



# Metastasis – recent scientific insights and challenging new therapeutic approaches

MARTINA DEŽELJIN  
MAJA HERAK BOSNAR

Laboratory for Molecular Oncology  
Division of Molecular Medicine  
Ruđer Bošković Institute  
Bijenička 54, 10002 Zagreb, Croatia

**Correspondence:**

Maja Herak Bosnar  
Laboratory for Molecular Oncology  
Division of Molecular Medicine  
Ruđer Bošković Institute  
Bijenička 54, 10002 Zagreb, Croatia  
E-mail: mherak@irb.hr

**Key words:** metastasis,  
epithelial-mesenchymal transition, cancer  
stem cells, metastasis suppressor genes,  
dormancy

## Abstract

*Majority of cancer patients never die from the original disease – primary tumor – but from the metastasis that disseminate throughout the body. Although they disseminate in millions only few of them succeeded in their journey and achieve their main goal – form a distant metastatic colony which is able to thrive in an inhospitable environment of an unrelated tissue. This review will summarize in brief some of the recent advances in cancer invasion and metastasis investigations and possibilities in using these findings for the benefit of cancer patients.*

## INTRODUCTION

Tumors kill the host when they start invading the surrounding tissue so 90 % of patients that suffer from solid tumors actually die from metastases which have a marked variety of clinical manifestations (1). In breast cancer patients, metastasis can occur many years after the appearance of the primary tumor while patients suffering from pancreatic cancer or melanoma often die before the detection of the primary tumor. Colon carcinoma patients live with detectible metastasis for decades, while childhood neuroblastoma metastases sometimes mysteriously disappear.

The risk of metastasis disease or metastatic recurrence can in some cases be predicted by histopathological parameters such as: tumor size or grade, presence or absence of certain gene/protein markers (2). However, in many cases this is not possible or is highly unreliable, therefore, the search for relevant prognostic markers is one of the main goals of researches in this field.

For decades it has been widely accepted that prediction of tumor outcome is of utmost importance for metastasis prevention and adequate surgical and/or other therapy. The latest developments in metastasis research brings new evidence in this field and opens new possibilities that could prevent metastatic spread but also offer new opportunities in metastasis treatment when/if they occur. This way we hope that even if a malignant tumor succeeds in spreading metastasis there will be tools available to eradicate them or keep them quiescent. This way the metastasis would, if not be cured at least be keep silent for many years. This would not only prolong the overall life span of cancer patients but also improve their quality of life.

## The metastatic cascade

Our organisms are highly evolved systems whose tissues and organs function in an extremely sophisticated manner; disobedience of a cell in

means of wandering around the body or growing in unfamiliar tissue without control is not to be tolerated. However, some of the cells manage to escape the multiple guardian mechanisms of the organism and fulfill this task adopting certain physiological and morphological changes which enable them to survive in circulation and invade unfamiliar microenvironments.

In order to fulfill its destiny the metastatic cell has to execute a complex series of events called the metastatic cascade: it has to detach from the original tissue (tumor cell dissociation), enter in the surrounding stroma (invasion), enter into the microvasculature, blood or lymph vessels (intravasation) after which it is passively carried by the circulation until trapped in a capillary bed when it begins to actively leave the circulation (extravasation) attacking the foreign tissue (invasion) (3). Although probably millions of metastatic cells detach from the original tumor tissue only very few of them actually manage to survive all of the mentioned steps which is why metastasis is considered to be a very ineffective process. During the metastatic cascade the cells can be destroyed in the circulation flow or trapped in small vessels and degraded. They could also be attacked by the immune system, or stay in a dormant state for many decades.

