Review

Epithelial plasticity, cancer stem cells and bone metastasis formation

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Abstract

Acquisition of an invasive phenotype of cancer cells in primary tumors is an absolute requirement for bone metastasis. The majority of bone metastases is derived from epithelial cancers, particularly those of the breast and prostate. Accumulating evidence suggests that transformed epithelial cells can activate embryonic programs of epithelial plasticity and switch from a sessile, epithelial phenotype to a motile, mesenchymal phenotype referred to as epithelial-to-mesenchymal transition (EMT). Induction of EMT can, therefore, lead to invasion of surrounding stroma, intravasation, dissemination and colonization of distant sites. In bone/marrow disseminated tumor cells can partially regain their original epithelial characteristics via a mesenchymal-to-epithelial transition (MET) as glandular structures in bone metastasis are frequently observed. To date, the importance of epithelial plasticity in cancer cells disseminated to the bone/marrow microenvironment has remained largely elusive. Interestingly, a number of growth factors that play a prominent role in EMT induction in the primary tumor have been identified as important stimulators of skeletal metastasis formation. Recent studies have demonstrated that EMT may render cancer cells with properties of stem cells, which in turn can lead to escape from immune surveillance, increased resistance to apoptosis, diminished senescence and, last-but-not least, therapy resistance. This review will discuss current concepts regarding the role of epithelial plasticity in the multistep processes of bone metastasis, the issue of minimal residual disease, cancer stem cells and the importance of EMT in the development of novel targeted drug therapy.

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Epithelial plasticity in embryogenesis, growth and epithelial homeostasis

The fact that complex, multi-tissue organisms can be formed from a single cell, the fertilized egg, has fascinated developmental biologists and pathologists for a long time [1]. With the publication of his book Virchow postulated the idea that each cell in each living organism, both plant and animal, originates from another cell and that the origin of disease can only be located in the cell. Ever since this important discovery, researchers identified that cells can assume various phenotypic states during development and post-natal growth and undergo a process that was termed differentiation. In 1985, Stoker and Perryman [2] discovered that embryonic fibroblast culture supernatant contained a scatter activity for epithelial cells and this discovery pointed to the characterization of hepatocyte growth factor (HGF; also known as scatter factor (SF)), the ligand of the c-met receptor, and it turned out to be the first validated inducer of EMT [2,3]. In 1995 the process of EMT was first described in a model of chick primitive streak formation [4]. At that time it was generally believed that EMT (initially termed epithelio-mesenchymal transformation) was an irreversible, differentiation process but later it turned out to be a transitional state in that reverse processes, mesenchyme-to-epithelial transition (MET), can also occur in the same cells.

An epithelial-to-mesenchymal transition (EMT) is nowadays defined as a biologic process that allows a polarized epithelial cell, which

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normally interacts with the basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of extracellular matrix components [5–10].

Following these interesting initial observations it became increasingly evident that many of the adult tissues and organs arise from a series of conversions of epithelial cells to mesenchymal cells or vice versa, through EMT or MET respectively.

Over the past three decades multiple EMT and MET effectors have been identified (and are being discovered) and a number their molecular pathways have been unraveled and are still being investigated (Fig. 1) [6–11]. These EMT and MET effectors thus play critical roles during embryonic development, postnatal growth and epithelial homeostasis but also are involved in a number of pathological conditions, including wound repair, fibrosis, inflammation and, last-but-not-least, cancer progression [6–12].

It is beyond the scope of this paper to describe the classification of EMT and all EMT/MET effectors in detail since this has been the topic of recent excellent reviews [6–12]. In brief, the first EMT occurs during implantation of the embryo and the initiation of placenta formation and this involves parietal endoderm [6]. Furthermore, the fertilized egg undergoes gastrulation, a process that will eventually yield three germ layers. The epithelial cells of the primitive streak of the epiblast express E-cadherin and exhibit apical–basal polarity. The epithelial-like cells of the epiblast undergo programmed changes dictated by specific expression of protein associate with cell migration and differentiation [6,13] leading to mesendoderm and subsequently mesoderm and endoderm formation via an EMT. These EMT processes are classified as type 1 EMT. Type 1 EMT is, therefore, associated with implantation and embryonic gastrulation (mesoderm, endoderm, mobile neural crest cells). Following EMT, the primary mesenchyme can be re-induced to from secondary epithelial via the reverse process of mesenchyme-to-epithelium transition (MET).

