Chapter 6
Metastatic Dissemination

Stefania Staibano

Abstract In spite of recent developments in diagnosis, staging and treatment, most patients with advanced prostate cancer will ultimately progress from androgen-sensitive to an irreversible castration-resistant disease. These androgen-independent cancers frequently give rise to widespread metastasis, dramatically reducing the median survival of patients (Tannock et al, N Engl J Med, 351(15):1502–1512, 2004) and accounting for more than 32, 000 deaths/year in USA (Jemal et al, CA Cancer J Clin, 60:277–300, 2010), which correspond to over 90 % of PC related mortality (Man, Gardner, Int J Biol Sci, 4(4):246–258, 2008).

It is a common belief that cancer metastasis result from a multi-stage nonrandom process characterized by intricate interactions between cancer cells and the host microenvironment, leading to the detachment of cancer cells from their tissue of origin, their dissemination through the bloodstream and to invasion of the target metastatic site (Patel et al, Future Oncol, 7(11):1285–1297, 2011).

Metastasis represents yet one of the most enigmatic aspects of prostate cancer pathogenesis, in which a cascade of proteolytic enzymes, inflammatory cytokines, growth factors, activated oncogenes, oxidative stress and hypoxia linked proteins and adhesion molecules, orchestrate a continuous loop that enable migrating cancer cells detached from the primary tumor bulk, to survive and proliferate in an adverse remote body microenvironment.

In this chapter, we discuss the nature and alterations of the signaling pathways involved in the development of prostate cancer metastasis, reporting the current status of knowledge on the changes occurring either in prostate cancer cells and in tumor-associated stromal tissue, with particular emphasis to the process of epithelial-mesenchymal transition (“phenotypic plasticity”) and to the role of cancer stem cells in prostate cancer progression and metastasis.

S. Staibano (✉)
Department of Advanced Biomedical Sciences, Pathology Section, Faculty of Medicine and Surgery, University of Naples “Federico II”, via S. Pansini, n.5, Naples, Italy
e-mail: staibano@unina.it

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We will highlight, also, the emerging data concerning new therapeutic targets for treatment of metastatic prostate cancer that, while deserving further inquiry, look very promising to improve our chances to successful approach the advanced disease or, even, primarily reduce the risk of metastasis from castration-resistant prostate cancer (Vashisht, Bagler, PLoS One, 7(11):e49401, 2012).

Metastases represent the most fearful evolution of advanced/systemic prostate cancer progressed into a castration-resistance state after first-instance deprivation therapy.

Before the onset of metastasis, prostate cancer is usually characterized by a long latency period, in which genetic (Nguyen and Massague 2007; Zhao et al. 2013) and epigenetic (Rodenhiser 2009) cellular alterations lead to changes in cancer cells molecular phenotype, with the gain of both cytoskeletal motility and the ability to detach from the tissue of origin. The acquired abilities of epithelial prostate cancer cells are critically boosted by activated prostatic stromal cells, as tumor-associated macrophages (TAMs), cancer-associated fibroblasts (CAFs) and endothelial cells (Wang et al. 2013).

Besides their role in tumor-associated angiogenesis, CD31/CD34-positive endothelial cells lining microvessels decrease upon castration, increasing instead when prostate cancer progress to castration-resistance. Recently, it has shown *in vitro* that endothelial cells secrete high levels of IL-6. This cytokine down-regulates AR and activates the TGFbeta/MMP9 signaling pathway in prostate cancer cells, contributing then to their invasive and metastasizing ability (Wang et al. 2013).

TAMs produce several migration-stimulating factors, as CXCL12, IL-6 and TNF (Allavena et al. 2008). Activated CAFs mostly exhibit a myofibroblastic phenotype induced either by the direct physical contact with cancer cells and *via* the hyperstimulation, by several tumor- and hypoxia associated growth factors, as EGF, FGF, IGF, VEGF. CAFs overproduce TGFbeta (Roodman 2004), which intervene in ECM remodeling (Allavena et al. 2008) and in the induction of epithelial-mesenchymal transition (EMT) (Yilmaz and Christofori 2009) of metastasizing cells.

