The epithelial-mesenchymal transition (EMT) phenomenon.

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How much do we know about cancer cells? Epithelial-mesenchymal transition (EMT) phenomenon. Pierre Savagner, IRCM U896 INSERM, CRLC Val d'Aurelle-Paul Lamarque, Montpellier France.

Keywords: Epithelium, differentiation, morphogenesis, invasiveness, apoptosis,

Abstract: The epithelial-mesenchymal transition (EMT) describes a rapid and often reversible modulation of phenotype by epithelial cells. EMT was originally defined in the context of developmental stages, including heart morphogenesis, mesoderm and neural crest formation. Epithelial cells loosen cell-cell adhesion structures throughout EMT. They modulate their polarity, cytoskeleton organization and typically express vimentin filaments and downregulate cytokeratins. They become isolated, mobile and resistant to anoikis. The epithelial-mesenchymal transition at least superficially resembles the evolution from normal to transformed cell phenotype during carcinoma progression. The relevance of the concept of epithelial-mesenchymal transition in this context was suggested by in vitro models using transformed epithelial cells. Transduction pathways typical for embryogenic EMT in vivo were also found to be activated during cancer progression. More recently, it has been found that such pathways suggest an increased plasticity linked to cellular stemness and ability to generate tumors. However, in the absence of direct evidence, a number of oncologists and pathologists remain skeptical about applying the EMT concept to human tumor progression. In fact, EMT concept appears to be fully relevant in some situations, but the concept has to be adjusted in other situations to reflect tumor cell renewal and plasticity during carcinoma progression and metastasis.

1) EMT semantics.

EMT consists of a rapid and often reversible change of cell phenotype. Epithelial cells loosen cell-cell adhesion structures including adherens junctions and desmosomes, modulate their polarity and rearrange their cytoskeleton: intermediate filaments typically switch from cytokeratins to vimentin. Cells become isolated, motile, and resistant to apoptosis. The epithelial-mesenchymal transition was initially defined as the cellular remodeling occuring during heart morphogenesis, but the concept was extended to analogous transformation occuring during the formation of mesoderm and neural crest [1, 2]. In the last twenty years, EMT has been studied in

detail in vitro allowing the characterization of numerous pathways, typically involving so-called "EMT master genes" a family of transcriptional factors including Snail (Snail1), Slug (Snail2), Twist, SIP1/Zeb, and E47 [3]. Based on the similarities between transducing pathways, the EMT concept has been extended to the physiological process of partial EMT taking place during wound healing and mammary tubulogenesis [4]. The link between EMT and stemness has been recently explored in several publications [5, 6]. Even though this link is somehow confusing when considering cell population renewal and dynamics, it emphasizes the plasticity of cells ongoing an EMT. In vivo, the EMT process generates poorly differentiated and potentially pluripotent cells rather than true fibroblasts, a differentiated cell type expressing tissue and organ specificity. This intermediate phenotype is linked to the expression of basal cytokeratins (CK5, CK14) and to some level of cell-cell adhesion allowing group cell migration. We call this intermediate phenotype a metastable phenotype to emphasize this transient plasticity [7].

Such EMT-like process is also evoked during tumor progression and metastasis emergence. However, due to the absence of direct clinical evidence for EMT, some pathologists are not convinced about the relevance of EMT in cancer progression [8]. We agree that a distinction must be made between EMT *sensu stricto* and the EMT-like phenotype observed in carcinoma . Since the poor differentiation typically expressed by tumoral cells can result from faulty differentiation process as well as EMT, it appears more appropriate to use the term of "EMT-like" to describe the phenotype observed in the tumors [9]. Many genes and pathways, have been implicated in inducing EMT in tumor cells. Typically, these pathways are also active in other processes including cell proliferation, apoptosis and differentiation during early developmental stages, tissue morphogenesis and wound healing. Their specific role during human tumor progression is usually not well understood.

