multiplying the degree of complexity of PCa. The cancer stromal reaction is often overlooked in PCa compared to other tumour types with a stronger fibrotic/immune infiltration component reactivity, such as pancreatic and hepatocellular carcinoma. Yet, the stroma of the prostate has clear trophic and inductive properties in directing prostate morphogenesis, specification, hormonal regulation, and pathologic manifestations (BPH, cancer) [5]. The components of the normal prostate stroma are fibroblasts, smooth muscle cells, endothelial cells, nerve cells, and extracellular matrix (ECM) proteins. Pro-tumourigenic properties of cancer-associated fibroblasts (CAFs) compared to normal prostate fibroblasts have been a subject of research for decades. AR signaling is active in CAFs, similarly to smooth muscle cells and fibroblasts in the normal prostate stroma [6]. Decreased stromal AR expression in PCa is associated with earlier disease progression and BCR, thus suggesting an antitumourigenic role of stromal AR during the early, hormone naïve stages of PCa [7, 8]. The tumour/stroma ratio and the expression of stromal markers represent valuable prognostic tools to determine PCa progression

and predict therapy response [9], highlighting the importance of the stroma in tumourigenesis. Many studies have demonstrated the clinical predictability value of stromal biomarkers, shifting the focus off of cancer cells *per se* towards their microenvironment; however, these have not been implemented in clinical practice.

At the early stages of carcinogenesis, the stroma plays a protective, tumour-suppressive role by acting as a barrier against epithelial cell invasion into the matrix, blocking epithelial proliferation and facilitating immune cell infiltration. However, contact with constantly evolving tumour cells during tumour progression dictates molecular changes to the stroma that favor tumourigenicity. Ground-breaking work has revealed the tumour-inductive properties of stromal cells isolated from cancerous tissue, sufficient to transform normal epithelial cells towards a malignant phenotype, highlighting the major influence of the surrounding microenvironment [10]. In this mini-review, we focus on the work of Prof. LWK Chung, a pioneer in the field of stroma/microenvironment contribution to prostate carcinogenesis and subsequent bone metastasis occurrence. He was one of the first to propose that prostatic fibroblasts exert a directive influence on their adjacent epithelial cells through a paracrine mechanism that determines epithelial growth and tumourigenicity *in vivo*. Here, we present the evolution of his work from the generation of experimental models for the study of tumour and stroma that are still invaluable to the field, and his contributions in advancing our knowledge on the role of stroma in androgen-dependent and -independent PCa and bone metastasis mechanisms.

## Highlighting a role for stroma by early experimental models for the study of primary and metastatic prostate cancer

In an era when the prostate field had a limited number of experimental *in vivo* and *in vitro* models, with no adequate functional properties maintained compared to human PCa, Prof. Chung contributed with the generation of novel *in vitro* and *in vivo* models. Among them, epithelial cancer cell lines of various stages, recapitulating androgen independent (AI) disease and model metastasis, *in vitro* 2D and 3D models of stromal cells (bone, prostate) and co-cultures to study the interaction among cancer-stromal cells.

His work was among the first to experimentally demonstrate how stroma-epithelium interactions impact androgen responses, growth induction, and prostate tissue specification. Specifically, the notion that the fetal urogenital sinus mesenchyme (UGM) can induce epithelial cell proliferation and prostate gland formation. This was shown in the orthotopic ventral lobe model of prostate hyperplasia [11], in cell inoculations where the urogenital epithelium from testicular feminised mice induced to form functional prostatic acini [12], as well as subrenal capsule inoculations [12]. These are among the most standardized and elegant *in vivo* models used to delineate the possible roles of mesenchymal or stromal mediators in normal and tumour epithelial growth and differentiation and have been utilized extensively to the contribution of the stromal cells in *in vivo* tumourigenicity. More importantly, a pro-tumourigenic role was played for the prostate reactive stroma, and specific signaling networks were identified using these experimental methods. Specifically, in the cell-cell recombination model, it was shown that *in vivo* co-inoculation of marginally tumourigenic epithelial cells and organ-specific mesenchymal cells (from the prostate and bone) is sufficient to promote solid tumour formation [13] providing a strong proof of the transforming role of the surrounding stroma. Strikingly, normal lung or kidney fibroblasts did not induce prostate tumour growth in contrast to the prostate or bone fibroblasts [13]. Carcinomas were mainly observed in male hosts, indicative of *in vivo* androgen sensitivity. Further application of these models led to the identification of fibroblast-derived growth factors isolated from the conditioned media of prostate fibroblasts, such as basic fibroblast growth factor (bFGF), which proved highly mitogenic specifically for prostate cell proliferation [13, 14]. Moreover, characterization of the heterogeneity of human prostate fibroblasts was made possible due to the derivation of stromal cell cultures from clinical radical prostatectomy (RP) specimens (matched normal and cancer-derived stromal clones) along with co-culture methods to study human epithelial-stromal interaction [15, 16].