Another process of EMT, type 2 EMT, is associated with tissue regeneration and organ fibrosis [6,14–18]. Inflammatory cells and stromal cells (e.g. fibroblasts) are capable of releasing of inflammatory agents as well as extracellular matrix proteins that include collagens, fibronectins, tenascin and elastins. Under pathological conditions, these stromal cells can stimulate normal epithelial cells to undergo an EMT leading extensive fibrosis of various organs and tissues include those of kidney, liver, lung and intestine [6,14–18]. Recent data also describe that endothelial cells associated with the microvasculature can contribute to the generation of fibroblasts during fibrosis, a process called EndMT [14]. Strikingly multiple bone active cytokines/growth factors and cell surface proteins drive these fibrotic (EMT/EndMT) processes. Of particular interest in this respect is the involvement of EMT inducers TGFβ, PDGF, FGF-2, multiple MMPs and αvβ3 integrins [6–12] while another member of the TGFβ superfamily, BMP7, functions as an inhibitor of TGFβ1-induced EMT, fibrosis and inflammation [19].

The last subtype of EMT, type 3 EMT or oncogenic EMT, is associated with cancer progression and metastasis. The importance of type 3 EMT in cancer progression and bone metastasis will be described in more detail in the following paragraphs.

**EMT and carcinoma progression at primary site in osteotropic cancers**

Over 80% of the malignant tumors are of epithelial origin and a number of these carcinomas are highly osteotropic. Normal epithelial cells are tightly cohesive and this property ensures the barrier function of epithelial organs in adults. Normal epithelial stem cells in epithelial tissues, from which bone-seeking cancers may arise, are critical for epithelial homeostasis and have been postulated to be the target cell for oncological transformation. Studies of these neoplastic tissues has provided substantial evidence for self-renewing stem/progenitor-like tumor cells, so-called cancer stem cells (CSCs) that are critical for initiation and maintenance of the primary tumor. Epithelial tissues are generally built according to a common set of architectural and hierarchical principles; relatively thin sheets of epithelial cells are separated from complex layers of stroma by a basement membrane. Normal epithelial cells are tightly cohesive and this property ensures the barrier function of epithelial organs in adults (Fig. 1). As described above, cohesion requires the development of intercellular adhesion by cadherins, in particular E-cadherin, which forms homophilic complexes and clusters at cell–cell interfaces in a region called the adherens junction. The formation and stabilization of E-cadherin clusters at adhesion junctions requires catenins: β-catenin,
which binds the cytoplasmic tail of E-cadherin, and α-catenin, which in turn binds β-catenin. In addition, actin filaments (F-actin) stabilize and immobilize E-cadherin clusters at adherens junctions [6,8]. By definition, carcinomas begin on the epithelial side of the basement membrane as hyperplastic and dysplastic growth with or without increased neovascularization in the underlying stroma. Cancerous epithelial cells are confined to the primary site by the continued expression of homotypic adhesion receptors and the intact basal lamina. In this pre-invasive stage of carcinoma in situ (or PIN in prostate cancer), acquisition of local invasiveness is considered the first step leading to dissemination/metastasis.

In order to acquire a mesenchymal migratory phenotype (motility and invasiveness), cancer cells must shed many of their epithelial characteristics, detach from epithelial sheets, and undergo a drastic alteration, which is referred to as type 3 EMT or oncogenic EMT [6,8]. It appears, therefore, that the EMT is a key developmental program that is often activated during cancer invasion and metastasis [6–12,20]. A major difference between carcinoma cells and normal epithelium is that cancer cells are often autonomous and less affected by paracrine, negative feedback mechanisms (in this case counteracting EMT) that normally prevent overgrowth, excessive migration, dissemination and organ colonization.