2) An example of cancer-linked EMT: mammary tumors include basal-like and luminal type of cancers.

Invasive breast carcinoma are characterized by their strong heterogeneity, reflecting tumor histology and response to therapy. Their clinical classification has been based on histological features including the presence of differentiated tubules, proliferation rate (mitotic index) and anisokaryosis, bases for the Nottingham and

Scarff Bloom Richardson grading system [10]. Other properties such as hormonal receptor status have been found to correlate with disease progression and are used as markers for diagnostic and prognostic purposes [11].

Therefore, due to this heterogeneity, it is likely that the contribution of a process like EMT in cancer progression depends on the tumor type. A limitation of the clinical studies is the impossibility to state if an undifferentiated phenotype reflects a lack of differentiation or an active EMT process during tumor progression. However, a classification of EMT-like phenotypes based on cell-cell adhesion status is possible and has been recently proposed, without presumptions about mechanisms responsible for this phenotype [9]. The best case for a complete EMT taking place during mammary tumor progression is carcinosarcoma or metaplastic carcinomas, which represent less than 1% invasive breast carcinomas, but carry a bad prognostic. In these tumors, an epithelial and a mesenchymal compartment can be distinguished based on the expression of, respectively, cytokeratins or vimentin intermediate filaments. Cytogenetic studies strongly suggest that these two compartments originate from a common precursor cell population undergoing a full EMT process giving rise to the mesenchymal component [12]. Recent studies show overexpression of Snail genes in these tumors, correlating with activation of Akt and b-catenin pathways [13]. A more prevalent mammary tumor, the infiltrating lobular carcinoma is also characterized by the lack of E-cadherin expression reflecting genomic and epigenetic silencing mechanisms [14, 15]. These tumors express significantly higher levels of a "classic" EMT-master gene, Twist [16], but interestingly still express cytokeratins. They provide an interesting case of partial EMT producing individualized cells. This phenotype results in a distinct, more insidious mode of invasion characterized by an “indian file” pattern, alignment of 3 to 10 cells following each other without adhering to each other. These tumors represent 10 to 15% of invasive breast carcinomas and tend to be detected later during tumor progression, resulting in a poorer prognosis.

More recently, expression profiling has provided new global approaches. Based on unsupervised clustering, most studies sort breast tumors in five groups including basal-like, ERBB2 overexpressing, luminal A and B and normal–like tumors [17]. Expression profiles and signatures characterize these groups, reflecting histological features and tumor phenotype. However, no consensus has been reached yet on their precise identity. Most of these studies have identified a group,

called the basal-like group. This group appears to be heterogeneous, probably encompassing several subtypes, such as “triple-negative (ER, PR, HER2/ErbB2) tumors”. Tumor cells in this group present a phenotype reminiscent of the elusive stem cell profile described for mammary gland. Several authors have suggested that basal-like cancers could be generated by mammary stem cells transformed at very early stages of differentiation [18]. This observation is also relevant considering the links established between EMT and emergence of stem cell-like cells. Several pathways activated along EMT models are also overactive in basal-like carcinomas. These include the oncogenic cMyc pathway, recently reported to activate Snail/GSK3 axis and induce EMT [19]. Also the expression of factors of the Snail family has suggested that EMT is controlling basal-like carcinomas progression [20]. It should be noticed however that basal-like tumor cells profile is distinct from a post-EMT profile. Indeed, basal-like cells typically express significant levels of vimentin, cytokeratins 5/6, and EGFR evoking an undifferentiated (basal) phenotype, but they also express typical epithelial markers such as CK8/18 and E-cadherin [18]. Overall, basal-like carcinomas are associated with poor relapse-free and survival. Another tumor group called the normal-like tumors is also characterized by the expression of some markers and pathways evoking early differentiation pathways [21]. In both cases, it is tempting to suggest that tumor cell phenotype could reflect low differentiation level from the original transformed tumor cell. Alternatively, initial transformation process could include a de-differentiation stage, possibly an EMT situation considering EMT-related pathways found to be activated during transformation and tumor progression. Among them, Snail genes have been studied in the context of breast carcinoma for one decade. A significant number of publications suggest an overexpression linked to tumor aggressiveness [10, 22]. Recent work using transplantations in humanized mouse mammary glands identified Slug among effectors of the Wnt pathway. In this basal-like carcinoma model, a lung metastasis signature was used to identify Wnt pathway role for tumor cell self-renewal and proliferation, linking again EMT, stemness and seeding capacity in human mammary tumor cells [23].

