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Disseminated tumor cells and dormancy in prostate cancer metastasis

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It has been reported that disseminated tumor cells (DTCs) can be found in the majority of prostate cancer (PCa) patients, even at the time of primary treatment with no clinical evidence of metastatic disease. This suggests that these cells escaped the primary tumor early in the disease and exist in a dormant state in distant organs until they develop in some patients as overt metastases. Understanding the mechanisms by which cancer cells exit the primary tumor, survive the circulation, settle in a distant organ, and exist in a quiescent state is critical to understanding tumorigenesis, developing new prognostic assays, and designing new therapeutic modalities to prevent and treat clinical metastases.

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Introduction

PCa will account for about one-quarter of new cancer diagnoses in men in 2015, with 202 800 estimated new cases and will be the second most common cause of cancer-related deaths in the United States with 27 540 estimated deaths [1]. Many patients with no evidence of metastatic disease undergo treatment for cure with surgery or radiation. Unfortunately, many of these patients develop a recurrence and ultimately succumb to their disease. Understanding how, when, and why these patients developed disseminated disease remains a high priority for the field.

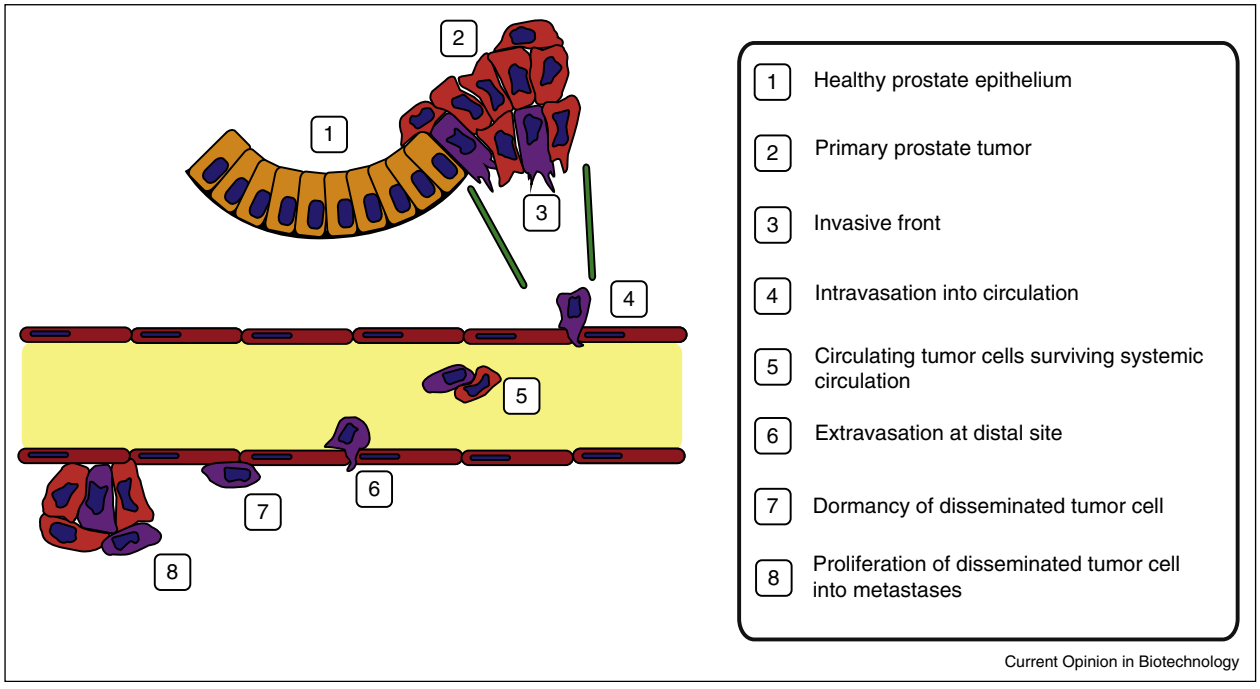
Cancer can theoretically metastasize to almost every organ of the body and these metastases play a central role in most cancer-related deaths. Tumor cells mainly travel to distant sites through the blood. Important steps in haematogenous metastasis in solid tumors include migration and invasion of those cells from the primary tumor into the blood vessels, circulation in the bloodstream (circulating tumor cells), dissemination to distant sites (disseminated tumor cells), and extravasation and eventual colonization in metastatic niches/sites [2••] (Graph 1).

