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REVIEW ARTICLE

Clinical significance of epithelial-mesenchymal transition and cancer stem cells

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Summary

Purpose: *Spread of cancer cells from the organ of the origin of them to another location, namely metastasis, is one of the most important factors that complicate the treatment of cancer. Therefore, research for the treatment of metastatic disease is gaining importance, especially for advanced cancers. This research focuses on the mechanisms that facilitate the metastatic tendency of cancer cells. Therefore, epithelial–mesenchymal transition (EMT) mechanism that helps the cells become metastatic and cancer stem cells (CSCs) present in the heterogeneous tumor mass are in the center of these researches.*

Key words: *cancer, epithelial mesenchymal transition, stem cell*

Introduction

Metastasis is very important for the clinical management of cancer, because the majority of cancer mortality is associated with disseminated disease rather than the primary tumor itself [1]. The first steps of metastasis require proliferation of the primary tumor and invasion through basement membranes and adjacent tissues. This process continues with the tumor infiltration of lymphatic channels or blood vessels, when individual tumor cells detach from the primary tumor mass and are carried to a distant target organ via the lymph or blood. Afterwards, tumor cells arrest in small vessels within the distant organ, extravasate into the surrounding tissue and proliferate at the secondary site. All of these steps must be performed while tumor cells avoid and survive apoptotic signals and host immune surveillance [2].

EMT has pivotal role in cancer cells as a molecular mechanism for tumor metastasis and invasion [3,4]. EMT plays an important role not only in tumor metastasis but also in tumor recurrence and that it is tightly linked with the biology of cancer stem-like cells or cancer-initiating cells [5]. Both the EMT and CSCs play a critical role in tumor metastasis, therapeutic resistance and recurrence; however, each one alone can not explain the sum of the cellular events in tumor progression, and the significance of EMT in regulating the stemness of CSCs has remained unknown until very recently. Balancing these two concepts has led researchers to investigate a possible link between EMT and the CSC phenotype [6].

In this review the clinical significance of EMT, CSCs and the relationship between them are discussed.

Epithelial-mesenchymal transition

EMT plays an important role in normal embryonic development, but it has also been linked to pathological conditions such as cancer cell metastasis and tissue fibrosis. Based on the biological context, EMT is classified into three types. Type 1 refers to the physiologic EMT that occurs

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during development; Type 2 EMT results from wound healing and chronic inflammation leading to tissue fibrosis; and Type 3 EMT refers to the trans-differentiation resulting in metastatic cells during oncogenesis [7].

EMT is a complicated process that endows epithelial cells with enhanced metastatic and invasive potential [8]. A hallmark of EMT is the loss of epithelial characteristics such as a decrease in the expression of the cell adhesion molecular E-cadherin and acquisition of a mesenchymal phenotype accompanied by increased expression of vimentin. EMT-related transcription factors such as twist, snail, slug, ZEB1 and ZEB2 orchestrate the EMT and enable the early steps of metastasis, which mainly consist of local invasion and subsequent dissemination of tumor cells to distant sites [9]. Commonly used molecular markers for EMT are increased expression of vimentin and N-cadherin, nuclear localization of β-catenin, and increased production of the transcription factors such as Snail1 (Snail), Snail2 (Slug), Twist, EF1/ZEB1, SIP1/ZEB2, and/or E47 that inhibit E-cadherin production. Phenotypic markers for EMT include increased capacity for migration and three-dimensional invasion, as well as resistance to anoikis/apoptosis [10].

Regulation of epithelial-mesenchymal transition

An important hallmark of EMT is the loss of expression of the cell-to-cell adhesion molecule E-cadherin. E-cadherin is a central component of cell- cell adhesion junctions and is required for the formation of epithelia in the embryo and to maintain epithelial homeostasis in the adult. Loss of E- cadherin is consistently observed at sites of EMT during development and cancer. This loss has been found to increase tumor cell invasiveness in vitro and contributes to the transition of adenoma to carcinoma in animal models [11].

Various mechanisms can lead to silencing of E-cadherin expression during tumor progression, but transcriptional repression has emerged as a fundamental mechanism. Several transcriptional repressors, including Snail, ZEB and bHLH factors (E47/E2A, Twist), have been found to inhibit the expression of the E-cadherin gene and to induce EMT [12]. ZEB1 is a key regulator of the EMT-factor network during tumorigenesis. Aberrant expression of ZEB1 in cancer cells induces EMT by repressing several cell-cell adhesion molecules, including E-cadherin [13,14] and Plakophilin 3 [15], as well as basement membrane components [16] and cell polarity factors [15-17].

EMT can be induced or regulated by different growth and differentiation factors, including TGF-®, growth factors that act through receptor tyrosine kinases, such as platelet derived growth factor, hepatic growth factor and fibroblast growth factor, and Wnt and Notch proteins [18].