Loss of functional expression of E-cadherin protein or its mRNA, and thus loss of cell polarity, is presently considered as the hallmark of all EMT types, both in embryonic development and in cancer progression. During cancer progression, E-cadherin expression can be either be functionally inactivated or silenced by different mechanisms. These mechanisms include somatic mutations (in rare cases), epigenetic down-regulation through promoter hypermethylation and/or histone deacetylation, and transcriptional repression. At the transcriptional level, several E-cadherin repressors have been characterized, acting via interaction with specific E-boxes of the proximal E-cadherin promoter. The best characterized E-cadherin repressors/EMT inducers are members of the zinc-finger transcription factors, Snail and Snail2 (formerly known as Slug), SIP1/ZEB-2 and 5EF-1/ZEB-1, E47, and helix-loop-helix family of transcription factors, (e.g. Twist E12/E47, E2-2A/E2-2B, Goosecoid, E2.2. and FoxC2) [Fig. 1] [8]. The cellular changes associated with EMT encompass the loss of E-cadherin expression and cell polarity.

In addition to a loss in epithelial characteristics, EMT frequently coincides with the acquisition of motility, invasiveness, changes in the cytoskeletal proteins (expression of vimentin, α-SM), altered adhesion receptor expression (switching from E- to N-cadherin or OB-cadherin/Cadherin 11) and proteinase secretion (e.g. MMPs) [Fig. 1] [6–12]. Tumors are, therefore, often viewed as corrupt forms of normal developmental processes and EMT is considered the most common fatal consequence in tumourigenesis [6,21–23]. For example, the microscopic appearance of prostate cancer by the so-called Gleason Grading system is used to predict prognosis of men with prostate cancer and guide therapy [24]. A Gleason score is given to prostate cancer based upon its microscopic appearance. Cancers with a higher Gleason score are more aggressive and have a worse prognosis. The Gleason patterns, ranging from 1 to 5, is indicative of the process of EMT. While Gleason pattern 1 represents a cancerous prostate that closely resembles normal prostate epithelial tissue (epithelial phenotype), prostate tissue with Gleason pattern 5 has no recognizable glandular structures and often contains sheets of invading, mesenchymal cancer cells (Fig. 2).

**Epithelial plasticity and circulating tumor cells**

Multiple *autocrine* and *paracrine* mechanisms have been defined that regulate epithelial plasticity in health and disease, most of which were initially identified by developmental biologists (Fig. 1). The initial stages of carcinogenesis are characterized by excessive proliferation and neovascularization (angiogenesis) [6,25]. The subsequent acquisition of an invasive mesenchymal phenotype by the cancer cells, leading to breakdown of the basal lamina and invasion of the underlying stromal compartment is frequently considered as the most critically important event in carcinoma patients as this switch facilitates dissemination and metastasis. The (epi)genetic control and molecular mechanisms underlying tumor invasiveness and systemic spread have been areas of intensive research [6–12].

The acquisition of an invasive mesenchymal phenotype by epithelial cancers can occur via somatic mutations and epigenetic alterations in the tumor cells themselves. However, this view appears too simplistic. In general terms, primary (and metastatic cancers) do not exist as isolated neoplastic cells but, rather, they closely interact with different cell types and the extracellular matrix constituting the stromal compartment (Fig. 3). It has also become increasingly clear that, in parallel with tumorigenesis, significant (epigenetic) changes in the tumour-surrounding stroma occur that drive cancer progression, including the acquisition of an invasive phenotype via EMT [Fig. 3] [6–12].

Cancer cells activate local stromal cells, such as fibroblasts, smooth muscle cells and adipocytes, and recruit endothelial- and mesenchymal progenitors, and inflammatory cells. In turn, this stromal activation leads to the secretion of additional growth factors and proteases, which further favour cancer cell proliferation and invasion [26–28]. Furthermore, various stem and progenitor cells, including endothelial progenitor cell and mesenymal stem cells, can contribute to the acquisition of an invasive phenotype via EMT [29].

The molecular determinants of the paracrine (stromal) support, however, have remained largely elusive but seem to encompass a number of stroma-derived EMT effectors like TGF-β (in addition to somatic alterations in cancer cells and autocrine processes in certain cancers). As a result the stroma of primary (and/or metastatic) cancers is no longer considered as an innocent bystander, but, instead, as a therapeutic target in addition to neoplastic cells (see below).

