

## JB Review

# Cancer early dissemination: cancerous epithelial–mesenchymal transdifferentiation and transforming growth factor $\beta$ signalling

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**Contrary to the long believed hypothesis, it is now evident that breast cancer cells can disseminate from the early phases of the oncogenesis; and that such early disseminated cells sometimes survive at the sites of dissemination and may outgrow after a long latency of years and decades. For cancer cells to leave their origin, they must at least transiently loosen their adhesion with adjacent epithelial cells and stroma, and become motile while avoiding anoikis. Such processes resemble epithelial-mesenchymal transdifferentiation (EMT), which normally takes place in situations such as embryogenesis and wound healing. Interestingly, the occurrence of an EMT-like process in breast cancer cells has been implicated in the generation of cancer stem-like cells, in which TGF $\beta$ 1 signalling often plays core roles. Here, I discuss the current knowledge regarding cancerous EMT and its signalling pathways with the aim to consider the possible mechanisms of early dissemination, and also the generation of cancer stem-like cells in mammary tumour.**

**Keywords:** cancer stem cells/EMT/p53/TGF $\beta$ /tumour dissemination.

**Abbreviations:** EMT, epithelial-mesenchymal transdifferentiation; ER, estrogen receptor; PRKD, Protein kinase D; RTK, receptor tyrosine kinases.

The concept of ‘systemic metastatic dissemination of cancer cells from the early phases of their oncogenesis’, which is referred to here as ‘cancer early dissemination’ for short, was established by pioneer works of C.A. Klein and colleagues. In their cohort studies of breast cancer patients, they provided the first evidence that breast cancer cells can disseminate into the bone marrow in a far less progressed genetic state than previously thought, and hence discussed that genomic aberrations that make them metastatic (i.e. outgrowing from the sites of dissemination) might be acquired after this step (1). This notion was confirmed experimentally using mouse models for human breast

cancer (2). Metastasis is the greatest threat of cancer. According to the model of ‘cancer early dissemination’, it is easy to understand the very late appearance of metastatic lesions, long after the resection of a primary tumour as well as its regional lymph nodes, as C.A. Klein has mentioned. Early disseminated tumour cells will acquire genome alterations independent of the primary site after leaving, that allow them to grow out. Thus, this model may also imply that therapies based on characters of the primary tumours have some limitations when treating metastatic lesions and recurrent cancers that have originated from early disseminated cells. This model also shed a new light on ‘cancer of unknown primary (CUP)’, which comprises ~7% of cancer patients bearing metastases, whose primary tumour remains unrecognized.

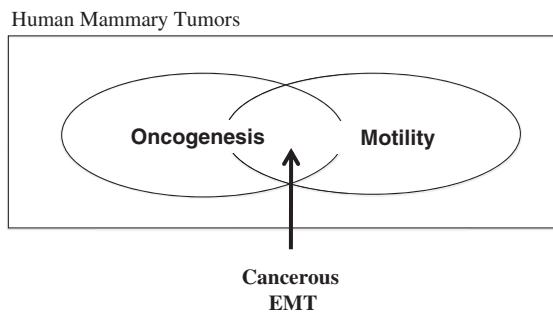
Mechanisms of cancer early dissemination are still largely unidentified. From a cell biological point of view, however, early dissemination is a phenomenon rather easy to understand because full-transformation is not necessary to move out (Fig. 1). Obviously, because of the lack of the full transformation, early disseminated cells do not outgrow immediately at their sites of dissemination, but instead sometimes become dormant, as has been demonstrated using untransformed mammary epithelial cells (3). Cancer is a tissue-specific disease, and studies discussed in this review are mostly carried out with breast cancers, while some parts of the intracellular mechanisms and signalling have yet to be confirmed in this cancer.