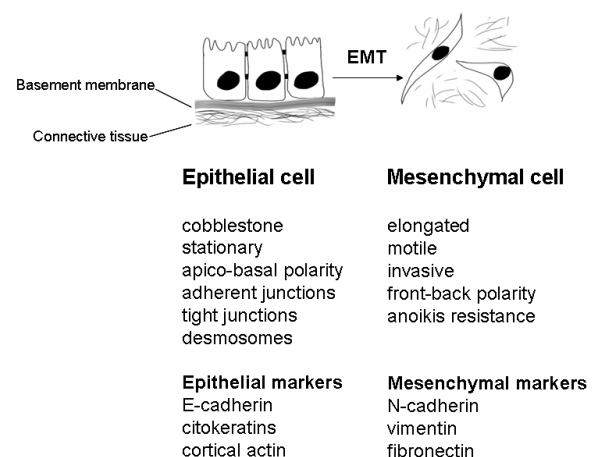
### Epithelial-mesenchymal transition

Carcinomas, malignancies of epithelial origin, encompass 80 % of all tumors. Epithelial cells embed internal organs or form upper skin layers protecting the body from injuries and infection. They form unicellular or multicellular layers which means they are tightly mutually connected and incapable of migration (4). To become invasive they have to adopt the ability to move which requires basic cellular transcriptional reprogramming which will eventually make them more similar to mesenchymal, fibroblast-like cells. This process is called epithelial-mesenchymal transition (EMT) (5). EMT is a normally active process in the body essential for wound healing (6) but even more important in embryogenesis during gastrulation (7) or the formation of the neural crest. In the case of tumor invasion it is, however, a pathological process driven by genetic and epigenetic aberrations leading to seriously deregulated cellular signaling. Transforming through the EMT the epithelial cell changes its shape to a fibroblast-like cell, becomes motile and invasive, loses its apical-basal polarity and changes a number of cellular markers (Figure 1). The crucial change that occurs in this transformation is the downregulation of E-cadherin. E-cadherin is a calcium dependent, transmembrane glycoprotein expressed at the basolateral membrane of the epithelial cell. It is a core point of adherens junctions which anchor the cell in the epithelial layer by forming homodimer connections between neighboring cells. With its intracytoplasmic tail E-cadherin is linked to the actin cytoskeleton through  $\alpha$ ,  $\beta$  and  $\gamma$ -catenins as well as p120. The loss of functional E-cadherin not only encourages the tumor cell to disengage from its original tissue and change its shape but also releases  $\beta$  catenin from the complex. Upon disinte-

gration from the complex the free  $\beta$  catenin translocates to the nucleus and activates the Tcf/Lef transcriptional program which initiates the EMT. The activation of the EMT program (8) induces the secretion of another member of the cadherin family, the N-cadherin. N-cadherin also forms homotypic connections between neighboring cells but also with the stromal cells underlying the epithelial layer. These connections are loose and enable the cells from the epithelial layer to escape their domestic tissue and by connecting with the stromal cells make a passage through the stroma to the blood or lymphatic vessel (9). Once available to the stromal factors (TGF  $\beta$ , TNF  $\alpha$ , EGF, HGF, IGF-1) the cells are constantly under their influence rendering them highly motile and invasive. The growth factors act either on their own or in synergism in different combinations and with the help of the mutated Ras oncogene maintain the EMT program. Although the complete mechanism of EMT is not yet revealed it is known that several transcription factors like Snail, Slug, Zeb and Twist can work as repressors of E-cadherin (10, 11). The activation of EMT transcription factors can also prevent apoptosis and anoikis (surface detachment induced cell death) or deregulate integrin expression.

The EMT program is not necessarily irreversible. Since EMT appears as a consequence of both genetical changes and the influence of the microenvironment, after disembarking in a distant tissue the altered microenvironment (growth factors, cytokines and components of the extracellular matrix) can lead to a reverse process – mesenchymal-epithelial transition (MET) which changes the cell back to an epithelial one (12).

Whether EMT is an obligatory process in turning a normal cell into a malignantly transformed one also has its skeptics (13). Tumor cells are a heterogeneous and changing population with different morphological and physiological properties. It is, however, probable that the



**Figure 1.** Epithelial-mesenchymal transition (EMT). A process characterized by loss of cell adhesion, repression of E-cadherin expression, and increased cell motility – a prerequisite for invasive phenotype.

cells on the „leading edge” must go through this transformation to be able to clear the way through the stroma to the blood or lymph vessel. It is nowadays suggested that the cells in the leading edge while passing through the extracellular matrix form a tunnel of least resistance which enables the other cancer cells to massively, collectively follow (14, 15). The collective is, therefore, composed of invasive, motile, integrin  $\beta 1$  expressing, *guiding cells* on the invasive front and a patch of tumor cells of various phenotypes that didn't, necessarily, experience the EMT on the rear (16, 17). Numerous studies have documented that the majority of circulating tumor cells (CTCs) that have been released in the blood stream do not form metastases. It is, however, possible that these cells, for example, have a role in forming a specific „niche” for potent metastatic cells. By secreting favorable growth factors during their voyage through the body CTCs condition specific tissues and make them convenient for growth of metastatic cells.