β-Catenin is an essential molecule both in cadherin-mediated cell adhesion and in canonical Wnt signaling. Numerous experiments have shown that the loss of cadherin-mediated cell adhesion can promote β-catenin release and signaling; this is accomplished by proteases, protein kinases and other molecules. Cadherin loss can also signal to several other regulatory pathways. Additionally, many target genes of Wnt signaling influence cadherin adhesion. The most conspicuous of these Wnt target genes encode the transcription factors Twist and Slug, which directly inhibit the E-cadherin gene promoter. Other Wnt/β-catenin target genes encode metalloproteases or the cell adhesion molecule L1, which favor the degradation of E-cadherin. These factors provide a mechanism whereby cadherin loss and increased Wnt signaling induce EMT in both carcinomas and normal embryonic development [19].

At the heart of TGFβ regulation of EMT is a nuclear reprogramming involving a set of transcription factors, i.e. the basic helix loop proteins Twist and E47, the zinc finger proteins Snail and Slug (also called Snail2), the zinc finger and homeodomain proteins ZEB1 (also called δEF1) and ZEB2 (also called SIP1) [12] , and FOXC2 [20]. These factors regulate each other in an elaborate manner. Thus, Snail upregulates Slug [21,22] and Twist [23], Snail and Twist induce ZEB1 [24,25] and Slug [26], and Snail induces ZEB2 [22].

Notch signaling pathway has been found to be a key regulator in the induction of EMT [27-30]. Notch activation in endothelial cells results in morphological, phenotypic, and functional changes consistent with mesenchymal transformation. These changes include downregulation of endothelial markers (VE-cadherin, Tie1, Tie2, platelet-endothelial cell adhesion molecule-1, and endothelial NO synthase), upregulation of mesenchymal markers (α-SMA, fibronectin, and platelet-derived growth factor receptors), and migration toward PDGF-B driven processes [31].

Cancer stem cells

CSCs possess several characteristics including pluripotency, self-renewal and tumorigenicity

and constitute a rare population in a tumor mass [32]. Circulating tumor cells are rare and difficult to isolate [33]. Established cancer cell lines contain CSCs which can propagate to form three dimensional (3D) tumor spheroids *in vitro* [34].

The self-renewal and differentiation ability of CSC gives rise to all tumor cell types, and thereby produce tumor heterogeneity. This relatively new perspective, the so-called "cancer stem cell" concept, casts new light on the origins of cancer [35].

Several signaling pathways and large number of molecules belonging to these pathways are important in terms of maintenance of CSCs.

The Wnt/β-catenin signaling pathway drives stem cell self-renewal and is involved in the pathogenesis of various types of cancer. Aberrant activation of the Wnt signaling pathway in normal stem cells can promote their transformation into cancer stem cells [36,37].

In the absence of Wnt signaling, cytoplasmic levels of β-catenin are tightly regulated by a multiprotein destruction complex. β-Catenin levels are kept low through phosphorylation, which leads to ubiquitinylation and subsequent proteosomal degradation. Binding of Wnt to the Fz receptors and LRP co-receptors allows β-catenin to be released from the multiprotein destruction complex. The free β-catenin is translocated to the nucleus where it acts together with either p300 or CBP as a transcriptional activator of Wnt-associated genes. Agents that inhibit Wnt/Fz binding and downstream events are in development [38].

Notch signaling has been reported to promote the self-renewal of CSCs in several malignancies and to participate in tumor-stroma and tumor-endothelium interactions in CSCs niches in primary and metastatic tumors [39,40]. There is increasing evidence that Notch signals are oncogenic in many cellular contexts, for example in T cell leukemia (T-ALL), colon and breast cancer [41-43].

Activation of the Notch receptor occurs following binding of membrane-bound Delta or Jagged ligands during cell-to-cell contact. Following absorption and proteolysis of the heterodimer Notch receptor (by ADAM and γ-secretase complex), a soluble fragment—the NICD—is released into the cytoplasm. The NICD translocates to the nucleus where it serves as a transcriptional activator of Notch-associated target genes, including HES, Myc and p21. Potential therapeutic inhibitors of Notch signaling target events such as γ-secretase complex proteolysis and transcriptional activation [38].

Data from many human tumors including breast cancer, glioblastoma, multiple myeloma, pancreatic adenocarcinoma, and chronic myeloid leukemia (CML) have suggested that Hedgehog (Hh) signaling moderates CSCs [44]. Self-renewal of CSCs is required for maintenance of the malignant clone, and reports studying mouse models of CML have provided evidence that Hh signaling regulates this property [45,46]. Active Hh signalling pathway has also been identified in glioblastoma CSCs, and pathway inhibition with cyclopamine or siRNA directed against pathway components results in the loss of tumorigenic potential [47]. Liu et al. demonstrated that the Hh signaling components Ptch1, Gli1 and Gli2 are highly expressed in normal human mammary stem / progenitor cells and that these genes are downregulated when differentiation is induced in these cells [48]. In multiple myeloma, CSCs have been found to display relatively higher levels of Hh signaling than the mature plasma cells [49], suggesting Hh signaling can act through multiple signaling modes within the same cancer and can mediate interactions between CSCs, differentiated tumor cells and the microenvironment [50]. Accordingly, the Hh pathway might play an important role in the continuous self-renewal of tissues from stem cells, which persists into postnatal and adult life [51].