### Cancerous EMT

EMT is a normal cellular process of epithelial cells in which their E-cadherin is downregulated and certain integrins are upregulated in order to move out of the epithelial cell layer, described originally by E. Hay (4). EMT occurring in normal cells involves many changes including an alteration in the expression of intermediate filaments, from cytokeratins to vimentin. However, cancer cells often express both cytokeratins and vimentin simultaneously (5), indicating that the EMT-like process which occurred in most cancer cells is not normal. Here I will refer to the EMT-like processes of cancer cells as ‘cancerous EMT’.

### Loss of E-cadherin: not simply the disruption of cell–cell adhesions resulting in cells to move-out

A long-standing question is whether the loss of E-cadherin-mediated cell–cell adhesion is enough to



**Fig. 1 Full transformation is not necessary to move out.** Genes, as well as their mutations involved in oncogenesis are overlapped with those regulating cell motile phenotypes. Therefore, cells under oncogenesis may thereby acquire motile phenotypes that are enough to move out from their original place, even if they are not fully transformed.

render cells to move-out. On the other hand, it is well known that E-cadherin associates with  $\beta$ -catenin, which is bifunctional and acts as a transcriptional co-activator, such as of Tcf/Lef in the nucleus. The current prevailing model suggests that association of E-cadherin with  $\beta$ -catenin suppresses  $\beta$ -catenin from entering into the nucleus to prevent transformation. A question is whether this is true.

Onder *et al.* (6) have addressed this issue by the use of HMLER cells, which are generated by immortalization of human primary normal mammary epithelial cells by introducing hTERT and SV40 large T antigen, followed by the introduction of H-Ras to make them tumourigenic (7). Although HMLER cells are highly tumourigenic, they are not metastatic on their own. Onder *et al.* have shown that the disruption of cell–cell adhesions by a dominant-negative form of E-cadherin is unable to facilitate their metastasis (6). On the other hand, loss of E-cadherin expression by shRNA renders HMLER cells to be invasive, anoikis resistant, and highly metastatic. Similar results has been shown by others (8). Gene expression profiles indicated that HMLER cells undergo EMT upon loss of E-cadherin expression. However, Onder *et al.* also show that the nuclear accumulation of  $\beta$ -catenin is not sufficient, although necessary, to induce the metastatic properties. Therefore, the loss of E-cadherin expression, but not simply the loss of E-cadherin-mediated cell–cell adhesions, appears to be a key for cancer cells to move out. Twist was originally found as an inducer of mesoderm formation in *Drosophila* (9); and is expressed upon the onset of EMT to suppress gene transcription including the *CDH1* (E-cadherin) gene in mammals (10). Twist is induced upon the loss of E-cadherin expression in HMLER cells (6). Twist may well contribute to cancer malignancy, perhaps even independently of the suppression of E-cadherin expression (also see later).

### TGF $\beta$ 1 signalling is the most frequent factor inducing mesenchymal and stem-like properties of breast cancer cells

TGF $\beta$  is one of the EMT-inducing factors. Under physiological conditions, TGF $\beta$  exhibits biphasic

roles in both the promotion and inhibition of branching morphogenesis of mammary gland ducts, depending on their concentration and cellular context (11). In cancer, TGF $\beta$  signalling also appears to be biphasic: it acts as a tumour suppressor during early oncogenesis, while in later stages it is often a tumour promoter (12, 13).