Derivation of experimental models that effectively mimic the natural history of the disease from orthotopic primary PCa to bone metastasis has always been challenging. Inoculation of epithelial cells with stromal cells from human

osteosarcoma led to derivation and establishment of a variety of cell line models (LNCaP, originally from lymph node PCa metastasis) that are extensively used up to date [17]. For instance, the C4-2 subline, a derivative of the LNCaP, when primed with human bone stromal cells (derived from human osteosarcoma) *in vivo*, it gave rise to bone metastases in castrated hosts, effectively mirroring *in vivo* the acquisition of androgen-independent metastatic phenotype [18].

Other significant contributions were *in vitro* modeling of epithelial-stromal cell interactions, the establishment of methods for three-dimensional cultures allowed *in vitro* maintenance of LNCaP cells and incorporation of prostate fibroblasts which grew as a mixed culture using microcarrier beads under microgravity-simulated conditions. The 3D co-culture model rotating-wall vessel (RWV) model was one of the earliest studies which showed that *in vivo* functional properties such as the growth response to DHT and upregulation of PSA are maintained in cells grown in 3D conditions, yet overall enhanced in co-presence of stromal cells. These mixed co-cultures gave rise to 3D structures, which were named "organoids" (one of the first times the term "prostate organoids" used in literature), referring to their ability to mirror the cell type composition of the originating organ/tissue [19] or later on termed epithelial prostatospheres [20]. Scientific focus on the organoid field in the last two decades has led to achieved major advancements by our laboratory [21] and others in developing models of normal and cancerous, murine, and human prostate [22].

Using the 3D co-culture RWV methodology, Prof. Chung's group demonstrated stable molecular and phenotypic alterations in PCa cells grown in 3D compared to monolayer, and even more when co-cultured with stromal cells [23]. Contact of LNCaP with prostate or bone fibroblasts as 3D organoids (RWV-2 and RWV-3 lines, respectively) led to chromosomal alterations compared to the parental line (loss of Y chromosome, telomere associations involving nonhomologous associations). In terms of lineage, these bone stromal cells (BMSCs) could be either multipotent mesenchymal stem cell (MSCs)/bone marrow stromal cells or more differentiated stromal or transitory fibroblasts [24]. From a functional perspective, the RWV-2 and RWV-3 lines acquired enhanced anchorage-independent growth loss of *in vitro* growth response to androgens and certain cytokines (bFGF, HGF, IGF1 but not EGF). *In vivo* intraprostatic growth under androgen-deprived conditions and bone metastases were significantly higher when LNCaP lines cultured in 3D (RWV system) were injected intraprostatically, compared to non-tumourigenic parental LNCaP line cultured in 2D monolayer [23]. Primary tumour growth was achieved at 100% occurrence in RWV-3 cells. However, the incidence of bone and lymph node metastases was similar in the presence or absence of stromal cells (62-87%) [23], emphasizing a role for the 3D epithelial cell organization as a sufficient stimulus for tumour cell growth *in vivo*.

Despite the expected changes in tumour cells, stromal cells are also reciprocally "transformed" and stably altered after direct contact with PCa cells [25]; human bone stromal cells ("MS" fibroblast-like cell line derived from human osteosarcoma) were genetically and morphologically altered after organoid 3D co-culture with PCa cells, in a reactive oxygen species-mediated mechanism [26]. The gene expression of the altered bone stromal cells was indicative of elevated chemoattractant chemokines and ECM proteins (versican, tenascin) and similar to cancer-associated prostate stromal fibroblasts [26]. In later studies, tenascin was detected in primary and metastasis PCa tissues [27, 28], in the circulation of PCa patients correlating with improved BCR prediction [29], and exhibited AR-responsive gene expression in the murine infiltrating stroma of subcutaneous tumours of bone metastasis PDXs (BM18, LAPC-9) [30]. In the context of epithelial-stromal cell interactions, recent research directions have demonstrated the occurrence of hybrid tumour-stromal cell fusions [31]. The above studies emphasize the bidirectional interaction of tumour and stroma and similarities among prostate and bone metastasis stroma.