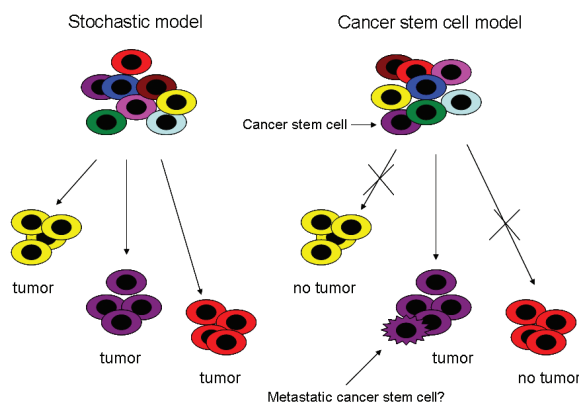
### Cancer stem cells – the origin of metastatic cells?

The origin of cancer cells was for decades thought to be a product of several or numerous mutations eventually acquiring features of a transformed phenotype: uncontrolled growth, morphological changes and in the end, certain migration and invasion potential. Widely accepted *stochastic model* defines metastatic cells as a subpopulation of cells that accumulate changes over time which enables them to detach from the original tissue and invade the surrounding stroma as well as give them selective advantages that made them robust enough to survive in circulation and colonize a distant tissue. The discovery of *cancer stem cells* shed new light to the possible origin of cancer and cancer metastasis (Figure 2). Cancer stem cells (CSC) are a rare subpopulation of cancerous cells that are defined by three major features: they give rise to tumors, they are capable of selfrenewal

and have a pluripotent nature (18). The *cancer stem cell model* proposes that only these particular cells are responsible for tumor renewal and seeding. Cancer stem cells were first identified in hematological malignancies (19) but are most extensively studied in breast, prostate and pancreatic tumors (20). CSCs in every particular tumor bear specific surface markers, for example, breast cancer stem cells are distinguished from the rest of the tumor population by  $CD44^+ CD24^{low}$  phenotype, whereas brain CSCs obtain a  $CD133^+ Lin^-$  phenotype. This is also one of the possible ways how CSCs can be isolated from the rest of the tumor cell population. These cells are considered to be resistant to chemotherapy which massively kills highly proliferating cells. CSCs are known to be quiescent and therefore escape conventional therapy. It is thought that this fact is actually the base for tumor relapse. If this hypothesis proves to be correct than CSCs are certainly candidate cells for seeding metastasis, as well (21). The recent work of Mani et al. demonstrated a link between the EMT and cancer stem cells (22). By inducing EMT in immortalized human mammary epithelial cells (HMLEs) treating them with TGF $\beta$  or inducing Snail or Twist transcription factors most of the cells acquire stem cell like characteristics including  $CD44^+ CD24^{low}$  phenotype. Overexpression of Snail or Twist together with V12H-Ras oncogene is sufficient to make them tumorigenic and capable of forming a small tumor. Their result suggests that initiating EMT (the earliest step in metastasis formation) in cells leads to CSC-like cells which could be a link between CSCs and metastatic cells. There is no direct evidence that this is really the case. It is, however, suggested that not all CSCs have the metastatic capacity but probably only a certain subpopulation (21).

### Metastasis suppressor genes

The group of metastasis suppressor genes (MSGs) were established after the identification of the first metastasis suppressor gene in 1988 (23) by Steeg and collaborators. Metastasis suppressor genes specifically regulate metastasis i.e. one or more steps in the metastatic cascade: detachment, invasion, migration, survival in circulation, invasion of the secondary site, colonization. Most MGS exhibit decreased expression in highly metastatic tumors vs. their non-metastatic primary tumors. Restoration of a MSG does not effect the primary tumor growth. Tumor cells expressing the MSG grow on the primary site but cannot colonize distant sites in the body (24). Based on this definition in the last couple of years as much as 30 or more metastasis suppressor genes have been identified. Nevertheless, mechanisms of action of these genes/ proteins are mostly still not understood. Metastasis suppressor are found to have different subcellular localizations while some of them are even present in the extracellular matrix (25). For instance the first identified MSG Nm23 (Nme) is mostly localized in the cytoplasm but can also be found colocalizing with several different intracellular structures (26). The E-cadherin is a plasma membrane bound molecule while MKK4, MKK7



**Figure 2.** Two different models defining tumor promotion, recurrence and secondary tumor appearance. The stochastic model implies that every tumor cell has the ability to form a new tumor while the cancer stem cell model presumes that not all tumor cells are tumorigenic but only the cancer stem cells (CSC) which are a small population within the tumor mass.

or RhoGDI2 are typical cytoplasmic signaling molecules. Many of the metastatic suppressors are multifunctional enzymes with clear functions that have up until recently had nothing to do with metastatic activity (caspase 8) (27). As already mentioned, MSGs suppress metastasis at one or more steps in the metastatic cascade. Some of them, like E-Cadherin, promote homotypic cell-cell adhesions and are, therefore, hindering tumor cell dissociation. Most of them suppress the invasion of local tissue and intravasation (gelsolin, RKIP, CD44, Claudin-4) while other impair survival in the circulation (caspase 8, DCC). Some of them exhibit their function in preventing the survival and proliferation of the micrometastasis (MKK4, MKK7, Smad7). Several metastasis suppressors are possibly involved in two or more steps in the metastatic cascade Nm23, KAI1, Kiss1, BRMS1, RhoGD1) (28).