Extracellular matrix proteins, facilitating either tumor growth and metastasis, continuously accumulate in tumor stroma. This is the case for versican, a large proteoglycan associated with metastasis and poor outcome of prostate cancer and several solid malignant cancers. It has been shown to regulate cancer cell adhesion, proliferation, migration, angiogenesis, invasion and metastasis mainly through physical interactions mediated by chondroitin and dermatan sulfate side chains; looking particularly attractive as a possible adjunctive therapeutic target for aggressive prostate cancers (Du et al. 2013).

Even a disturbance of the interplay between the electrical and metabolic activity of prostate cells seems to play a role in the gain of propensity to metastasize of prostate cancer. It has been, in fact, recently reported that an altered expression of connexins, which form intercellular channels involved in gap-junction-mediated intercellular coupling, might be correlated with the invasive potential of cancer cells (Czyż et al. 2012). This finding, however, deserves further investigation.

The acquired EMT ability of prostate cancer cells leading to the detachment from the bulk of primary cancer, is conditioned by the dramatic loss of adhesion proteins, as E-cadherin (Yates 2011; Lazari et al. 2013) and their regulating transcriptional inducers, as the SAM Pointed Domain ETS transcription Factor (SPDEF) (Pal et al. 2013), and by the increase of their transcriptional repressors, as the Wilms' tumor gene (WT1) (Brett et al. 2013). Recently, the altered expression of the human metastasis-associated gene 1 (MTA1) has been found strictly associated with the pAkt/E-cadherin pathway regulation and with metastatic prostate cancer (Wang et al. 2012), and, a combined testing strategy for detecting MTA1 and E-cadherin, has been proposed for selecting high-risk prostate cancer patients (Fan et al. 2012).

Before permeating blood vessels, detached tumor cells have to escape anoikis and gain survival benefits (Hu et al. 2012). The anoikis-resistance and EMT properties of prostate tumor cells are mediated by several molecular players, including members of the Notch signaling pathway, as well of the Akt survival pathway including the early-recruited focal adhesion player tallin. Tallin mediates integrin activation and induces downstream survival pathways resulting in the promotion of cancer cells progression to metastasis (Desiniotis and Kyprianou 2011).

Both Notch-related proteins and tallin appear, then, as promising candidate as either prognostic markers and therapeutic targets in metastasizing prostate cancers.

To survive in the bloodstream, prostate cancer cells activate multiple survival pathways, comprising the overexpression of several members of the anti-apoptotic Bcl-2 protein family, combined with the inactivation of the FADD death receptor pathway, or the lack of expression of pro-apoptotic effector proteins as Bax and caspases (Igney and Krammer 2002).