Circulating tumor cells

As early as 1869, Asworth noted that cancer cells could be found in circulating blood [3]. Although this was almost 150 years ago, the identity and role of circulating tumor cells (CTCs) in cancer metastasis remains unclear. Over the last decade, research on developing CTCs as minimally invasive multifunctional biomarkers has become the 'Holy Grail' of the cancer community. The detection, capture, and characterization of CTC's in peripheral blood as a 'real-time liquid biopsy' continues to be developed as an alternative to standard biopsies. A benefit of the liquid biopsy is the fact that it can be conducted repeatedly with low risk for side effects, monitoring cancer progression and response to therapy [3,4••,5].

The fate of CTCs remains unclear. What percent of cells survive transit in the blood stream to a target organ? Are they passively sloughed into the circulation or do they actively migrate out of the tumor? When do they start leaving the primary tumor? What determines how frequently they lodge in one distant site versus another? This has made the characterization of disseminated tumor cells (DTCs) critically important. Even less, however, is known about the character of DTCs. The presence of a DTC in a PCa patient, for example, does not necessarily mean that he will develop a clinically evident or overt metastasis. While it is still not possible to directly prove that DTCs initiate metastases, there is indirect evidence that DTC's can develop into overt clinical metastases. Disseminated epithelial cells are rarely found in healthy persons/individuals, and their presence in the bone marrow of patients with prostate cancer significantly reduced metastasis-free survival [6].

Graph 1



Overview of metastatic cascade and seeding of metastatic sites.

Prostate cancer metastasis

PCa cells mainly metastasize to bone sites. The majority of men with clinically localized PCa who develop these bone metastases do so many years after the resection of the primary tumor. This demonstrates a delay between the initial treatment and the biochemical recurrence (BCR), the first sign of future overt metastasis — suggesting that cancer cells escaped early in the disease (prior to surgery or radiation) and are able to stay dormant in the bone marrow for years before switching to a proliferative phenotype and eventually causing metastatic progression [7,8]. This data makes PCa a good target to investigate the role of DTCs in cancer dormancy and metastasis. The questions arise, why do certain cancers recur after long periods of time, while others remain dormant? What happens with the DTC's while they stay dormant and what causes the dormant DTC's to start proliferating? [8,9••] (Box 1).

Different types of cancer dormancy

There are many theories to explain how DTCs are kept in a dormant state before they emerge as a clinically evident metastasis. Cancer dormancy may be divided physiologically into 'cellular dormancy' or 'tumor mass dormancy'. The latter can be subdivided into 'angiogenic' and 'immunologic' dormancy. It is increasingly appreciated that the microenvironment has an important role in conferring and maintaining these states [10,11•] '**Cellular dormancy**' is a state in which individual cells are quiescent and halted in the G0 phase of the cell cycle. One of the major causes for cancer cells to enter this type of dormancy appears to be hypoxia of the microenvironment. Dormant cells can re-enter the cell cycle (and thus exit the G0 arrest) and resume proliferation when the circumstances are favorable, for instance with the addition of growth factors, cytokines and nutrients. The second mechanism, '**angiogenic dormancy**', is caused by the lack of angiogenesis, and thereby nutrients, which prevent cancer cells from proliferating. The tumor mass is kept constant and at a limited size, due to a balance between proliferation and apoptosis of cells. The third mechanism which can cause dormancy is 'immune surveillance', where the immune system keeps a proliferating tumor mass limited to a constant size via persistent cytotoxic activity; that is, '**immune-mediated dormancy**' [9••,11•,12] (Figure 1).