### Tumor dormancy

Once anchored in a distant site the metastatic cell can experience several different fates: a) it can die of apoptosis; b) remain in a quiescent (G0/G1) state as a single solitary cell (cellular dormancy); c) remain dormant as a micrometastatic cluster (metastatic/angiogenic dormancy) or d) proliferate and form a detectible metastatic colony (29).

In general, cancer dormancy is a stage in tumor progression in which tumors remain in dormant microscopic and clinically asymptomatic stage for a long time. Dormant tumors are often only a few millimeters in diameter but they can switch to highly proliferating growing lesions which can become clinically relevant and lethal in a very short period of time. It is of great clinical significance that recent studies display that cancer can produce disseminating cells in a very early stage of primary tumor development and that dormant tumors (micrometastases) can actually be present very early in tumor progression (30). It is even hypothesized that most of the tumors disseminate in a very early stage but the disseminated cells stay dormant for decades (31). The escape of tumors from dormancy is still a puzzle but it is widely accepted that the main reason for this phenomenon lies in the fact that the dormant mass of tumor cells fails to reconstruct functional vasculature. The mass grows to a certain size but the tumor expansion relies on sufficient nutrition supply so cells, although possibly highly proliferative, die of apoptosis due to malnutrition. The transition from the unvascularized lesion to a highly vascularized growing tumor mass is called the *angiogenic switch*. The reason why these small primary or metastatic tumors fail to form functional vasculature remains to be investigated but it is known that dormant tumors secrete relatively high levels of potent angiogenic inhibitor called *thrombospondin* (32). It is, however, to be considered that there are other mechanisms that can contribute or even be crucial for tumor dormancy. Tumor development is a complicated multistage process which depends on a well tuned series of biochemical and biological events. It has been shown that large proportion of cells remains in

distant locations as single dormant cells. This is probably due to inhospitable environment of the host tissue which does not support the proliferation of the unrelated cell due to interactions with the extracellular matrix components and stromal cells. Solitary tumor cells or small tumor masses can also be a target of immunosurveillance or be influenced by hormonal control or autophagy. Dormancy can be induced by activation of metastasis suppressor genes responsible for this step in the metastatic pathway. The proposed mechanisms of action of these suppressors include either activation of p38/MAPK or inhibition of ERK1/2 MAPK pathways. These mechanisms can be linked to angiogenesis as a major control mechanism but can possibly also be unrelated (33).

### New directions in therapy

Treatment of metastatic cancer is one of the major challenges in tumor management. Unlike primary tumor lesions, metastasis are often inoperable and thus the choice of treatment is dependent on tumor type and stage and includes chemotherapy, hormonal therapy, targeted therapy and radiotherapy. Due to the cascade nature of metastatic spread, targeting any of the distinct steps would successfully prevent overt metastatic disease. Unfortunately, at time of diagnosis most cancer patients already harbor disseminated tumor cells in lymph nodes, circulation, bone marrow or distant organ sites (34). Thus, development of treatment should be targeted at the colonization step of metastasis, preventing growth of solitary disseminated cells or micrometastasis into clinically significant metastasis (35, 36). Major effort to develop cancer drugs was dedicated to eradicate tumor cells using chemotherapy and radiotherapy adjuvant to surgery, but disseminated cancer cells are often resistant to such therapy due to dormancy at distant sites or stem-cell like properties (36, 37). In addition, therapeutics targeted at specific pathways in primary tumor cells may not be efficient for managing metastasis due to genetic and expression differences. During the initial seeding of tumor cells at a distant organ site and development of micrometastasis, cells undergo changes in order to adopt to the new microenvironment, rendering them different from primary tumor cells and thus resistant to targeted therapy directed against the primary tumor (38). Broadening the knowledge of metastatic cells specific properties is essential for designing novel targeted therapies. Novel imaging techniques as well as better detection and evaluation of metastatic gene and expression signatures led to improvement of detection of distant metastasis and disseminated tumor cells in circulation and bone marrow. This enabled assessment for clinical usefulness: diagnostic, prognostic and therapeutic potential (34, 38, 39). As stated, the colonization step is the most promising target process in metastasis. Since the discovery of nm23, the first characterized metastasis suppressor gene, this group of proteins pose an intriguing opportunity to develop anti-metastatic therapy. The most interesting members include KISS1, KAI1, MKK4/7 and Nm23, which can promote dormancy (33). In animal metastatic models,