Among the different signalling pathways, TGF $\beta$  signalling appears to be most frequently involved in inducing the mesenchymal and cancer stem cell-like property of primary human breast cancers. The gene expression profile of CD44<sup>+</sup>/CD24<sup>-</sup>/low breast cancer cells resembles that of stem cells (14). Moreover, CD44<sup>+</sup> breast cancer cells are negative for estrogen receptor (ER) even in some ER<sup>+</sup> cancers and are refractory to current adjuvant hormonal therapy (15). In these cancers, ER is expressed in CD24<sup>+</sup> cells. Polyak and colleagues have studied gene expression and genetic profiles of cancerous and normal breast tissues of humans, and identified that direct interaction networks built around the lin<sup>-</sup>/CD44<sup>+</sup>/CD24<sup>-</sup>/low breast cancer cell-specific genes is centered around TGF $\beta$ 1 (15). Correlation of high expression of gene signatures specific for CD44<sup>+</sup> cancer cells, as well as for the TGF $\beta$  pathway of these cells, with metastasis-free survival of breast cancer patients was also demonstrated. Interestingly, CD24<sup>+</sup>/CD44<sup>-</sup> cancer cells may also exhibit abnormal localization of E-cadherin and nuclear accumulation of  $\beta$ -catenin, while their *TGF $\beta$ 2* gene expression is epigenetically silenced. Apparently paradoxically, moreover, CD24<sup>+</sup> cancer cells are dramatically higher in distant metastases regardless of their sites, while positivity for CD44 expression is not consistently different among metastatic sites (15). The reason for this remains unclear.

Consistent with above results primarily based on clinical samples, Weinberg and colleagues have shown *in vitro* that TGF $\beta$ 1 induces EMT, and this EMT conversion induces cancer stem cell properties of HMLE cells (16). HMLE cells are generated by immortalizing human primary normal mammary epithelial cells (HMECs) by introducing hTERT and SV40 large T antigen. This group has previously shown that Twist is expressed at high levels in invasive lobular and ductal carcinomas, and that Twist appears to be one of the master regulators of the mesenchymal morphogenesis and the metastatic activities (10). Expression of Twist alone can induce mesenchymal and stem cell-like properties in HMLEs. They also described that the *CDH1* gene is not the sole target of Twist (16). In contrast to HMLEs, in the case of HMECs expression of Twist is not sufficient: Twist expression only partially induces EMT, and the significant but incomplete decrease of E-cadherin and the modest increase of vimentin (17). However, co-expression of H-RasV12 (or ErbB2) with Twist1 (or Twist2) in HMECs triggers complete EMT and induces invasive properties (17). Twist can override oncogene-induced premature senescence during EMT, most likely by abrogating p53- and Rb-dependent pathways (17). Taken together, HMLER cells (HMLEs expressing Ras) are non-mesenchymal and non-invasive as far as their *CDH1*

gene expression is not suppressed and hence Twist is not expressed, while HMLE/Twist and HMECs/Ras/Twist cells are mesenchymal and invasive. It should be remembered that SV40LT, used in HMLE cells, interferes with the normal function of p53 and Rb. From these data, oncogenic Ras signalling and abrogating the normal function of p53 and/or Rb seem to be involved in processes that make mammary epithelial cells able to undergo EMT and become cancer stem cell-like, in response to TGF $\beta$  or Twist.

### Balancing proliferative and cytostatic programs: roles of wild-type p53

p53 functions both in the nucleus and the cytoplasm, and its mutation is very common in cancer (18). Upon accumulation in the nucleus, wild-type p53 participates in transcriptional regulation of a number of genes via specific DNA elements, in which each set of expressed gene products is thought to be engaged either in the control of cell cycle arrest, differentiation, senescence or apoptosis. Wild-type p53 thereby exhibits cytostatic and tumour suppressive properties. Wild-type p53 also appears to be important in the regulation of polarity of cell division in mammary stem cells, and its loss is coupled with the symmetric division of cancer stem cells and may hence contribute to tumour growth (19).

Ras/MAPK pathways, activated downstream of growth factor receptor tyrosine kinases (RTKs), generally exert growth-promoting effects in normal cells, while these pathways may well act towards tumour suppression dependent on wild-type p53 (20, 21). Moreover, RTK signalling crosstalks with TGF $\beta$  signalling, affecting TGF $\beta$ -induced growth arrest of mammalian cells, as well as mesoderm development in vertebrate embryos (22). Piccolo and colleagues (23) have shown that wild-type p53 is phosphorylated at Ser6/Ser9 by casein kinase 1  $\epsilon/\delta$ , which are activated downstream of Ras, and this phosphorylation induces the association of p53 with Smads to promote cytoskeleton specification in *Xenopus* embryos. Therefore, this appears to be a mechanism to compromise the growth promoting effects elicited by RTKs with the cytostatic signalling by TGF $\beta$ , in order to limit neoplastic transformation.