An important property of dormant DTCs as opposed to senescent DTCs is the fact that they retain the capability to proliferate, but that they are by definition currently not dividing. This is determined by the lack of proliferating markers (for example Ki-67) when DTCs are profiled at the single-cell level. This makes them resistant to chemotherapies targeting cell division [13,14]. Multiple other factors in different studies are suggested to contribute to the development of chemotherapy resistance in PCa patients; that is, ABCG2 activation, inhibition of apoptosis, overexpression of P-glycoprotein and multidrug resistance gene 1, and mutational alterations in the tubulin

Box 1 Important terms

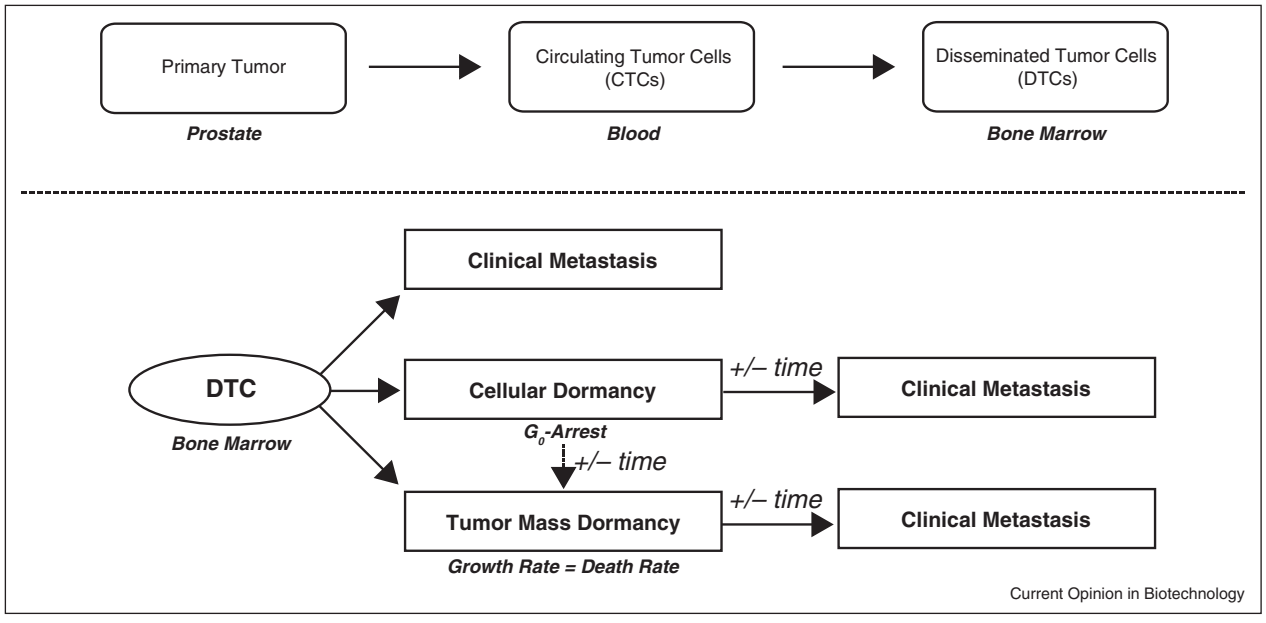
Circulating tumor cell	Cancer cell found in the circulation.
Dormant cell	Cancer cell that remains in a quiescent state but has the potential to proliferate.
Disseminated tumor cell	Cells that separated from the primary tumor and that have spread through the circulation to other locations of the body.
Dormant disseminated tumor cell	Cells that persist within foreign microenvironments; they are (reversibly) growth-arrested and they resist targeted and cytotoxic treatment.
Minimal residual disease	Tumor cells that remain after treatment, but cannot be detected by current methods in routine clinical testing.
Micrometastasis	Lesion derived from a disseminated tumor cell, that grows in another location than the primary tumor. They are too small to be detected by current methods.
Tumor mass dormancy	DTCs that are able to proliferate at the metastatic site, but due to insufficient angiogenesis and/or active immune surveillance' they are not able to progress to a clinically apparent metastasis.
Difference between senescence and quiescence	Similar to senescence, quiescence is a stable, non-proliferative state, but in contrast to senescence it reversible.

gene [15]. A recent study by Hao *et al.* demonstrated in a preclinical model the role of both CD44 and CD147 in the enhancement of metastatic capacity and chemoresistance of PCa cells. They propose that selective targeting both factors alone or combined with docetaxel may limit PCa metastasis and increase chemosensitivity [16].