Although the involvement of EMT processes in the metastatic cascade is still a subject of debate, an increasing number of studies demonstrate their involvement in increased cell migration, invasion and intravasation. These processes are mediated through three major intrinsic modifications that are induced in epithelial cells [9]. First EMT weakens cell-cell cohesion due to reorganization of epithelial intercellular junctions [8,9]. Accordingly E-cadherin is a major target repressed by EMT-induced transcription factors (see above). Furthermore cell-cell adhesion complexes and their transcriptional regulators are strongly regulated by a number of classical EMT-regulated pathways (including TGF-β, HGF, PDGF, Notch, Wnt, IGF) many of which also seem to play key role in skeletal metastasis. Moreover hypoxia and microRNAs have also been implicated in cell invasiveness via inhibition of cell-cell adhesion (Figs. 1 and 3) [6–12,30–34]. As a result of reorganization of adherens, tight junctions and desmosomes, new pathways can be initiated that promote EMT. For instance, after adherens junction recombination (e.g. E-cadherin loss), β-catenin can accumulate and translocate to the nucleus and–via binding of LEF/TCF transcription factors–activate EMT target genes like vimentin and EMT regulators such as Twist and Snail [6–12,35–37]. In addition to weakening cell-cell adhesion, EMT stimulates focal, proteolytic degradation of extracellular matrices, thus favoring invasion of stroma and intravasation. Again a number of E-cadherin repressors, such as Snail and ZEB factors, indeed induce basement membrane and stromal matrix degradation via matrix metalloproteinases (MMPs) and plasminogen activator pathways [9,38–42]. The modification of the cytoskeleton during EMT also contributes to migration. Intermediate filaments of epithelial cells such as cytokeratins are responsible for maintaining cell structure, stiffness and integrity. During EMT epithelial cells downregulate cytokeratins and upregulate vimentin by EMT transcription factors to allow migration and invasion [6–12,23,43–46]. In addition to invasiveness, EMT also potentiates angiogenesis and intravasation. While angiogenesis is generally
suppressed in the adult, EMT can contribute to the “angiogenic switch” favoring the ingrowth of new vessels into the tumor. A number of pro-angiogenic factors, including VEGF-A and MMPs, are induced by E-cadherin suppressors like Snail and nuclear translocation of β-catenin upon E-cadherin downregulation [9]. Moreover, VEGF-A, in turn, can trigger EMT via upregulation of Snail repressors thus linking EMT and angiogenesis. In addition to stimulating neovascularization, migratory carcinoma cells that have undergone EMT have acquired a number of specific properties that allow them to interact with endothelial cells and to enhance transendothelial migration. For instance TGFβ-induced EMT caused upregulation of N-cadherin and VE-cadherin [9,47].

**Fig. 2.** The Gleason Grading system of prostate cancer is indicative of EMT. The microscopic appearance of prostate cancer is used to predict prognosis of men with prostate cancer and guide therapy. Prostate cancers with a higher Gleason score are more aggressive and have a worse prognosis. While Gleason pattern 1 represents a cancerous prostate that closely resembles normal prostate epithelial tissue (epithelial phenotype), prostate tissue with Gleason pattern 5 has no recognizable glandular structures and contains often just sheets of invading, mesenchymal cancer cells. The immunohistochemical staining represents the E-cadherin repressor Snail1. In normal prostate glandular epithelium Snail1 expression is low/absent (upper left panel). Upon acquisition of an invasive, mesenchymal phenotype (from low grade to high grade) Snail1 expression is induced leading to nuclear translocation and potential repression of E-cadherin (source of immunostaining: http://www.proteinatlas.org).

**Fig. 3.** Epithelial plasticity during carcinogenesis and bone metastasis formation. EMT occurs at the primary site and allows epithelial cancer cells to invade the surrounding stroma, intravasate, circulate and extravasate to distant sites. Upon colonization of bone marrow the cancer cells frequently can regain their original epithelial phenotype by a MET thus resembling the primary tumor. Tumor-stroma interactions are critically important in the subsequent steps of the metastatic cascade. A number of growth factors, including TGFβ, PDGF and IGFs, stimulate EMT in the primary tumor and have also been identified as stimulators of bone metastasis formation, presumably via the acquisition of an invasive phenotype of cancer cells in micrometastases. CTCs = circulating tumor cells. DTCs = disseminating tumor cells MICs = metastasis-initiating cells.
A number of studies have shown that breast cancer cells that have undergone EMT upregulate Mena, a member of the Ena/VASP family that controls the geometry and assembly of F-actin networks. Mena plays a role in cell migration [48]. More recently, an invasion-specific splice isoform MenaINV has been implicated in EGF-driven carcinoma cell motility, invasion and metastasis [49,50]. Strikingly, the major isoform of MenaINV expressing CTCs in the bloodstream, display a mesenchymal phenotype indicative of EMT, and also acquired a breast cancer stem cell phenotype CD24+/CD44+/lin−.