### Conversion of TGF $\beta$ 1 signalling to be pro-malignant: roles of oncogenic p53 and Ras

Mutations of p53 often gain oncogenic activities, rather than simply abrogating its tumour suppression function (24). Human pathological analyses have indicated that mutant-p53 expressing tumours are often very aggressive and associated to poor-prognosis, especially in Her2-positive and basal-like breast cancers (25, 26). The malignant future of mutant p53 was confirmed experimentally by generating mutant-p53 knock-in mice: tumours emerged in these mice exhibited very aggressive and metastatic that were never detected in tumours developing in a p53 null

background (27, 28). Like p53, mutations of Ras can also be oncogenic. In breast cancer, however, Ras mutations are relatively rare (29), while overexpression of non-mutated Ras as well as hyper-activation of RTKs signalling such as EGFR are frequent events (30–32). In a mouse model, mutant p53 and oncogenic Ras are cooperative in enhancing malignant cancer development (33, 34).

Given that TGF $\beta$ 1 signalling is the most frequent among the different types of signalling involved in the mesenchymal and stem cell-like properties of human breast cancers, while on the other hand it can crosstalk with wild-type p53 and Ras to be cytostatic, an important question is how TGF $\beta$ 1 signalling becomes able to induce such malignant properties in cancer cells. In other words, what kinds of cancerous contexts convert TGF $\beta$ 1 signalling to induce EMT without stopping cell-cycle progression? Oft *et al.* have shown that oncogenic H-Ras signalling cooperates with TGF $\beta$ 1 signalling, especially with activated Smad2, to induce mesenchymal and invasive/metastatic properties of already proliferative breast, colon and skin cancers (35–37). Piccolo and colleagues have demonstrated a way by which mutant p53 enhances TGF $\beta$ -induced invasive and metastatic activities (38). In this pathway, oncogenic mutant p53 forms a complex with Smads, when mutant p53 is phosphorylated by casein kinase 1  $\epsilon/\delta$  activated under Ras signalling, and this mutant-p53/Smads protein complex then binds to p63, a p53 family-member. p63 can oppose TGF $\beta$ -induced metastasis. Incorporation of p63 into the mutant-p53/Smads complex antagonizes the transcriptional functions of p63, and hence appears to enhance TGF $\beta$ -induced metastasis. They identified that the expression of *Sharp-1* and *Cyclin G2* genes are downstream of p63 that are essential to antagonize TGF $\beta$ -induced invasive/metastatic activities. Decreased expression of *Sharp-1* or *Cyclin G2* genes was validated clinically with relation to a significant high probability to develop recurrence of breast cancers (38). Of note, *Sharp-1* and *Cyclin G2* are not contained in the previously known gene signature for breast cancer metastasis (38).

### Molecular machineries required for cancerous EMT

Sharp-1 and Cyclin G2, as well as p53 and Smads, are all basically engaged in the regulation of gene expression, while they each regulate groups of genes rather than a certain single gene. Moreover, their target genes are not fixed intrinsically, but are altered depending on cellular contexts and other transcriptional factors they may work with. Therefore, while understanding the status of such factors that regulate gene expression and genome status is very important, attempting their understanding may sometimes be frustrating, like seeing shadow pictures. Another aspect towards the substantial understanding would be to understand the molecular machineries that actually accomplish the properties of cancer malignancy. Below I briefly

discuss the molecular machineries that execute cancerous EMT and the moving out.