The haematopoietic stem cells niche

Critical to understanding dormancy in the bone marrow microenvironment is the haematopoietic stem cell niche. The tumor microenvironment consists of many different cell types — tumor cells, cancer stem cells (CSC), endothelial cells, target organ cells, immune inflammatory cells and fibroblasts. Studies have demonstrated that CTC's target the same niche in bone marrow (BM) that houses haematopoietic stem cells (HSCs) and that DTCs co-localize with HSCs in the BM. Competition transplant studies have demonstrated that DTCs compete directly with HSCs for occupancy of the niche. The result of these factors is that those 'niche-engaged' PCa cells are more resistant to therapeutic intervention, either by becoming quiescent or through other protective mechanisms of the bone marrow environment [17••,18]. Factors known to drive and maintain haematopoietic stem cell (HSC) quiescence (for example, CXCR4, stem cell factor-1 (SDF-1) and angiopoietin 1 (ANG1)) may also act on DTCs in the bone marrow [19]. PCa cells express CXCR4, a member of the seven-transmembrane G-protein-coupled chemokine receptors. The stromal cells in the HSC niche, in particular the osteoblasts secrete SDF-1, the ligand for CXCR4. The migration of cancer cells and the adhesion to niche cells are regulated by the interactions between

Figure 1



Overview: from primary tumor to clinical metastasis.

these 2 factors. It has been shown in different studies that inhibition of CXCR4 and application of the granulocyte-colony stimulating factor (G-CSF) in mice releases BM-engrafted disseminated prostate cancer cells into the circulation, which in turn demonstrated that HSCs and PCA cells are tethered to niches in BM by the same signal [20••,21].

A study by Taichman *et al.* showed earlier that the HSC niche in the BM was able to promote cellular tumor dormancy. They demonstrated that prostate cancer cell lines express the growth-arrest specific 6 receptors (GAS-6, derived from osteoblasts) Axl, Tyro3 and Mer. In vivo studies showed that when Axl levels predominate, the prostate cancer cells became growth arrested and remained quiescent (in response to GAS-6), compared to the PCa cells that express a low Axl/tyro ratio, which were able to escape from dormancy. This study showed a possible association of the expression ratio of Axl and Tyro3, with the ability of PCa cells to switch between a dormant and proliferative phenotype in the metastasis process [22••,23].

Different cell intrinsic factors and signals from the microenvironment (secreted as part of their normal activity) are associated with an alteration of the molecular and cellular pathways of the DTCs, which can result in a switch between a dormant and proliferative phenotype [24•]. For example, endothelial-derived thrombospondin 1 (TSP1), bone morphogenic protein 7 (BMP7), transforming growth factor-β2 (TGF-β2) and growth arrest-specific 6 (GAS6) can mediate DTC quiescence in bone marrow. BMP7 is a TGF-β family member and is secreted by bone marrow stromal cells. This protein is capable of inducing inhibition of ERK and p38 activation that can induce dormancy in PCa tumor cells [12,25•]. Bragoda *et al.* demonstrated in a head and neck squamous cell carcinoma model that stromal cells in the BM can produce TGF-β1, which can upregulate the p38/ERK ratio in DTCs, which then remain dormant. To induce cell growth arrest, TGF-β2-induced dormancy was required; this was achieved by TGF-β receptor 1, TGF-β receptor III and SMAD1/5 activation. Systemic inhibition of TGF-β receptor 1 or p38 activates dormant DTCs, which lead to metastasis [26]. In addition, a recent patient-derived xenograft (PDX) model, showed in vitro that cellular adhesion of prostate cancer cells with each other and with BM stroma activates the cells to proliferate and promotes them to escape from dormancy. Furthermore, global gene expression showed a downregulation of TGF-β2 in the PCa PDX lines when proliferating, compared to the cells that were not proliferating; thus this could be a possible mechanism for PCa cells to escape from dormancy as well [27].