Thirdly, EMT renders enhanced resistance to apoptotic signals and may, thus contribute to the survival of circulating tumor cells (CTCs) in the hostile bloodstream environment and, eventually, distant sites of metastasis. Moreover, Induction of the EMT program can lead to chemo- and radiotherapy resistance in a variety of cancers (reviewed in [9]).

Taken together, EMT may play a decisive role in the acquisition of an invasive phenotype and contributes to intravasation, survival and therapy resistance of CTCs.

Bone colonization: a continuing story of cancer stem cells and epithelial plasticity?

A number of studies have provided strong evidence that carcinoma cells acquire a mesenchymal phenotype after an EMT and that these cells are typically seen at the invasive front of primary tumors. It is also believed that these invasive, mesenchymal carcinoma cells are the first to enter into the subsequent steps of the metastatic cascade [6–12,23,43,51–56].

The final stages of the metastatic cascade involve adhesion to the bone marrow endothelium of the sinusoids vessels, extravasation and colonization of bone marrow (Fig. 3). Strikingly, upon successful colonization of bone marrow, many morphological similarities exist between primary tumor and metastatic bone lesions in that distant metastases are largely composed of cancer cells showing a mixed epithelial–mesenchymal phenotype closely resembling the primary tumor [11].

It appears, therefore, that a proportion of disseminated tumor cells (DTCs) in bone marrow can reacquire certain properties through a mesenchymal to epithelial transition (MET) and no longer exhibit mesenchymal migratory phenotypes ascribed to metastasizing carcinoma cells [6]. The tendency of DTCs to undergo MET likely reflects the local bone marrow microenvironment that they encounter after extravasation. This indicates that malignant progression is based on dynamic processes, which cannot be explained solely by irreversible genetic alterations but rather temporal transitional states that are strongly affected by the tumor microenvironment [56].

Brabletz and coworkers demonstrated that invasion and metastasis in colorectal cancer is driven by EMT and MET [55,56]. EMT in the primary tumor was found to lead to β-catenin nuclear translocation due to down-regulation of the epithelial homotypic adhesion receptor E-cadherin (see above) while, in matching metastases, β-catenin nuclear translocation may again be impaired due to re-expression of E-cadherin via an MET. In line with the observations made in colorectal cancers, a limited number of reports describe detectable expression of E-cadherin in phenotypically mixed epithelial–mesenchymal bone metastases [12,57]. At present the relative importance of EMT and MET in skeletal metastasis is not fully clear.

Various studies have provided strong evidence that growth factors, that are produced in or released from the bone/bone marrow microenvironment (e.g. TGFβ, IGFs and PDGF in bone matrix) alone or in combination with hypoxia [23,34,43–46,58–66] can stimulate the formation of bone metastasis (Fig. 3). A number of major bone active cytokines, including TGFβ, PDGF, IGF-I are potent EMT effectors and have been shown to be released from the microenvironment, both from the extracellular bone matrix upon osteoclastic bone resorption and/or secreted by the bone marrow stromal cells [23,34,43–46,58–66]. In line with this notion, evidence from clinical and experimental studies supports the concept that the rate of bone remodelling is directly related to the occurrence and progression of bone metastases [23,34,43–46,58,67–74].

These EMT effectors may, therefore, stimulate quiescent micro-metastatic deposits in bone marrow to acquire an invasive phenotype after EMT and, subsequently, grow invasively leading to clinically overt bone metastasis (Fig. 3) [23,43–46].