3) Mouse mammary tumor models: Oncogenic pathways can induce EMT.

Mouse models have been used to decipher the links between cell phenotype, EMT and oncogenic pathways. Recent expression-profiling analysis has established

interesting links between mammary-specific tumor promoting pathways and resultant phenotype and dominant active pathways [24]. These profiles evoke human classification, with notable differences. Basal-like, normal-like, luminal-like and mixed phenotype-based groups were identified by a clustering based on a 866 gene signature designed by unsupervised tumor sample clustering. These phenotype-based groups were found to associate tumors models including MMTV-Wnt1, p53 null transplant, DMBA, BRCA1xP53/irradiated (basal-like phenotype); MMTV-Neu, MMTV-PyMT, WAP-Myc, Wap-Int 3 (luminal-like phenotype); WAP-Tag, WAP-T121 (mixed phenotype). These phenotypes appear to result from distinct inductive pathways converging to generate a differentiation status that may also reflect transformed cell origins. Similarly to human mammary basal-like carcinomas, tumor cells from basal-like group expressed cytokeratin 5 and c-Kit. A separate group called « mesenchymal » was also identified and clearly evoked a large scale ongoing EMT among tumors expressing significant amounts of vimentin and Snail among other EMT-related genes. These tumors were mostly composed of dissociated cells. Slug was also found to be overexpressed in this group and in the basal-like tumors, as reported for human mammary basal-like carcinomas. This work suggests that EMT-like phenotype can result from oncogenic controlled activation. This was demonstrated more clearly in an intricate mouse model. MMTV-Cre and FSP-Cre (fibroblast specific protein) strains were engineered to express LacZ under the control of an epithelial (MMTV) or mesenchymal (FSP)-specific promoter [25]. These strains were intercrossed with mouse mammary tumor models: WAP-myc, MMTV-neu, and MMTV-PyMT. LacZ expression was monitored in heterozygous mice and reflected MMTV or FSP promoter activity used as a cell marker for epithelial or mesenchymal cell origin. Mice from MMTV-Cre/WAP-myc showed a large expression of LacZ, indicating a strong MMTV promoter activation within most tumor cells, but also in mesenchymal-like cells located outside the histological tumor border. This clear localization illustrated an extensive EMT process affecting a significant proportion of tumor cells. In this model, a good proportion of the peritumoral stroma was actually generated by EMT from the original mouse mammary epithelial cells. Interestingly, no such epithelial-derived mesenchymal cells were obtained when crossing MMTV-myc mice with MMTV-neu, or MMTV-PyMT mice. This work represents a clear demonstration of an EMT process involved in tumor progression and emphasizes the specific role of some oncogenic pathways such as myc and ras in triggering this

process. This is reminiscent from the role of the myc pathway mentioned before in human basal-like mammary carcinomas. Recent work on p21CIP1 explores links between Myc and Ras induced tumor progression, EMT and tumor cell stemness [26].