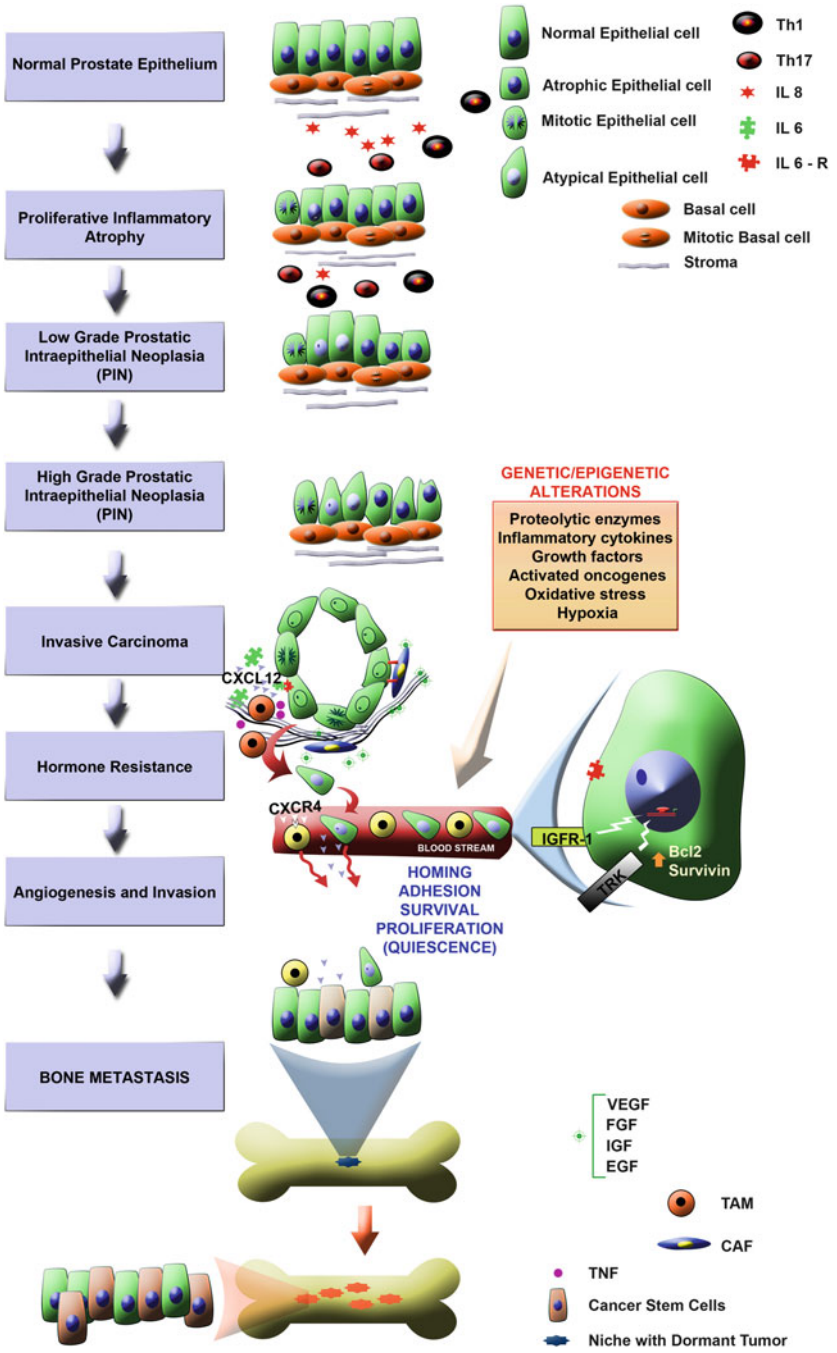
Moreover, circulating prostate cancer cells may activate survivin expression and undergo to autophagy, to survive in the absence of sufficient extracellular nutrients (Roca et al. 2008).

While metastasizing prostate cancer cells may optionally variously localize in several body sites, as lung and liver, they invariably hit the bone (Osanto and Van Poppel 2012) (Fig. 6.1).

In bone-metastasizing cancers, the CG-protein-coupled calcium sensing receptor (CaSR), which is primarily involved in the feedback regulation of extracellular free ionised calcium (Ca2+), may act as an oncogene, associating also with cancer progression. In prostate cancer, its altered expression seems to facilitate bone metastasis (Brennan et al. 2012).

Disseminated prostate cancer cells (DPCC) reaching the bone marrow occupy the same bone niche in which hematopoietic stem cells reside in a quiescent state (Taichman 2005). This has led to the concept that DPCCs behave as "parasites" of the hematopoietic niche.

DPCCs which evade immune attack and/or chemotherapy cytotoxicity may outlive for a variable time in bone marrow of patients after radical prostatectomy or chemotherapy (Morgan et al. 2009; Pfitzenmaier et al. 2007), in an auto-induced reversible state of growth arrest, the so-called "tumor-dormancy" (Townson and Chambers 2006; Aguirre-Ghiso 2007; Shiozawa et al. 2008b; Joyce and Pollard 2009). This underlies the troublesome unresolved phenomenon of "minimal residual


Normal Prostate Epithelium
Proliferative Inflammatory Atrophy
Low Grade Prostatic Intraepithelial Neoplasia (PIN)
High Grade Prostatic Intraepithelial Neoplasia (PIN)
Invasive Carcinoma
Hormone Resistance
Angiogenesis and Invasion
BONE METASTASIS
Normal Epithelial cell
Atrophic Epithelial cell
Mitotic Epithelial cell
Atypical Epithelial cell
Th1
Th17
IL 8
IL 6
IL 6 - R
Basal cell
Mitotic Basal cell
Stroma
GENETIC/EPIGENETIC ALTERATIONS
Proteolytic enzymes
Inflammatory cytokines
Growth factors
Activated oncogenes
Oxidative stress
Hypoxia
CXCL12
CXCR4
BLOOD STREAM
IGFR-1
TRK
Bcl2
Survivin
HOMING
ADHESION
SURVIVAL
PROLIFERATION
(QUIESCENCE)
VEGF
FGF
IGF
EGF
TAM
CAF
TNF
Cancer Stem Cells
Niche with Dormant Tumor