### Osteomimicry, tumour and stroma adaptation

Several mechanisms have been proposed for the preferential metastatic growth of PCa cells to the bone site, such as the hemodynamic model and Paget's "seed and soil" models [32].

The permissive and specialized bone microenvironment has also been attributed a role for the preferential growth to the bone. Two types of niches have been identified that allow homing, dormancy, and reactivation of disseminating tumour cells: the perivascular and endosteal niche. Multipotent BMSCs are key cells found in both niches that can give rise to structural bone lineages like osteoblasts, chondrocytes, adipocytes, and specialized pericytes in the perivascular niche [33]. Pericytes/BMSCs around vascular structures of the bone marrow permit homing of tumour cells by expressing C-X-C motif chemokine ligand 12-(CXCL12), which specifically interacts with the tumour-derived CXCR4 receptor [34]. Osteoprogenitors and differentiating osteoblasts within the endosteal niche also express CXCL12 [35], and secrete osteoclast-activating interleukin(IL)-6 that contributes to initial osteolysis. Metastasis to the bone is promoted by signals produced by the tumour host stroma, such as inflammation mediator prostaglandin E2 (PGE2), which osteoblasts produce after cell-cell interaction with tumour cells and, in turn, affects osteoclast activity and bone lesion formation [36]. Increased prostaglandin signaling or overexpression of molecules involved in its signaling and production (EP receptors, cyclooxygenases) is implicated in tumour angiogenesis and invasion [37, 38]. Moreover, PGE2-EP4 overexpression is mediated via AR activation and implicated with castration-resistant PCa phenotypes, while antagonism of its function via the EP4 receptor inhibits bone metastasis growth [36, 39]. Additional molecular and cellular steps in the early cascade of bone metastasis include stimulation of osteoblast differentiation uncoupled from bone resorption, secretion of growth factors favorable for tumour growth (i.e., IGF-1, ILs), and activation of osteoclast-mediated bone resorption via the RANK-RANKL axis [40].

Another mechanism facilitating bone metastasis is osteomimicry, a process recognized by the team of Prof. Chung, among others. PCa cells progressively acquire osteoblast gene expression as a preparatory mechanism to enhance survival into the bone [41]. Expression of non-collagenous bone matrix proteins such as osteopontin (OPN), osteocalcin (OC), and bone sialoprotein (BSP) has been found in PCa cells. Interestingly, higher OPN, OC, and BSP protein expression was found in the most proliferative and tumourigenic androgen-independent LNCaP sublines C4-2 and C4-2B than the parental line. Instead, in the context of bone remodeling, higher expression of OC and BSP protein correlates with later stages of osteoblast differentiation and lower proliferative capacity. The above suggests that the acquisition of an osteomimetic phenotype by PCa cells provides a pro-tumourigenic advantage as well as camouflage and survival benefit inside the bone environment [41]. OPN has been identified as a paracrine and autocrine mediator of PCa growth by exerting its function by ligand binding to CD44 receptor and interacting with αvβ3 cell surface integrin heterodimer [42]. The expression signatures of bone ECM proteins were also identified in localized PCa; OPN is expressed both at the RNA and protein level in primary PCa as assessed in RP and transurethral resection of the prostate (TURP) specimens, while absent in benign prostatic hyperplasia (BPH) [42]. Similarly, BSP was found to be expressed in localized PCa. Higher expression was possibly linked to biochemical relapse rate [43], indicating that osteomimicry phenotype is initiated at the primary PCa stage by cancer cells and likely also by stromal cells. In fact, stromal signatures of prostate-specific osteoblastic bone metastases genes have also been found to be expressed in primary PCa clinical specimens [44] and in the stroma of the host in subcutaneous bone metastasis PDXs [30].

The adaptation of PCa cell surface repertoire has been thought to facilitate adhesion to cellular and matrix bone components and possibly explain the high rate of osteoblastic micro- and macrometastases found in PCa. Interestingly, integrin expression differs among osteo-tropic C4-2B and parental borderline tumourigenic LNCaP [45]. However, assessment of the early cell interactions among PCa and osteoblast or bone marrow-derived endothelial cell lines indicated an inverse correlation between metastatic/aggressive PCa lines and adhesion to osteoblast lines [45]. In fact, interaction with bone stromal cells rather than endothelial cells favors PCa cell growth [46]. Thus, not necessarily the initial adhesion, but mainly the ability of PCa cells to survive and colonize the bone after complex interactions with the bone niche, are responsible for metastasis occurrence.