In a study about the formation of bone metastatic lesions in breast cancer (BCa) patients, an important role has been found for VCAM-1, a cell adhesion molecule that recruits osteoclast progenitors and elevates the local osteoclast activity. They showed in a bone metastatis dormancy model that the expression of VCAM-1 promoted a metastatic outgrowth in the bone in breast cancer cell lines [28]. Although PCa bone metastases are mostly caused by osteoblastic activity, a study of Morrisey *et al.* showed a subset of PCa bone metastases samples obtained from rapid autopsy, a variety in osteolytic activity. Since osteoblastic and osteoclastic activities are often coupled, this suggests that there may be an important role of VCAM-1 for the prevention and inhibition of metastatic recurrence in the bone of PCa patients [29] (Figure 1).

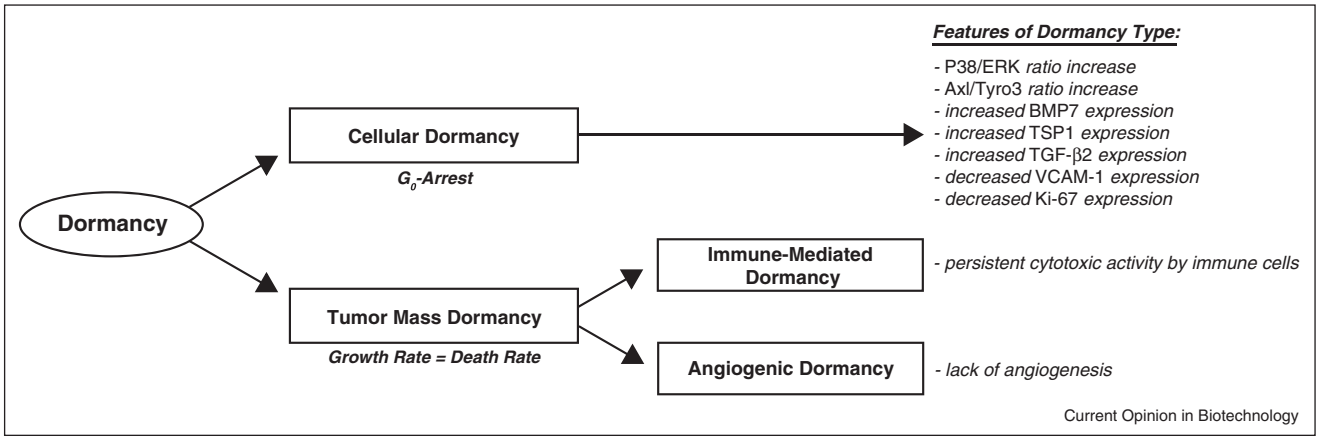
Detection of circulating and disseminated tumor cells

Many different assays have been developed for the detection of CTCs in whole blood samples, including microfluidic chips and methods based on size-exclusion, red blood cell lysis, and density separation. A novel method that does not rely on any single protein strategy is the Epic CTC platform. With this method, all nucleated cells are retained as a blood smear on 'sticky' slides and stained with fluorescent antibodies, for example, against cytokeratins, CD45 and DAPI, and subsequently imaged with a high definition scanner. An advantage of this system is the capability to analyze multiple parameters for the characterization of CTCs without enrichment [30]. Another novel non-invasive method to monitor CTCs and tumor evolution over time, based on density separation, is the Rarecyte system which also saves nucleated cells on positively charged slides for analysis [31•].