disease", responsible for most cases of prostate cancer recurrence and therapy failure. The overall regulation of this process is still under active investigation but it has been now accepted that it involves prostate cancer stem cells (PCSC). From the first appearance on the scenario of cancer metastasis of solid tumors, in 2003 (Al-Hajj et al. 2003), a definitive consensus about their origin and specific markers has not been reached yet.

Several putative surface markers, in fact, are shared also by normal stem cells (Patrawala et al. 2007; Collins et al. 2005) as well as by different solid tumors. This is the case for CD133/prominin-1 and CD44 (Patrawala et al. 2007) that have been found expressed in CSC of lung, breast, colon, ovarian and head and neck squamous cell carcinomas (Cui et al. 2011; Chu et al. 2009; Shi et al. 2010). As well, CD133, CD44, integrins, Sca-1, and breast cancer resistance protein (BRCP) are expressed either in PCSC and in normal prostate stem cells (Yu et al. 2012; Tang et al. 2007); Oct-3/4, beta-catenin and SMO are stemness markers expressed by most of normal and neoplastic stem cells (Patrawala et al. 2006). In addition, there is still a considerable variance among the different antibodies available for the detection of stem cells markers and this may explain, almost partially, the presence of some overlaps or discrepancies between the many existing studies on this topic.

Encouraging results indicate that ALDH1A1, a member of ALDH family of proteins involved in the intracellular production of retinoic acid, could be considered as a promising marker of stemness for prostate cancer cells (Li et al. 2010). ALDH1A1 overexpressing prostate cancer cells display, in fact, high migration and clonogenic ability *in vitro* and metastatic ability *in vivo* (van den Hoogen et al. 2010). As well, a second member of the ALDH family, ALDH7A1, seems to be involved in the bone metastasis formation (van den Hoogen et al. 2010), since its knockdown results in inhibition of experimentally induced intra-bone metastasis.

Fig. 6.1 The signaling pathways involved in the development of prostate cancer bone metastasis. Metastasis result from interactions between cancer cells and the host microenvironment that enable them to detach from the primary tumor bulk, disseminate through the bloodstream and invade of metastatic site. These steps are regulated by a cascade of proteolytic enzymes, inflammatory cytokines, growth factors, activated oncogenes, oxidative stress and hypoxia linked proteins, and adhesion molecules. Activated stromal cells, as tumor-associated macrophages (*TAMs*), cancer-associated fibroblasts (*CAFs*), and endothelial cells, favor the entire process: TAMs produce several migration-stimulating factors as CXCL12, IL-6, and TNF; CAFs intervene in ECM remodeling and in the induction of epithelial-mesenchymal transition (EMT) of metastasizing cells. Circulating cells activate multiple survival pathways, as the overexpression of several members of the anti-apoptotic Bcl2 protein family and the activation of survivin expression. Disseminated prostate cancer cells (DPCC) reaching the bone marrow occupy a bone niche and, when evade immune attack and/or chemotherapy cytotoxicity, may outlive for a variable time in an auto-induced reversible state of growth arrest, the so-called "tumor-dormancy". Dormant DPCCs give rise to bone metastatic lesions by re-entering the cell cycle and proliferating. The causative factors leading to this process are still a matter of investigation, but it seems involve prostate cancer stem cells (PCSC)

It has become clear, however, that the existence of a single reliable marker of PCSCs doesn't exist and a definite combination of markers expression may, instead, identify the metastatic profile of PCSC (Eaton et al. 2010). To support this idea, it has been shown that the co-expression of CD166 (epithelial stem cell marker) (Dalerba et al. 2007), CD151 (marker of stem-like tumor stromal cells) and the tumor rejection antigen/TRA-1-60 (Draper et al. 2002) identifies prostate cancer cells with high ability in sphere formation *in vitro* and generating, *in vivo*, tumors capable of self-renewal and differentiation, consistent with stem cells properties.