Core events for EMT are the activation of integrin and the inactivation of E-cadherin. Integrins are activated in two ways. One is the conformational change, which has already been discussed extensively in other reviews (39). Another aspect is their *de novo* synthesis and the recycling. It is well known, for example, that transcription of the  $\beta$ 1 integrin gene is induced during TGF $\beta$ 1-induced EMT of mammary epithelial cells (40), although the transcriptional control is not discussed here. On the other hand, integrin actively recycles between the plasma membrane and early endosomes, recycling which has been demonstrated to constitute a basic machinery driving cell migration (41). Moreover, functions of integrin in cancer cells include phagocytosis of degraded ECM components, which also involves endocytosis.

Arf6 is primarily involved in the recycling of plasma membrane components (42), and its signalling greatly contributes to invasive and metastatic activities of many breast cancer cells (43–46). Arf6 colocalizes with  $\beta$ 1 integrin at endosomes and is implicated in serum-induced recycling-back of  $\beta$ 1 integrin (47, 48).  $\beta$ 1 integrin forms a complex with several Arf GTPase-related proteins, to be under the control of Arf6. It has been shown that ACAP1, a GTPase activating protein for Arf6, forms a complex with  $\beta$ 1 integrin, and this complex formation is regulated by Akt to be dependent on external stimulations (49). In breast cancer,  $\beta$ 1 integrin associates with AMAP1 (Onodera, Y. and H.S., unpublished observations), an effector for Arf6 (44). Protein kinase D (PRKD) regulates intracellular traffic as well as cell proliferation and apoptosis (50), and is an essential component of invadopodia (51, Onodera, Y. and H.S., unpublished observations). Yeaman *et al.* (52) have shown that PRKD1/2 is involved in basolateral protein sorting, including  $\beta$ 1 integrin and E-cadherin. Norman and colleagues have shown that PRKD1 binds to the  $\beta$ 3 integrin subunit, and this binding is involved in the platelet derived growth factor-induced recycling-back of  $\alpha$ v $\beta$ 3 integrin (53, 54). Our results indicate that  $\beta$ 1 integrin binds to PRKD1, and PRKD1 then binds to AMAP1, and through this complex  $\beta$ 1 integrin becomes under the control of Arf6 as well as RTKs in invasion of breast cancer cells (Onodera, Y. and H.S., unpublished observations). Interestingly, p53 oncogenic mutation has been shown to also promote integrin recycling and invasion, by inhibiting p63 (55).

Rab GTPases are also important. The above mentioned PRKD1-mediated recycling-back of  $\beta$ 3 integrin appears to be dependent on Rab4, but not Rab11 (53, 54). On the other hand, Rab11, but not Rab4, has been shown to be involved in the endosomal colocalization of  $\beta$ 1 integrin with Arf6, and its recycling (48). Moreover, Rab11 is involved in the trafficking of  $\beta$ 4 integrin in hypoxia-induced invasion of some breast cancer cells (56). Rab GTPases consist of more than 80 isoforms in humans, while Arf6 is the sole member among the Arf-family GTPases to be involved in recycling. The primary functions of Rabs appear to be the

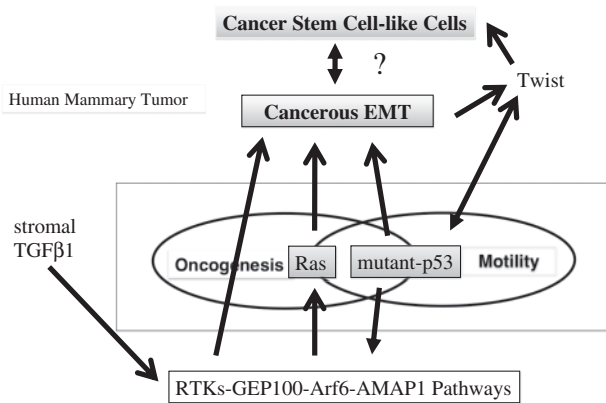
sorting of cargos and fate determination of endosomes, which are accomplished by interaction of Rabs with their cognate binding partners. Comprehensive identification of Rabs involved in integrin trafficking, as well as identification of their binding partners are necessary for the fine understanding of the mechanisms regulating the intracellular traffic of integrins, essential for EMT and cell motility.