In the inactive state of the Hedgehog signaling pathway, the absence of Hh leads to inhibition of Smo by the transmembrane receptor Ptch, while Gli1/2 are phosphorylated and removed from the cytoplasm through proteosomal degradation. In the active state, Hh is secreted by an adjacent cell and binds to Ptch, allowing Smo activation. Gli1/2 are released from the Smo protein complex and translocate to the nucleus, leading to transcriptional activation of Hh-associated genes. New therapeutic agents have been developed that target Hh and Smo activation and downstream proteins, such as Gli [38].

Epithelial-mesenchymal transition, cancer stem cells and drug resistance

Several studies have demonstrated that loss of epithelial phenotype through EMT can promote the acquisition of a stem-like phenotype and drug resistance [5]. Notch signaling regulates both the formation of CSCs and the acquisition of the EMT phenotype, which are associated with drug resistance [52,53].

Morel et al. demonstrated that $CD44+CD24^{-/low}$ stem-like cell signatures could be generated from $CD44^{low}CD24^{+}$ cells, non-tumorigenic mammary ep-

ithelial cells, through activation of the Ras/MAPK signaling pathway. In addition, they also found that CD44+CD24$^{-/low}$ cells displayed an EMT phenotype as characterized by the loss of E-cadherin expression and gain of vimentin expression. They hypothesized that the induction of EMT could be responsible for switching CD44^{low}CD24^{+} cells to CD44^{+}CD24$^{-/low}$ stem-like cells. To this end, CD24^{+} cells treated with TGF-β, a potential inducer of EMT, led to CD24^{-} cell appearance 8 days after treatment, concomitant with enrichment of mesenchymal phenotypic cells as characterized by the loss of E-cadherin and the gain of vimentin expression [54].

There are several molecular mechanisms that may account for CSCs resistance to therapy. Many CSCs are not cycling and are in G0 phase and thus resistant to cell cycle-specific chemotherapy agents [55]. They express several ATP binding cassette (ABC) transporters [56]. Expression and activity of ABC-transporters leads to multiple drug resistance (MDR), and this is a major obstacle to antineoplastic therapy [57]. CSCs express higher levels of antiapoptotic proteins, such as members of the Bcl-2 family and inhibitors of apoptosis [55]. Increased tolerance to radiation-induced DNA damage and enhanced DNA repair activity enables the CSCs radioresistance [58].

The specific ABC transporter pump expressed in the CSCs determines the specificity of chemoresistance. ALDH1 is a cytosolic enzyme, whereas other isoforms can localize to the mitochondria as well as the cytosol. The efficacy of chemotherapeutic drugs such as cyclophosphamide is reduced in ALDH expressing CSCs, as these drugs are substrates for these enzymes. Pro-survival protein BCL-2 binds to proapoptotic proteins BAX and BAK, preventing the release of the apoptogenic factor cytochrome C from the mitochondria. Aberrant activity of BCL-2 and other prosurvival BCL-2 family members utilize this mechanism to prevent chemotherapy-mediated apoptosis. Following DNA damage, ATM and ATR recognize breaks in DNA and activate CHK2 and CHK1, respectively. CHK2 and CHK1 can impair cell cycle and promote DNA repair. Activation of these DNA repair proteins in CSCs can impair the efficacy of DNA interstrand cross-linking (ICL) agents [59].

Not all the Snail targets are related to EMT. As mentioned above, besides having been associated to tumor invasion, EMT and Snail have been related to other cancer hallmarks such as the gain of unlimited replication potential, a greater resistance to apoptosis and even with the evasion of immunosurveillance. For instance, cell lines that overexpress Snail show lower apoptosis when exposed to ionizing radiation, genotoxic drugs or proapoptotic cytokines [60-62]. Repression of proapoptotic genes such as PTEN, p53, Bid or DFF40 have been associated to this resistance [61,62]. Moreover, Snail also enables breast cells to become tumor-initiating cells [54,63] and promotes immunosuppression in melanoma cells [64].

EMT is associated with therapy resistance in some cancers [65,66]. EMT markers are enriched in pancreatic cancer cell lines that are resistant to gemcitabine, 5-fluorouracil, and cisplatin [67]; chemoresistance to 5-fluorouracil correlates with the expression of mesenchymal markers in breast cancer cells [68]; and non-small cell lung carcinoma cells induced to undergo EMT with either epidermal growth factor (EGF) or transforming growth factor β (TGF®) show enhanced resistance to cisplatin and paclitaxel [65].