Moreover, it has been reported that the signature for stem cell markers may also vary between metastasis and primitive tumors with different Gleason grade (Castellón et al. 2012).

Traditionally, prostate CSC have been thought to derive from the basal cel layer, which express most of the known markers of stemness, as CD133, CD44, CD117, Tert, p63 (Tsujimura et al. 2002). Several findings support this hypothesis. As an example, it emerged that normal basal cells of human prostate can initiate prostate cancer in immunocompromised mice (Goldstein et al. 2010) and primary cells FACS-sorted confirmed the basal cell origin for prostate cancer (Goldstein et al. 2010; Lawson et al. 2010).

On the other side, there are line of evidence that, in several instances, PCSC could have originated from prostate luminal-cells. For instance, a genetic lineage-marking study has shown that rare prostate luminal cells express the androgen/AR-regulated transcriptional co-activator Nkx3-1 in absence of androgens (castration-resistant Nkx3-1-expressing cells, CARNs). CARNs show stem-cell properties, as they are self-renewing and reproduce prostate ducts in renal graft, and cause HGPIN and cancer following Pten deletion (Wang et al. 2009).

Besides their origin, PCSC are considered as the guest actors in the bone marrow metastasis phase (Colombel et al. 2012). They have, in fact, the necessary characteristics for survive and reproduce in the bone microenvironment.

The bone marrow niche, in turn, is critical for the progression from localized disease to distant metastases (Chung et al. 2005; Cher et al. 2006; Morrissey and Vessella 2007; Karlou et al. 2010). The niche is composed by the endothelia of sinusoids (Kiel and Morrison 2008; Doan and Chute 2012), osteoblasts, adipocytes, mesenchymal stem cells, and contains a soluble extracellular matrix rich in growth factors, cytokines (Bussard et al. 2008) and nutrients, useful for cancer cell survival. In addition, it contains adhesion molecules (Taichman 2005; Yin and Li 2006; Arai et al. 2009) as annexin II (Shiozawa et al. 2008a), which interact with tumor cells and local osteoblasts and fibroblasts to provide the framework for the stable homing of prostate cancer cells (Shiozawa et al. 2008b).

Among the several cytokines actively secreted by osteoblast, a pivotal role seems to be played by CXCL12, also known as stromal cell derived factor-1, with its receptors CXCR4 and CXCR7. These two receptors are strongly expressed by DPCC. The binding of CXCR4 and CXCR7 of prostate cancer cells with CXCL12 induces the expression of several adhesion molecules, which enhance their binding to the bone niche (Sun et al. 2005, 2007).

This finding may have relevant implication on therapy, as it has been shown that molecular antagonists of the CXC4R, as the small molecule AMD3100 and the G-CSF analog Filgrastim is able to mobilize metastasizing prostate cancer cells from the bone marrow niche.

Another protein responsible for the reversible cell-cycle arrest of DPCC is the fibroblast secreted annexin II (Anxa2) which operates with its receptor Anxa2R in a manner similar to the CXCL12/CXCR4-CXCR7 pathway (Jung et al. 2007; Shiozawa et al. 2008a).

The degree of expression of either CXCR4 and CXCR7 by prostate cancer cells has been found to correlate with a poor outcome of patients (Sun et al. 2003; Wang et al. 2008; Shiozawa et al. 2008b; Mai et al. 2000). All these considerations have rendered the targeting of bone marrow niche molecules a particularly active and attractive research field.

Several reports indicate that the alteration of multiple other signaling pathways accounting for the tumorigenic potential of PCSC may be used to control them.

For instance, targeting NF-kB with small molecule inhibitors may block sphere generation *in vitro* and tumor-initiation *in vivo*, by purified naïve stem-like human prostatic cells (Rajasekhar et al. 2011), thus supporting the reported adverse prognostic significance in terms of biochemical recurrence risk of the presence of NF-kB stained cells in positive margins of radical prostatectomy specimens (Ross et al. 2004). Similarly, the therapeutic use of WNT inhibitors has been shown to reduce the self-renewal of PCSC and improve the outcome of patients harbouring tumors co-expressing Wnt3a, nuclear beta-catenin, keratin 18, CD133 and CD44 (Bisson and Prowse 2009).