These studies provide a rationale for assessing the therapy effects at the cellular level (e.g., bone changes due to radiation or castration that favors tumour growth) and developing more specific approaches such as dual targeting of both components [47] for therapeutic purposes.

### Stroma and androgen-resistance acquisition

A number of studies have shown the hormone regulation properties of the prostate stroma with increasing evidence on its role in ADT acquisition and CRPC progression. PCa stromal cells do not acquire genetic mutations [48]; however, it has become evident that the prostate ECM undergoes molecular alterations that are indicative of cancer formation, and stromal cells can significantly contribute to the development of castration-resistant disease (CRPC) [49, 50]. Human PCa CAFs can enhance the growth and tumourigenicity of benign prostatic hyperplasia (BPH) cells and metastasis potential of non-aggressive prostate epithelial cells, in contrast to normal fibroblasts [51]. Secreted factors from stromal cells impact androgen resistance acquisition [52]. CAFs have active AR signaling, but it has been shown that AR binds to unique genomic sites in CAFs, different from PCa cells, thus having distinct genomic targets in different cell types [53]. Consequently, reduced AR signaling activity following ADT increases CAF-mediated secretion of inflammatory cytokines, enhancing PCa cell motility [53].

Interactions with stromal cells (derived from human bone) not only induced the tumourigenicity of LNCaP (C4 subline but also led to spontaneous androgen-independent growth (C4-2), sustained androgen-independency and induced metastasis when implanted in castrated mice [17], representing one of the first models recapitulating the natural history of PCa from primary (C4-2P) to lymph node (C4-Ln) and bone metastasis (C4-2B subline) [18]. The short time frame (4-5 weeks) of acquisition of AI growth after the castration, that led to the derivation of the new LNCaP lines, supports the hypothesis that the contact with the bone stromal cells [13] led to a fast adaptation of PCa cells and enhanced AI phenotype [17]. Once the aggressive AI phenotype was acquired, such as in the case of the C4-2, serial passaging in castrated hosts no longer required co-inoculation with stromal cells [17].

Similar to the phenotype induced by bone stromal cells, contact of LNCaP cells with human primary PCa fibroblasts led to cytogenetic changes in primary tumour formation (as opposed to parental LNCaP) in intact and in castrated hosts. The most inductive fibroblasts were from the peripheral (PZ) and transitional zone (TZ) rather than the central zone [51]. The derived LNCaP sublines primed with the PZ or TZ fibroblasts (T4-2 and P4-2) were tumourigenic in castrated hosts and led to bone micrometastases after intraprostatic injection, modeling the transition from primary PCa to bone metastasis. The outcome of these early studies highlights an active role for stroma in the ADT-response and the acquisition of androgen resistance. As seen in recent studies [30, 52], this concept is now revisited and should be further explored.

### Conclusions

Tumour cells initiate or hijack molecular cues to instruct their surrounding microenvironment to acquire tumour-promoting properties. CAFs from the prostate tumour microenvironment and bone marrow-derived stromal cells, as opposed to fibroblasts from other tissue origins such as dermis, support the growth of prostate cancer cells. This tissue specificity of surrounding stroma from specific sources can induce the aggressive phenotype of non-tumourigenic cells, enhance the aggressiveness (androgen independence, bone metastasis) of tumour cells, and indicate the selective osteotropism PCa. Characterization of tumour's cellular and molecular properties and their surrounding stromal cells, especially at the early disease stage and in a time-dependent manner, is crucial for understanding the evolution of the tumour-stroma interactions from primary to metastatic disease progression. In our view, the field of the tumour microenvironment is moving in the direction of developing more complex modeling tools to facilitate the study of tumour-tumour microenvironment interplays, such as microfluidic organ-on-chip systems [54], multicellular co-culture systems (including immune and endothelial cells) [55] and macrofluidic models [56]. We anticipate that future directions in the prostate field will focus more on patient/derived *in vitro* methodologies and more precise *in vivo* transgenic mouse models to understand prostate and bone interactions that lead to metastasis.

## Disclosure of conflict of interest

None.

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