Targeting cancer stem cells and epithelial mesenchymal transition

CSC-targeted therapies are aimed at destroying them, either directly or indirectly. Direct approaches are destruction therapies targeting pathways or mechanisms essential for their survival. Destruction therapies include self-renewal pathways alterations that target NOTCH, Hedgehog, WNT, Polycomb, HOX, and PTEN/PI3K/Akt signaling pathways, and modulation of chemoresistance that target ABC-transport proteins, anti-DNA repair mechanisms, and inhibition of antiapoptotic pathways. Other mechanisms of destruction therapies are telomerase inactivation, modulation level of reactive oxygen species (ROS) and inhibition of tumor vasculature. Indirect approaches are differentiation therapies that force the CSCs out from their stem status into more differentiated, proliferating cells that can then be destroyed using more conventional therapeutic approaches carried out with the help of various chemical substances such as vitamin A and epigenetic alterations [69].

There are many therapeutic approaches targeting EMT in benign and malignant processes [70]. In liver, inhibition of STAT 3 phosphorylation with the use of sorafenib causes decrease in TGF-β signaling, apoptosis and fibrosis [71]. In kidney (tubular epithelium), targeting ALK3/6 receptors and Smad5 with recombinant BMP-7 causes antagonistic ALK receptor activation/Smad1 signaling, therefore E-cadherin expression increases [72,73]. In colorectal cancer, knockdown

of siRNA targets FGFR4. Therefore, reduction of Src and MEK1/2-ERK1/2 signaling causes reduction in tumor formation and targeting antibodies cause reduction of cell growth [74]. Targeting ILK with kinase-inactivated ILK (S343A) causes reduction of Akt signaling in hepatocellular carcinoma, so that this cancer becomes more sensitive to anti-EGFR therapy [75]. Use of sorafenib in lung adenocarcinoma targets HAT/HDAC. This causes increase in HAT expression and decrease in HDAC expression, probably via inhibition of Ras/Raf/MAPK and ErbB signaling. Thus changes in histone acetylation and transcriptional repression of EMT-related genes occur [76]. Tumor associated antigen and transcription factor Brachyury is targeted in lung adenocarcinoma. Administering of Brachyury-specific T cells to the patient provide T-cell mediated cytotoxicity and this causes lysis of brachyury-positive tumor cells [77]. Inhibition of Axl phosphorylation with targeting Axl RTK by SGI-7079 causes decrease in growth of mesenchymal NSCLC xenograft tumors [78]. In breast cancer, targeting LYN kinase by dasatinib inhibits LYN kinase activity and this inhibition contribute to a decrease in invasion [79]. Metformin targets EMT master gene expression and as a result - while expression of Twist1, ZEB, Slug, TGF-β 1–3, MMP-3, MMP-9 decreases - E-cadherin expression increases [80]. Also in a study performed by Topcul et al. it was shown that there was a significant decrease in cell proliferation, mitotic index and labelling index values after administration of metformin to MCF-7 cells *in vitro* [81]. Sorafenib also targets urokinase plasminogen activator (uPA) expression and inhibits Ras/MAPK signaling urothelial carcinoma *in situ* (UCIS). Inhibition of this signaling decreases uPA levels and increases E-cadherin levels [82]. In pancreatic cancer, Gli1 and Ptch genes that belong to Hedgehog signaling pathway are targeted by cyclopamine (IPI-269609). With inhibition of Hedgehog signaling, while Snail levels and metastasis decrease, E-cadherin levels increase [83,84]. Resveratrol targets EMT master gene expression and as a result of this expression Slug, Snail, ZEB1 and migration/invasion decrease [85]. Knockdown of siRNA targets Axl RTK and therefore inhibition of MAPK and PI3K/AKT kinase signaling causes decrease in GTP-bound Rho/Rac, Slug, Snail, Twist, MMP-9 and migration/invasion [86].

Conclusions

Although some types of cancer can be treated nowadays, spread of cancer cells (namely metastasis) reduces the efficacy of therapy and may cause death of the patient. Clinically, avoiding this undesirable situation is the focus of attention for many researchers. Multiple mechanisms contribute to metastasis formation which is not a simple process. Elucidation of these mechanisms and molecules in this mechanism is important for the development of new therapies. Among these mechanisms EMT and CSCs are important. Unlike cancer cells, CSCs that can not be destroyed easily are one of the most important factors that complicate the treatment of cancer. Since EMT contributes to generate CSCs as well as metastasis formation, it causes persistence of cancer. Therefore, a therapy targeting this mechanism and particular cell type is very important in terms of increasing clinically the efficiency of therapy.

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