Moreover, the colonization of the skeleton by prostate cancer cells is mediated also by collagen type I, the most represented bone protein, mainly through the binding with the increased expression of integrin $\alpha(2)\beta(1)$. This integrin has been found elevated in PCa bone metastatic lesions compared to either primary tumors or their soft tissue metastases suggesting it is needed for the selective metastatization to the bone (Sottnik et al. 2013).

Dormant DPCCs give rise to bone metastatic lesions by re-entering the cell cycle and proliferating. The causative factors leading to this process are still a matter of investigation.

The striking propensity to localize to the bone is shared also by other “big killers”, as lung and breast cancer (Patel et al. 2011). However, these other cancer types give rise to osteolytic (bone resorbing) bone marrow metastases, while prostate cancer can produce predominantly osteoblastic lesions (Zetter 1990; Jacobs 1983; Chappard et al. 2011), via the inhibition of osteoblast apoptosis and the increase of osteoblast proliferation and metabolism, induced by parathyroid hormone (PTH), PTH-related protein and bone morphogenic proteins BMP (Keller et al. 2001). In addition, the expression of BMP may lead to the osteoblastic differentiation of bone mesenchymal stem cells creating an autocrine and paracrine feedback loop between the prostate cancer epithelial cell and the bone microenvironment.

In contrast to the rapid progress being made in the development of anti-osteolytic therapies, the treatment of osteosclerotic MBD remains restricted to palliative radiotherapy for symptomatic solitary lesions and systemic taxane-based chemotherapy for widespread multiple lesions (Sturge et al. 2011). Thus, new therapeutic strategies focused on the complex pathology of osteoblastic bone-forming metastases of prostate cancer are urgently needed and promising results start to emerge from current preclinical studies.

The "lethal phenotype" of metastatic castrate-resistant prostate cancer depends, then, from the bi-directional action of cancer epithelial cells in the bone and host stromal response to tumor cells (Loberg et al. 2005).

Elucidating the bidirectional interactions between the cancer cell and host bone microenvironment is now an important area of prostate cancer research (Efstathiou and Logothetis 2010).

By a clinical point-of-view, these osteoblastic metastases cause bone pain, and are constituted by disorganized neo-synthetized, unstructured "woven" bone which, similarly to that observed also for osteolytic lesions, frequently give rise to painful fractures (Roudier et al. 2003, 2008; Eastham 2007).

The progressive filling of bone marrow by metastatic prostate cancer cells cause myelophtisis, leukoerythroblastic anemia (Eriksson et al. 1972; Shamdas et al. 1993), up to bone marrow failure (Spivak 1994). These phenomena are thought to be caused, at least in part, by the physical displacement out of their bone marrow niches of hematopoietic stem cells by prostate cancer cells. HPCs displaced in the bloodstream might then undergo to forced, but incomplete, differentiation into lineage-specific nonfunctional progenitors (Shiozawa et al. 2011).

Patients with bone-metastatic prostate cancer experience a significative higher risk of death for disease when compared with patients without skeletal involvement (Norgaard et al. 2010).

The rationale for this bone-forming activity could reside in its possible contribute to support availability of bone niches for the successful homing and expansion of metastasizing prostate cancer cells.

The last decade has registered significant advancement in the identification of the steps involved in the multilayered process of prostate cancer metastasis but further translational studies are needed, to shed new light on several fundamental questions:

– Do hormone receptors have a relevant role in the induction and establishment of prostate cancer metastasis?

  Mounting evidence indicates that androgen receptor (AR) signaling continues to play a critical role in the growth of advanced PC despite androgen deprivation (Zheng et al. 2013). Recent data indicate that convergence of oncogenic and hormone receptor pathways promotes the metastatic phenotype (Augello et al. 2013). However, the downstream AR target genes involved in progression of castration-resistance are largely unknown. It has been reported that cyclin D1b, a splice variant of cyclin D1 exerting a highly oncogenic function in human

cancers, promote AR-mediated activation of genes associated with metastatic phenotype in tumor xenograft models of prostate cancer (Augello et al. 2013).