The only FDA-approved assay for CTC detection in patients with metastatic breast, prostate and colon cancer is the Cellsearch system; an immunomagnetic system for the enumeration of circulating tumor cells of epithelial origin. It defines a CTC according to its size, lack of the leukocyte maker CD45, and positivity for EpCAM and CK. The enrichment of CTCs is achieved by using immunomagnetic antibodies against EpCAM [4••,5,32,33].

There are no FDA-approved assays to detect DTCs, which make it difficult to standardize detection of PCa DTCs as the field attempts to determine the significance of disseminated cells at the time of primary therapy [34]. CTC systems are being adapted to isolate and detect DTCs in the BM [24•]. Another technique used for the detection of DTCs is the reverse transcriptase polymerase chain reaction (RT-PCR) to detect PSA. For this technique there is no enrichment required, due to high reaction sensitivity. Disadvantages of this technique include lack of 'seeing' the DTCs (not allowing for characterization) and the fact that some PCa DTCs do not express PSA (with the result that the presence of DTCs could be underestimated) [9••].

Figure 2



The different types of dormancy and potential important factors that can keep the disseminated tumor cells in a dormant state.

Strategies to treat dormant disseminated tumor cells

Given the recent data about DTCs and their role in formation of metastases, the question arises whether it would be better to target these DTCs while they are still dormant. There are theoretical strategies to 'treat' dormant DTCs. One idea is the 'chronic dormancy maintenance therapy' to keep the dormant tumor cells quiescent. Another strategy is the use of 'niche-targeted agents' to sensitize the DTCs to cytotoxic treatment, thereby killing the dormant DTCs. Either strategy could result in metastasis prevention (Figure 2).

Chronic dormancy maintenance therapy

This treatment strategy could be achieved by the chronic activation of several pathways which drive tumor cells in a dormant state (i.e. P38) or by the use of small molecules that mediate DTC quiescence (i.e. TSP1) [35]. Possible drawbacks of keeping DTCs dormant include long-term effects of chronic systemic induction of these factors on physiologic processes in the body. TSP1 has, for example, anti-angiogenic potential besides its anti-tumor function [18]. Moreover, when supplementing niche-derived factors, it is still possible that dormant DTC may eventually switch to a proliferative phenotype; so this type of treatment will may give the patient a feeling of false security. Current strategies for chronic dormancy maintenance are only in preclinical testing.

'Niche targeted agents'

Existing chemotherapies are not sufficient to eradicate disseminated prostate cancer cells, once they are established in the bone. A critical component of the 'targeted niche therapy' is that agents that induce HSCs to leave the niche also stimulate cell cycle progression [12]. If similar agents can release PCa DTCs out of the BM niche, then the DTCs should become more sensitive to the chemotherapeutic agents that target cells in cell cycle. In this way, DTC can be mobilized and targeted with existing therapies. These types of niche eviction strategies are in clinical trials.

'Indirect targeting'

A recent clinical trial by Banys *et al.* in patients with breast cancer showed that zoledronic acid (an inhibitor of osteoclast-mediated bone resorption) is able to contribute to DTC eradication in the bone marrow. They propose that the positive influence of the bisphosphonates on survival in the adjuvant setting may be due to their effects on DTCs. More research is needed to answer the question whether zoledronic acid really has impact on DTCs in PCa patients and if this treatment can maybe prevent these patients for developing metastasis [36].

Conclusions and future perspectives

Much remains to be learned in the fields of CTCs, DTCs, and cancer cell dormancy. In vitro models and standardized methods to detect and isolate CTCs and DTCs are needed. It appears that many autocrine and paracrine signals are needed to induce a proliferative switch in dormant prostate cancer DTCs. Potential treatment strategies are in development to target dormant DTCs or their environment, with the aim to keep them dormant or to eradicate them from the BM. Since which patient will eventually develop metastatic disease cannot be currently predicted, it is difficult to determine which patients will receive a benefit from targeting dormant cancer cells. Future research must be done to determine if all early-stage patients with detected DTCs in the BM, but with no other signs of disease, should be treated.

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