Although speculative at present, bone resorption inhibitors have indeed been shown to inhibit real-time TGFβ signaling in bone metastasis [75]. Research from our group has demonstrated that another member of the TGFβ superfamily, namely BMP7, can antagonize TGFβ signalling, counteract EMT responses or even induce MET, leading to strong inhibition of bone metastasis [43–46].

It appears, therefore, that metastatic carcinoma cells still display profound cellular plasticity extremely in that bone metastasis seemed to be stimulated by various potential EMT effectors while systemic administration (or forced overexpression) of BMP7 counteracts EMT mediated responses [43–46].

Interestingly, recent evidence suggest that the EMT program also promotes the self-renewal capability of breast cancer cells. [6–14,20,76,77]. Factors like TGFβ, that induce E-cadherin repressor like Snail and Twist, have now been implicated in the generation of cancer cells with stem cell properties that are capable of tumor initiation and maintenance [20,76]. This important observation implies a direct link between EMT and cancer stem cells as critical cells for tumor initiation and maintenance. Evidence is mounting that cancer stem cells are also involved in colonization and (bone) metastasis formation, dissemination and (bone) metastasis formation [78–81]. Furthermore, classical chemo- or endocrine therapy generally targets more differentiated epithelial cells but, via yet unidentified mechanisms, may cause substantial proportional increase in tumor cells with stem/progenitor phenotypes [81].

For instance, residual breast cancer cells after conventional chemotherapy (doxetaceal) or endocrine (lerezole) therapy display mesenchymal (vimentin, MMP2) as well as tumor-initiating features (CD24-/low/CD44+) [81].

Future perspectives

Despite multiple sophisticated attempts to improve treatment, the development of novel therapies for osteotropic tumors in patients has been limited and generally failed to improve patient overall survival. Too often initial beneficial responses in (bone) metastases are temporal and followed by re-growth of therapy resistant malignant lesions [82,83]. Once cancers have spread to the skeleton and developed into clinically overt bone lesions, treatment options are predominantly limited to palliation, prevention of pathological fractures and irradiation.

The search for new targets for drug discovery programs is hampered by both the marked cellular heterogeneity found within the same malignant tumor as well as among the cancers of individual patients. The acquisition of therapy resistance in patients with metastatic bone disease is indicative of a number of important processes in tumor biology. Evidence is mounting that therapy resistance is caused by the presence of a resistant subpopulation(s) of cancer cells with stem/progenitor characteristics, capable of driving tumor progression. Indeed, data are beginning to emerge that many standard-of-care chemotherapy, endocrine therapy and irradiation therapy are less effective in promoting cell death or cytostasis in tumor-initiating cancer stem cells (TICs, CSCs) and metastasis-initiating cells (MICs). Furthermore, an emerging concept suggests the stroma is not just an innocent bystander, but rather the site of primary dysfunction, which may be critical for carcinogenesis [26–29,54,84]. Therapeutic targeting of these metastasis-initiating cells, or
their supportive microenvironment, should be considered a high priority in the continued pursuit of more effective cancer therapies. Pathological EMT can be regarded as a reactivation of developmental programs in the adult [6–14]. Accumulating evidence shows that EMT is involved acquisition of an invasive, metastatic phenotype in epithelial cancers. Furthermore, EMT promotes tumor progression and metastasis via angiogenesis/intravasation, resistance to apoptosis or senescence, escape from immunosurveillance, therapy resistance, and—last but not least—acquisition of cancer stem cell properties by more differentiated cancer cells.

Because of the pleiotropic effects of EMT on tumor progression and bone metastasis, novel EMT-targeted therapeutic strategies can contribute to the prevention or the treatment of metastatic bone disease. For this, effective therapeutic targeting of EMT in tumor cells or targeting of the supportive stroma, that can dictate EMT type responses in cancer cells, is required. Alternatively, the tumorigenic and metastasis-initiating ability of critical cell subpopulations in human osteotropic cancers can be potentially affected by so-called differentiation-inducing agents like the bone morphogenetic proteins (or TGF-β inhibitors) [23,43–46,58,83,85].

To date, the exact role of epithelial plasticity in bone metastasis has remained largely elusive. New research is essential to address the role of epithelial plasticity in the multistep processes of bone metastasis, the issue of minimal residual disease, cancer stem cells and the importance of EMT in the development of novel targeted drug therapy.

References