4) EMT in carcinoma: a current vision

Both human tumors and mouse models of breast tumorigenesis show evidences of EMT or partial EMT. Besides the modification of the phenotype, EMT also results in the acquisition of other properties involved in carcinoma progression, such as an increased capability to migrate, a higher resistance to apoptosis and the already mentioned acquisition of stemness properties. In vitro and in vivo model systems have allowed the characterization of various pathways leading to EMT and EMT-like phenotypes. Such pathways are referred to as EMT pathways in this review, without assuming functional specificity. Five main pathways have been found to trigger EMT-associated processes [1, 3]: tyrosine kinase receptors (epidermal growth factor, fibroblast growth factor, hepatocyte growth factor, platelet-derived growth factor, insulin-like growth factor), integrins, Wnt, NF-KB, and TGF-β pathways. These pathways activate Akt, GSK3, Rho-GTPases, and SMAD signaling pathways. A distinct EMT pathway has also been recently described involving the protein tyrosine phosphatase Pez. In direct association with cancer progression, several molecules including tyrosine phosphatase Pez [27] , ILEI [28], RKIP [29], and CXCR447 appear to control EMT-like phenotypes and tumor metastasis in various mouse models [3]. Transcriptional down-regulation of junctional components accompanies the EMT process in most EMT models [1, 3] and may be either a cause or an effect of EMT-like events. Down-regulation of E-cadherin is linked to cell-cell dissociation and invasion in various mouse cancer models [30]. Typically, EMT master genes negatively regulate E-cadherin expression [31, 32] and presumably display overlapping functional redundancy, in part through their common recognition of E-box sequences. Their overexpression in epithelial cell lines usually induces an EMT [1, 16, 22, 33]. At the same time, detailed mechanism(s) of their effects remain unclear. Cellular co-expression of Snail, Slug and E-cadherin has been described in breast and colon carcinoma cells by several groups [10, 34]. In addition, these transcription factors clearly do more than repressing E-cadherin regulation and inducing EMT. For

example, members of the Snail family have been shown to be involved in cell motility, proliferation control, differentiation, and apoptotic regulation in vivo and in cell models [22, 35]. Distinct pathways inducing EMT have been uncovered recently, emphasizing functional links between EMT-like phenotypes and inductive pathways specifically activated during tumor growth and progression. Tumor cell growth requires an increase in local vasculature to provide metabolites and oxygen. Cells adjust to a nutritionally impoverished and hypoxic environment by activating specific pathways associated with hypermetabolism, glycolysis and resistance to acidosis-induced toxicity, and neoangiogenesis. Hypoxia genes have been found to be expressed locally within solid tumors, probably contributing to tumor heterogeneity [36]. The link between hypoxia and EMT has been recently strengthened by the observed activation of Snail and Twist expression by HIF-1, a key hypoxia effector [37]. Another hypoxia related gene, lysyl oxydase, was found to interact directly with Snail [38]. Another specific feature of tumor microenvironment is the stromal reaction through which epithelial-mesenchymal interactions activate or regulate several pathways involving integrins, cytokines, and growth factors that are critical for tumor growth and metastasis [39]. Inflammatory cells play a major role in secreting activating factors, and NF-KB, a key regulator of the inflammatory response, has been found to regulate Slug and Snail [40]. A putative role of macrophages in supporting the movement of post-EMT individualized cells from mammary tumors into the bloodstream has recently been suggested in striking movies [41].

In conclusion, the concept of EMT has been very fruitful in emphasizing new pathways controlling cell fate and tissue morphogenesis. Based on clinical observations, it appears more appropriate in most cases to describe the emergence of an EMT-like phenotype during tumor progression. This descriptive term does not necessarily imply an active dedifferentiation process but emphasizes an intermediary phenotype resulting from tumor cell renewal and adaptation to specific microenvironments. Several transcription factor families have proved powerful regulators of cell phenotype, also involved in apparently unrelated cell processes such as apoptosis and acquisition of stemness properties. In vivo studies show functional links between these processes along developmental stages, stress response and indeed carcinoma progression. Although many questions remain to be answered, the remarkable advances during the last years in the mechanism

controlling EMT opens new hopes about the use of inhibitors of this process as an antitumoral drugs, alone or in combination with other compounds targeting epithelial cells [2, 42].

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