cancer cells with induced expression of these genes disseminate but do not develop overt metastasis. KISS1 and KAI1 promote cellular dormancy while Nm23 and MKK4/7 support dormancy at the micrometastasis level (40–43). Lack of MSG function in metastatic cells is usually due to downregulation, rarely to mutation. That is why main therapeutic approach should include re-establishment of MSG expression in disseminated cells (44). The benefit of promoting metastatic dormancy by inducing transcriptional activation of Nm23 by medroxyprogesterone (MPA) is currently being tested in phase II clinical trial. The main objective of the trial is determining the benefits of MPA monotherapy and MPA and low dose cyclophosphamide and methotrexate in postmenopausal patients with refractory – hormone receptor-negative metastatic breast cancer. During the trial, expression of Nm23 will be evaluated in biopsies of primary tumor, metastasis and skin to assess potential correlation between Nm23 and growth of metastases (45). Beside metastatic cells' intrinsic mechanisms, tumor associated microenvironment at distant sites offers valuable targets for metastasis therapy. Fibroblasts, endothelial cells and macrophages secrete soluble factors (cytokines, growth factors) that can either promote growth or induce dormancy. Illustrative example is the interplay between disseminated cells and bone microenvironment in development of bone metastasis. Tumor cells secrete osteoblastic and osteoclastic factors that increase bone turn-over and release of growth factors from bone matrix that stimulate tumor growth creating a positive feedback loop (46). Based on preclinical data, osteoclast inhibitors that may interrupt that cycle are developed. Osteoclast inhibitors: denosumab (RANKL inhibitor) and zoledronic acid (bisphosphonate) are already used to treat overt metastasis (47), and currently ongoing clinical trials assess the possibilities of using these drugs adjuvant to primary tumor treatment in order to prevent or delay development of metastases (48).

Another useful target is angiogenesis. Rationale for anti-angiogenic therapy in metastatic cancer is to prevent vascularization of micrometastasis thus inhibiting growth or to prevent intravasation and secondary spread of cancer. Currently, inhibitors of VEGF pathway combined with chemotherapy are approved by FDA for treatment of metastatic breast cancer, metastatic renal carcinoma and colon cancer (49). Bevacizumab, monoclonal anti-VEGF antibody and receptor tyrosine kinase inhibitors: sunitinib and sorafenibin are shown to increase disease-free and overall survival in most clinical trials (50). However, after initial growth inhibition by VEGF inhibitors, regrowth of tumor or distant metastasis is often observed (50–52). In addition, recent preclinical studies show that administration of these drugs can enhance more aggressive and metastatic phenotype (53, 54). Thus, anti-angiogenic therapy for treatment of early disseminated cells and micrometastases should be carefully reassessed, taking into consideration both the benefits and limitations, depending on tumor type, stage and accompanying therapy. Apart from the already mentioned ap-

proaches there are numerous perspective drugs developed for targeting other processes connected to metastasis: EMT (55), invasion, intravasation/extravasation, dissemination and colonization (56), immune axis (57), microenvironment and tumor stem cells (37, 47, 58). Considering the evidence we have today, it is clear that therapies aimed specifically at prevention of clinically significant metastasis needs to be administrated at the time of cancer diagnosis, either adjuvant or neo-adjuvant to primary treatment. Although metastatic cells eradication is the considered favorable treatment end-point, inducing or prolonging dormancy of metastatic cells and micrometastasis could represent a new treatment strategy that would turn cancer into a chronic disease and reduce mortality.

## CONCLUSION

Cancer represents one of the major health challenges in the 21 century. In spite of great financial imputes in the last decades we are still in the process of searching for answers how to manage this disease. Lately, the scientific community has done significant contributions in understanding the mechanisms not only of cancer onset but on its dissemination and metastatic colonization. The findings we represented in this review are only a part of the extensive studies which demonstrate that many of the postulates that have been taken for granted in the past should probably be redefined. Still, much effort should be put in proving the novel concepts *in vivo*. However, concerning the fact that cancer patients mostly die from metastasis it seems rational to focus particularly on this step in carcinogenesis which will hopefully bring us valuable new therapeutic approaches.

*Acknowledgements:* This work is supported by grant 098-09824642513 to MHB from Ministry of Science, Education and Sports of the Republic of Croatia.

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