Moreover, Jin HJ and colleagues showed that the AR pathway induces prostate cell growth also via the induction of the synthesis of FoxA1 (Jin et al. 2013). However, this protein, which is a transcription factor essential for the prostate lineage-specific gene expression, inhibits cell motility and epithelial-to-mesenchymal transition (EMT) through AR-independent mechanism opposite to the action of AR signaling, thus behaving as an inhibitor of prostate cancer metastasis. In orthotopic mouse models, FoxA1 has been found up-regulated in localized prostate cancer and down-regulated in EMT bearing metastatic prostate cancer cells. Then, FoxA1 may be considered an AR-independent metastasis inhibitor that, following mutations, can contribute instead to prostate cancer progression.

WNT7B, as a direct AR target gene highly expressed in castration-resistant prostate cancer (CRPC), suggests that AR-regulated WNT7B signaling is critical for the growth of CRPC and development of the osteoblastic bone response characteristic of advanced PC (Zheng et al. 2013). WNT7B is necessary for the growth of PC cells and this effect is enhanced under androgen-deprived conditions; it promotes the androgen-independent growth of CRPC cells likely through the activation of protein kinase C isozymes, induces osteoblast differentiation *in vitro* through a direct cell-cell interaction, and is upregulated in human PC xenografts that cause an osteoblastic reaction when grown in bone. Contrasting data still exist about the real significance of AR reactivation in castration-resistant prostate cancer cells and its relevance for prostate cancer stem cell biology (Miki et al. 2007; Collins et al. 2005; Rajasekhar et al. 2011; Patrawala et al. 2006).

– MicroRNAs (miRs) function as either oncogenes or tumor suppressor genes in cancer (Zhu et al. 2013). Early reports suggest that in androgen-dependent prostate cancer cells, they may play a role in tumor development, progression, evolution to metastasis, response to therapy, and prognosis (Qu et al. 2013) In prostate epithelial EP156T cells, miR-182 and miR-203 have been really shown to induce MET features and growth factor independent cell growth.

  On the opposite side, elevated serum levels of miR-141 have been found related with the presence of bone prostate cancer metastasis, without significant correspondence with either Gleason score of primary tumor or PSA value. By converse, miR-141 showed a positive correlation with serum alkaline phosphatase levels (Zhang et al. 2013).

  However, more data are required before we reach a comprehensive knowledge about their definite roles in androgen-independent, bone metastasizing prostate cancer (Brennan et al. 2012).

The better understanding of the molecular phenotype of PCSC and DPCC could provide novel therapeutic strategies, allowing the targeting of bone metastatic prostate cancer cells, before they exit dormancy and become lethal (Patel et al. 2011).

Early profiling studies have evidenced the role of miRNA expression in prostate CSC (Liu et al. 2011), revealing that they specifically target several stem cells markers in prostate cancer. As an example, the overexpression of miR-34A leads to the decrease of CD44+ prostate cancer cells, inhibiting tumor development and metastasis, thus appearing as a promising potential new therapeutic tool for neutralize the killing potential of PSCS.

– Recently, it has been suggested that infiltrating immune cells facilitate tumor stem cell proliferation. Moreover, it has been proposed that aberrant immune cell infiltration preferentially associates with tumor capsular areas showing distinct degenerative alterations. Tumor-associated lymphocytes might cause focal disruption of prostate cancer capsule, favoring, then, tumor cell budding and metastasis (Jiang et al. 2013).

  This finding deserves further evaluation, as it may have a relevant impact on our knowledge of the prostate cancer metastasis causative events. It suggests, in fact, that the aberrant immune cell infiltration may have the same destructive impact of cancer cells on the lining capsule, offering in turn a selective proliferative advantage to prostate cancer stem cells proximal to these focal disruptions.

  Moreover, it will be also clarified if the selective tumor-associated immunoreactive infiltrate may have a causative role even for the early onset of aggressive prostate cancer at young ages, typically originating in healthy men with morphologically normal prostate (Man and Gardner 2008).

Overall, the understanding of the molecular background of prostate cancerogenesis has already changed our way to look at prostate cancer.

The growing flow of information concerning the bidirectional interactions between the epithelial cancer cells, tumor-associated stroma, and host bone microenvironment has become an impressively active area of prostate cancer research (Efstathiou and Logothetis 2010). The stromal-interacting pathways represent exciting targets for new molecular niche-directed therapies, which in the next future will guide our efforts to fight metastatic prostate cancer.

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