Inactivation of E-cadherin is also accomplished in two different ways. One is suppression of its gene transcription, as already mentioned. The other is the regulation of endocytosis, in which again, Arf6 plays an important role. It has been shown that expression of an inactive form of Arf6 blocks hepatocyte growth factor-induced endocytosis of E-cadherin-based junctional components, while expression of a constitutively active form of Arf6 causes disassembly of adherens junctions (57). The results of siRNA-mediated knock-down of GEP100 and of *Arf6*<sup>-/-</sup> mice also support this notion (58, 59). Moreover, activation of Arf6 by GEP100 disrupts E-cadherin adhesion, while activation of Arf6 by other GEFs does not (45). We have moreover shown that Git1 is responsible for the inactivation of Arf6 at the plasma membrane in order to prevent E-cadherin from being endocytosed (60). In this mechanism, Git1 was found to be activated by EphA2, whose dysfunction is frequently observed in malignant cancers. We have moreover shown that EPB41L5 is induced during EMT and is essential for EMT conversion, by facilitating E-cadherin endocytosis (61).

## Perspective

In this review, I have discussed mainly the intracellular signalling of breast cancer cells, which most frequently arises from luminal epithelial cells. However, it should be noted that the mammary gland is composed of several components, including luminal epithelial cells, myoepithelial cells and the basement membrane. Stromal cells, such as fibroblasts, are also present around the ducts; and they may all participate in the regulation of the mammary gland. In particular, myoepithelial cells have recently been recognized as regulators of normal mammary gland development and function (62), besides their contractile role in expelling milk from the ducts. Interestingly, myoepithelial cells act to suppress tumour growth, invasion and angiogenesis (63, 64), while stromal fibroblasts act to enhance tumour malignancy (65). Such functions of myoepithelial cells and fibroblasts have been shown to be effective even in the absence of detectable genome alterations in the tumour epithelial cells. In these events, TGF $\beta$  is again involved as a key player (65).

As discussed, oncogenic mutation of p53 and activation of Ras (or aberrant activation of RTK) appear to be prominent events during the processes that result in breast cancer cells to be reactive to TGF $\beta$  and undergo EMT, and sometimes also become stem cell-like cells. Oncogenic mutation of p53 as well as RTK signalling may also be a key in activating the GEP100-Arf6-AMAP1 pathway in response to TGF $\beta$  stimulation of breast cancer cells (Hashimoto, A.,



**Fig. 2** Our current model as to how GEP100-Arf6-AMAP1 pathway is involved in cancerous EMT and perhaps also 'early dissemination' of primary tumours of the human breast. An outstanding question of this model is to clarify detailed mechanisms as to how TGFβ1 stimulation activates RTKs in cells under oncogenesis, that are linked to the GEP100-Arf6-AMAP1 pathway.

Hashimoto S., and H.S., unpublished observations: also see Fig. 2), that induces EMT-like properties by activating β1 integrin and inactivating E-cadherin adhesion. TGFβ is produced in adults such as upon wounding and inflammation in order to facilitate wound repair while suppressing some immune responses; and can be produced both by breast cancer cells and the stroma cells. Taken together, an important point in understanding the processes initiating cancer early dissemination would be the identification of what conditions (or even genome status and alterations) trigger production of TGFβ in what types of cells within the microenvironment wherein precancerous epithelial cells already have p53 mutations, and aberrant Ras- or RTK-signalling. Another important aspect is the clarification of whether p53 mutations and excess Ras signalling are really the minimal requirements for luminal epithelial cells to undergo EMT in response to TGFβ. It may also be interesting to examine whether certain types of p53 mutations are more prone than other mutations of p53 in inducing cancerous EMT.

#### Conflict of interest

None declared.

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